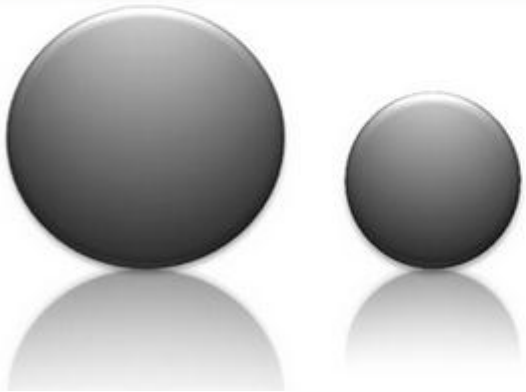
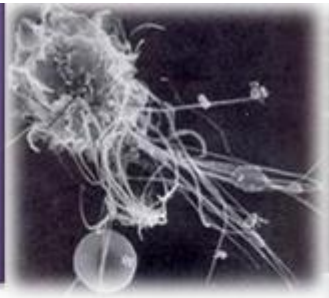
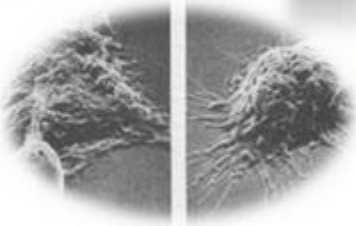
Biomolim

LABORATOIRE DE BIOLOGIE MOLÉCULAIRE APPLIQUÉE ET IMMUNOLOGIE







UT Université de Tlemcen

Synapses Immunologiques

Par:

Professeur Mourad ARIBI

Définition – Structure des Synapses Immunologiques*

- Supramolecular adhesion complex (SMAC)

- Composées d'anneaux concentriques, composé chacun des clusters définissant les groupes suivant:

1. **c-SMAC (central-SMAC)**: isoforme (θ) de la proteine kinase C , de CD2, CD4, CD8, CD28, Lck, et Fyn

2. **p-SMAC (peripheral-SMAC)**: la protéine LFA-1 (lymphocyte function-associated antigen-1) et la proteine du cytosquelette protéine taline sont agrégées

3. **d-SMAC (distal-SMAC)**: enrichi en molecules CD43 et CD45

*Nom dérivé des synapses du système nerveux

Rôles

1. Régulation de l'activation des lymphocytes

2. Interaction CPA/CMH-peptides–TCR/lymphocytes T

3. Induction de la sécrétions de cytokines ou de granules lytiques

Différents contacts cellule-cellule

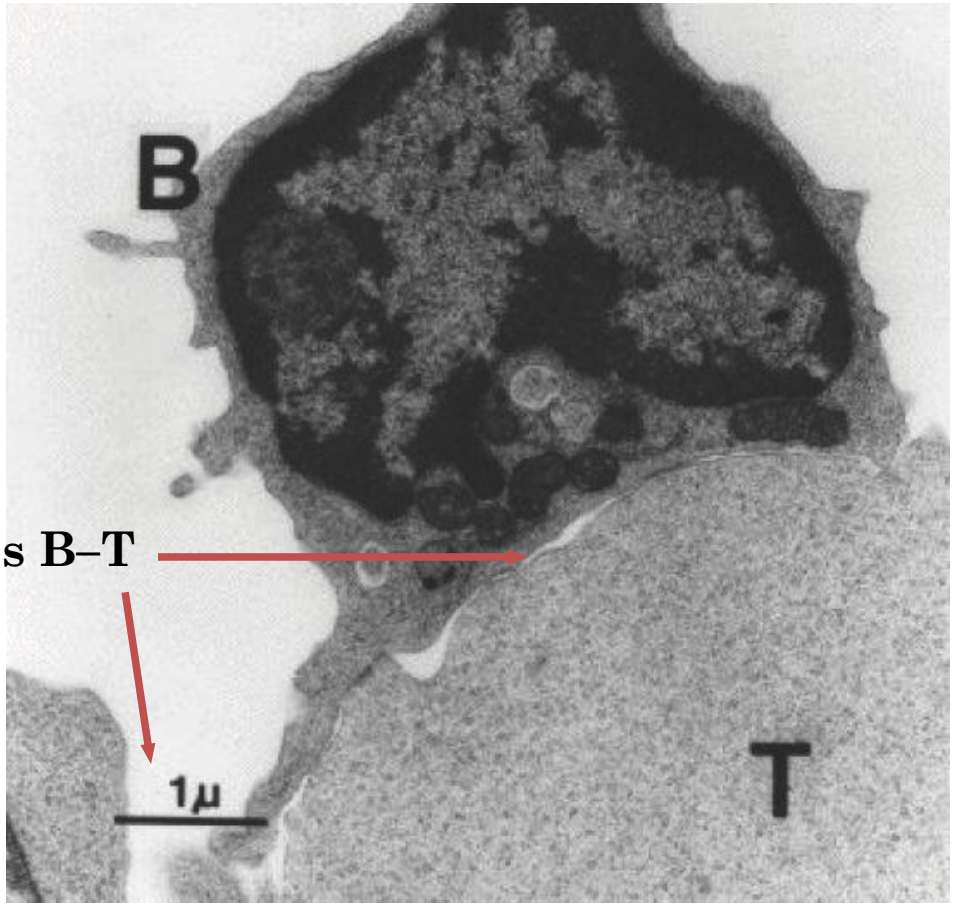
Les contacts cellule-cellule sont nécessaires aux réponses humorales (production d'anticorps) et cellulaires.

Différents contacts:

- Th1–CPA professionnelle (notamment DC, mais aussi certains MØ)
- Th2–Cellules B
- CTLs–Cibles
- NK–Cibles

Observation microscopique des interactions cellule-cellule

De telles interactions peuvent être observées à l'aide du Microscope Électronique, mais également à l'aide de certains Microscopes Optiques dédiés à l'Imagerie Cellulaire.



Interaction cellule B–cellule T observée à l'aide de la micrographie électronique. *La barre = 1 µm. (d'après J. W. Uhr)*

Contacts CPA-cellule T

Les contacts d'une CPA avec une cellule T engagent:

1. Plusieurs pairs de CMH-peptides–TCR.
2. Pairs de molécules de costimulation.
3. Pairs de molécules d'adhésion.
4. Pairs de cytokines et récepteurs de cytokines.

- TCR-MHC interaction is not strong
- Accessory molecules stabilize interaction
  - CD4/MHC class II or CD8/MHC class I
  - CD2/LFA-3
  - LFA-1/ICAM-1

Contacts CPA-cellule T

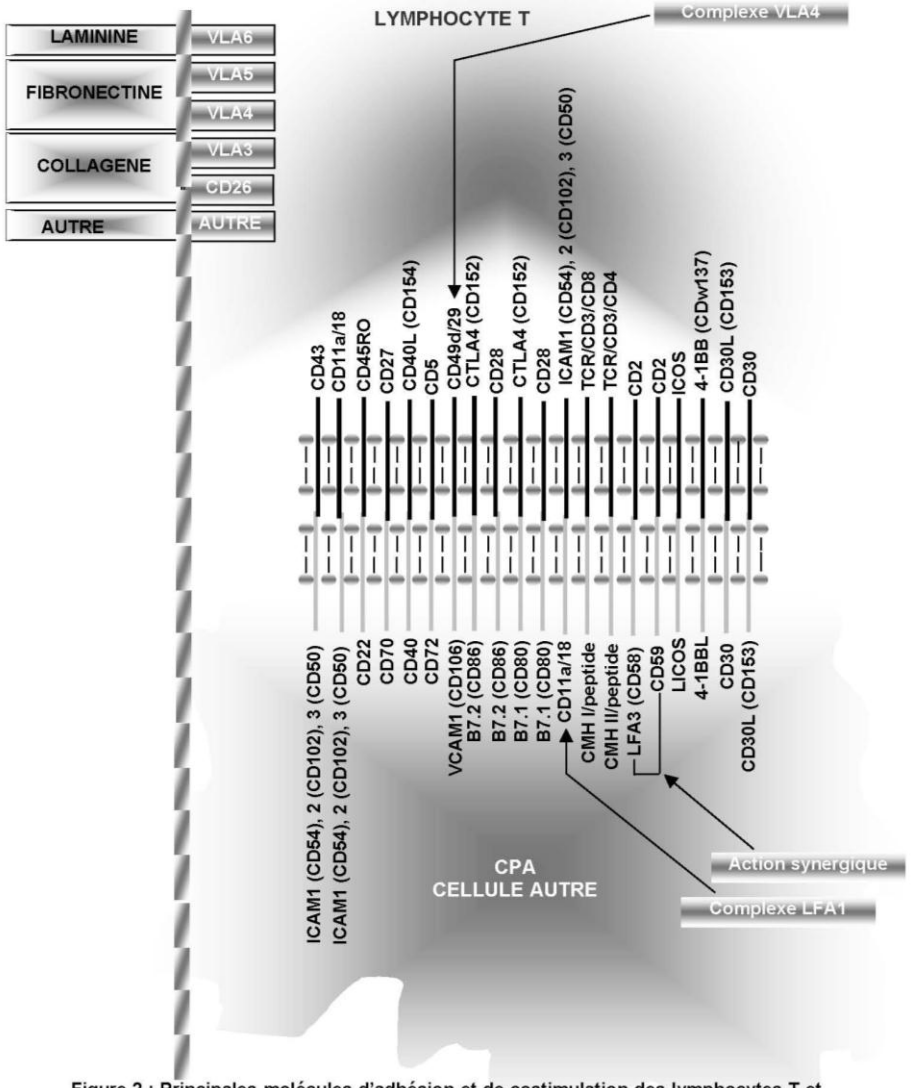


Figure 2 : Principales molécules d'adhésion et de costimulation des lymphocytes T et des cellules associées (adapté de Monier *et al.*, 2005 ; Boitard, 2000). *Les principales*

molécules de costimulation sont impliquées dans les interactions : B7-CD28, B7-CTLA-4 (CD152), CD40-CD40L (CD154), CD8-CMH I, CD4-CMH II, 4-1BB (CDw137)-4-1BBL, LFA3 (CD58)/CD59-CD2, LAF1 (CD11a/18)-ICAM1 (CD54), LAF1-ICAM2 (CD102), LAF1-ICAM3 (CD50), ICOS (inducible costimulator)-LICOS (ligand ICOS). L'interaction CD40-CD40L (famille des récepteurs TNF), active le lymphocyte T, mais aussi active la cellule présentatrice de l'antigène et la stimule à exprimer la molécule B7. Cette interaction serait bidirectionnelle (Janeway et al., 2001). B7.1 (CD80) aurait un effet dans la régulation de l'activation des lymphocytes T et intervient dans l'expression du gène de l'IL-2. B7.2 (CD86) aurait un effet régulateur en se liant aux ligands appropriés (CTLA-4 et CD28), induisant la régulation de la production d'IL-2 et la prévention d'anergie. La molécule CD28 présente beaucoup moins d'avidité pour les molécules B7 par rapport à CTLA-4. Elle aurait un effet modulateur sur la stabilité de l'ARN messager de cytokines lymphocytaires T, et donc sur la production de lymphokines, comme l'IL-2 (Olive et Cerdan, 1999). Cette production d'IL-2 serait diminuée suite à l'interaction CTLA-4-B7 qui transduit un signal négatif au lymphocyte T. Le CD 28 pourrait augmenter la résistance des lymphocytes T à certains agents immunosuppresseurs et prévient l'apoptose et l'anergie. Il a été ainsi montré à l'aide d'anticorps anti-CD28, mais aussi des ligands B7.1 et B7.2, que CD28 peut transduire un signal apoptotique impliquant l'induction de l'expression de FasL ainsi qu'une mort cellulaire dépendante directement de Fas, ou via la production d'IL-2 et l'entrée en cycle de la cellule. De plus, l'effet de CD28 a été corrélé avec l'induction de deux facteurs de survie (Collette et al., 1998) : Bcl-2 (B-cell chronic lymphocytic leukemia 2), molécule régulatrice de la survie cellulaire (Srivastava et al., 1999), et la molécule antiapoptotique Bcl-XL (Leo et al., 1999 ; Gross et al., 2000). L'antigène CD28 existe à la surface des thymocytes triples positifs (CD3+CD4+CD8+) et des lymphocytes T périphériques CD8+ et CD4+ à pouvoir cytotoxique surtout. Il ne transduit pas de signal stimulateur qu'après association avec un signal de transduction délivré initialement par le TCR. La molécule CTLA-4 joue un rôle essentiel dans l'homéostasie lymphocytaire et s'exprime tardivement après l'activation (Monier et al., 2005). La molécule 4-1BBL est localisée principalement à la surface des cellules dendritiques, des macrophages et des lymphocytes B activés. En se liant à la molécule 4-1BB localisée à la surface des lymphocytes T, elle augmente son activation. L'interaction 4-1BB-4-1BBL serait également bidirectionnelle (Janeway et al., 2001). L'antigène CD2 active le lymphocyte T antigène spécifique en se liant à LFA3, mais active aussi les cellules NK dépendamment de l'interaction avec le CD16. La transduction du signal par CD2 est indépendante du complexe CD3/TCR. Par ailleurs, le CD2 est responsable de la formation de rosette GRM, en adhérant avec les molécules LFA3 des globules rouges du mouton (GRM). La molécule LFA3 est fortement exprimée sur les macrophages, et est présente sur les cellules B du centre germinatif, les thymocytes médullaires, les cellules T mémoires. Le CD59 aurait une action synergique avec le CD58, et est exprimée sur l'endothélium vasculaire, le placenta, les cellules T, mais peu sur les cellules B. Le complexe LFA1, en se liant au ligand ICAM1, ICAM2 (CD102) ou ICAM3 (CD50) stimule la prolifération des cellules T et B, la cytolyse dépendante des cellules T, et de l'interaction des leucocytes avec les autres tissus (Monier et al., 2005), leur permettant de migrer à l'intérieur du tissu cible, comme avec l'endothelium (Issekutz et Issekutz, 1992 ; Van Kooyk et al., 1993). La molécule ICOS induit la production d'IL-10 et permet l'activation des lymphocyte T en se liant à la molécule LICOS localisée à la surface des cellules dendritiques, des monocytes et des cellules B activées.

Contacts CPA-cellule T

1. PAIRS DE CMH-PEPTIDES–TCR

Le TCR se lie à une molécule du CMH d'une CPA:

- Pour les cellules T CD4 +: les molécules du CMH sont de classe II, et la liaison est facilitée par le corécepteur CD4.

- Pour les cellules T CD8 +, les molécules du CMH sont de classe I, et la liaison est facilitée par le corécepteur CD8.

- Les molécules de TCR sont attachés par des filaments d'actine dans le cytoplasme.

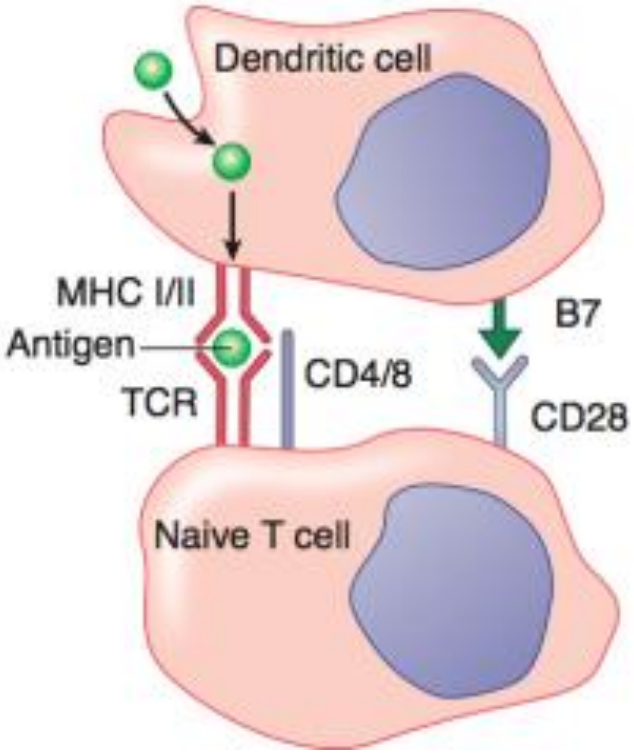
- Cas d'une cellule T naïve: Plusieurs centaines de paires de CMH-peptide/TCR sont nécessaires pour stimuler une cellule T naïve à la mitose.

- Cas d'une cellule T mémoire: seulement environ 50 paires de CMH-peptide/TCR sont nécessaires pour activer une cellule T mémoire pour faire devenir une cellule T effectrice.

Contacts CPA-cellule T

2. PAIRS DE MOLÉCULES DE COSTIMULATION

- CD28 (cellule T) se lie à
- son ligand B7 (CPA).



Contacts CPA-cellule T

2. PAIRS DE MOLÉCULES DE COSTIMULATION

L'interaction CD28 (cellule T) – B7 (CPA) est nécessaire pour activer complètement la cellule T

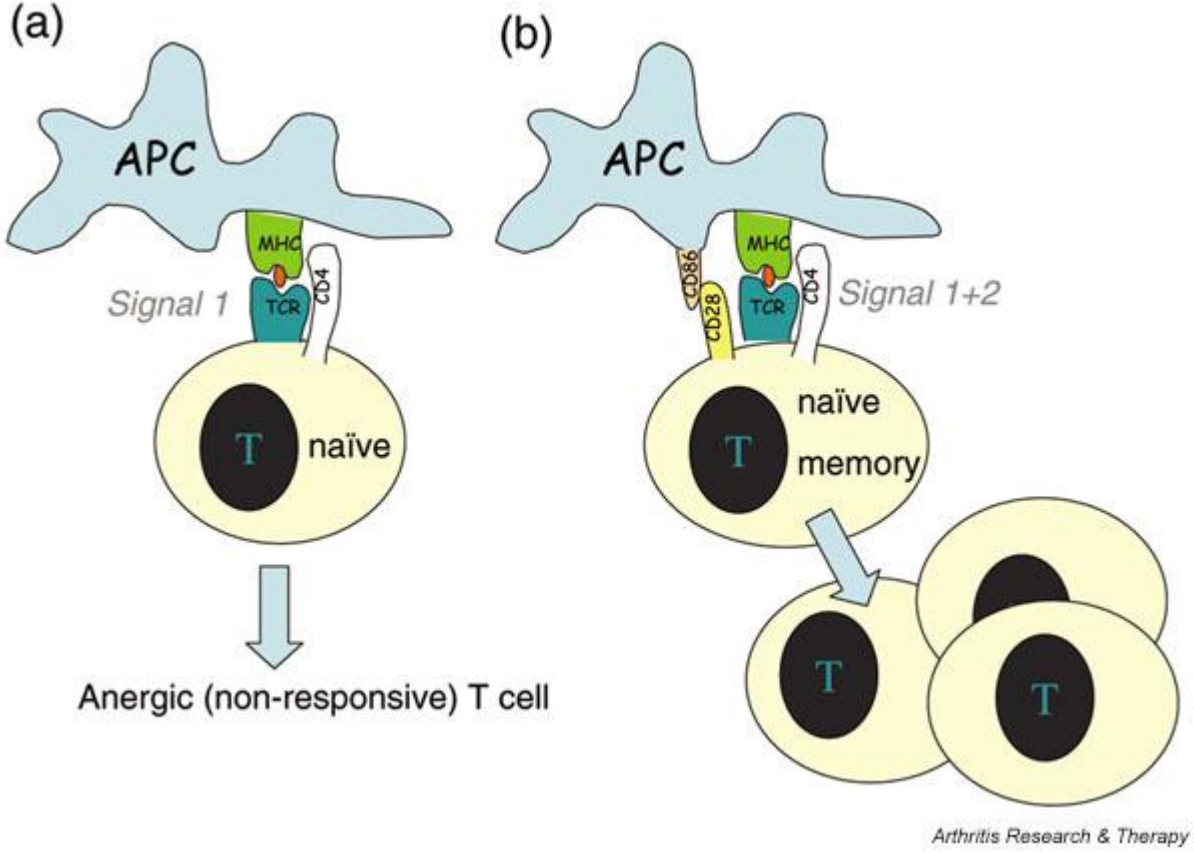


Figure 1 (d'après Malmström V, Trollmo C et Klareskog L. Arthritis Res Ther. 2005;7 Suppl 2:S15-20.)

Activation of naïve T cells requires (a) T cell receptor (TCR)–peptide–MHC interaction (signal 1) and (b) co-stimulation (signal 2) for full activation. This can be provided by so-called professional antigen-presenting cells (APCs; i.e. dendritic cells, macrophages and B cells). In the absence of co-stimulation the T cells will become anergic.

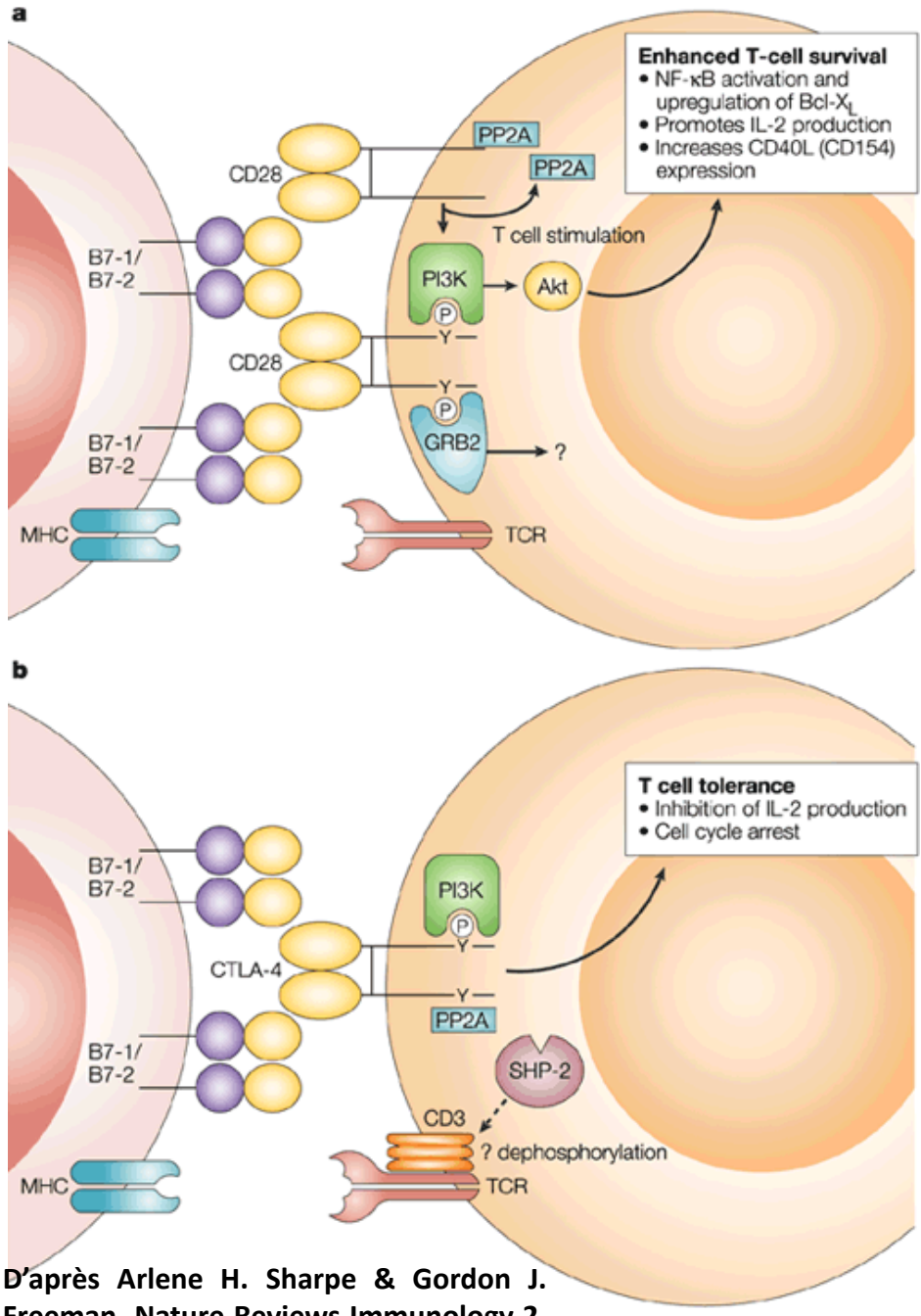
Contacts CPA-cellule T

2. PAIRS DE MOLÉCULES DE COSTIMULATION

B7 possède aussi un autre ligand autre que CD28: CTLA4 ==> Désactivation de la cellule T:

- Protection contre l'autoimmunité
- Mais prometteur des cancers

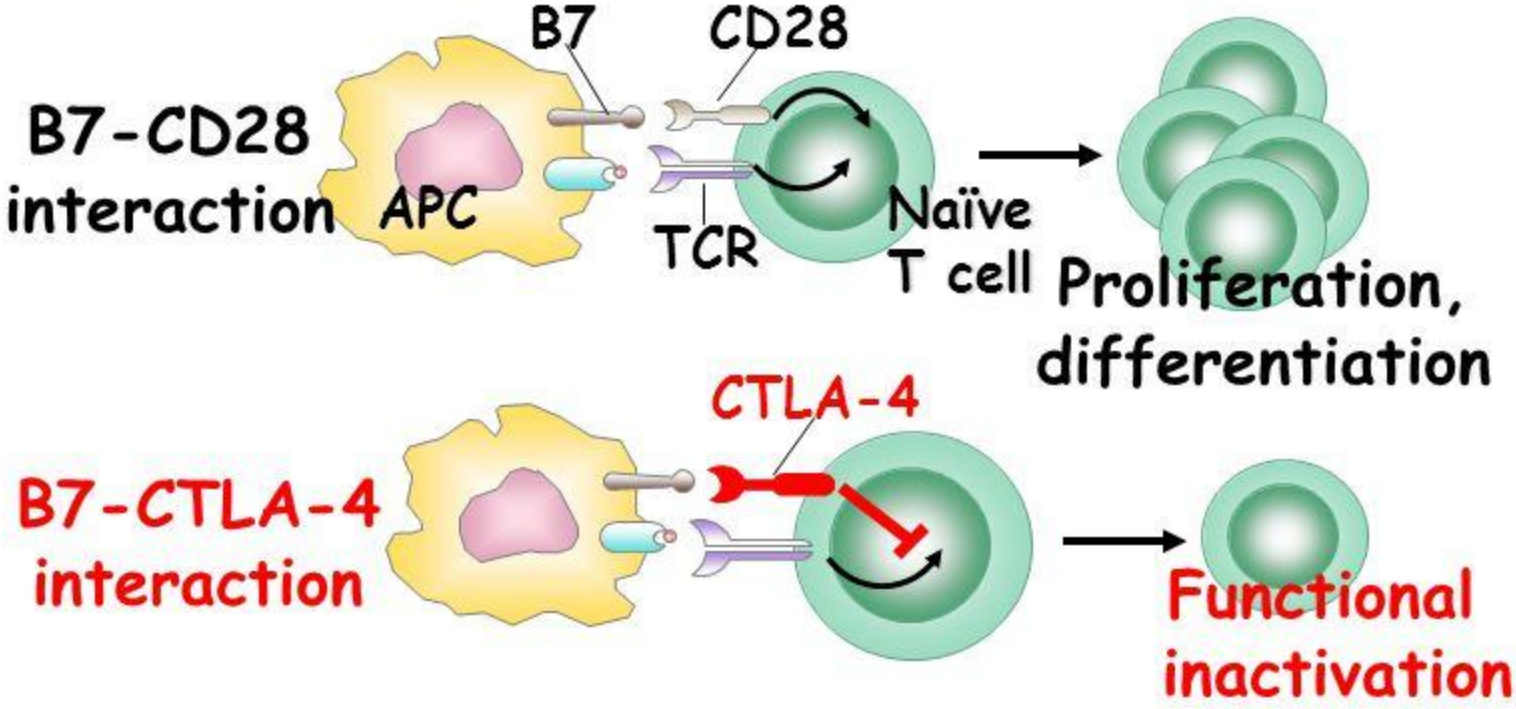
FIGURE 2 | B7-1 and B7-2 form back-to-back, non-covalent homodimers that interact with covalent homodimers of CD28 or CTLA-4 (RefsEach CTLA-4 dimer can bind two independent B7-1/B7-2 homodimers. The crystal structure of B7-1/B7-2–CTLA-4 indicates that a linear zipper-like structure might form between B7-1/B7-2 and CTLA-4 homodimers117, 118. Such a structure might promote the recruitment of inhibitory signalling molecules. The opposing outcomes of CD28 engagement and CTLA-4 engagement are illustrated. Signalling through CD28 promotes cytokine (IL-2) mRNA production and entry into the cell cycle, T-cell survival (at least in part by induction of Bcl-XL), T-helper-cell differentiation and immunoglobulin isotype switching. Signalling through CTLA-4 inhibits IL-2 mRNA production and inhibits cell cycle progression. Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen 4; MHC, major histocompatibility complex NF-kappaB, nuclear factor kappab; PI3K, phosphatidylinositol 3-kinase; PP2A, protein phophatase 2A; TCR, T-cell receptor; Y, tyrosine.



D'après Arlene H. Sharpe & Gordon J. Freeman. Nature Reviews Immunology 2, 116-126

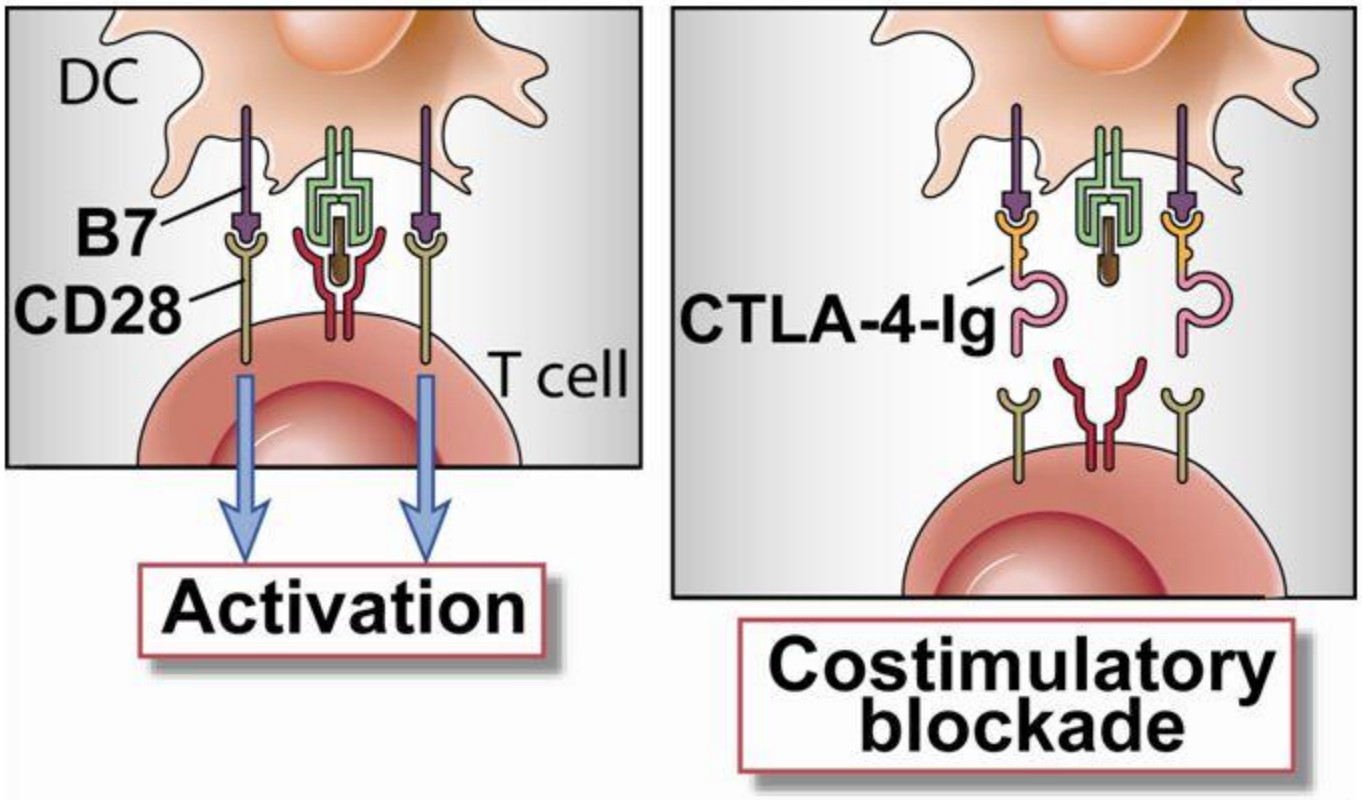
Nature Reviews | Immunology

The opposing functions of CD28 and CTLA-4



Inhibitory pathways function normally to prevent responses to self antigens: demonstrated by the finding that blocking or eliminating these inhibitors (CTLA-4, PD-1) causes autoimmune disease

Therapeutics based on the B7:CD28/CTLA-4 family
1. Costimulatory blockade



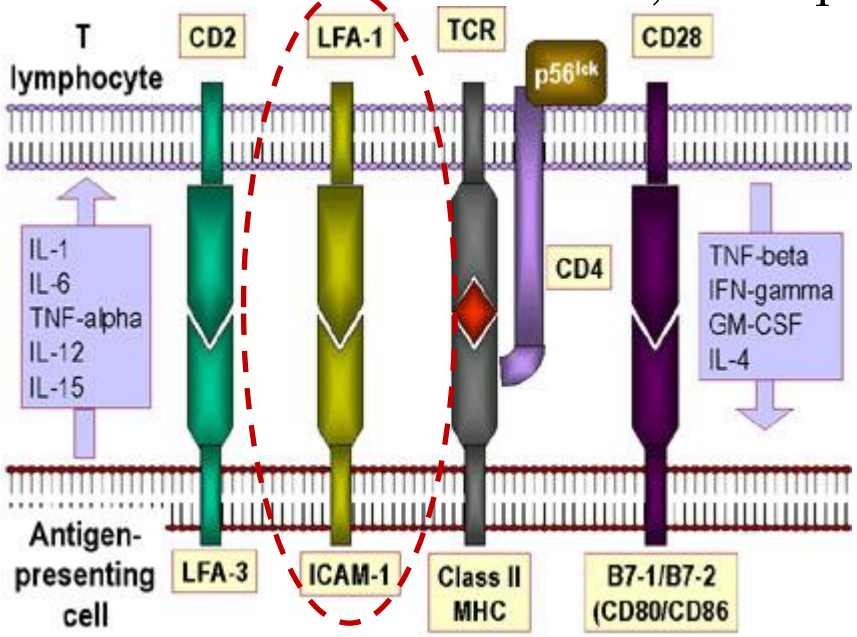
CTLA-4.Ig is being tested in diseases caused by excessive T cell activation -- rheumatoid arthritis (FDA approved), psoriasis, graft rejection (approved)

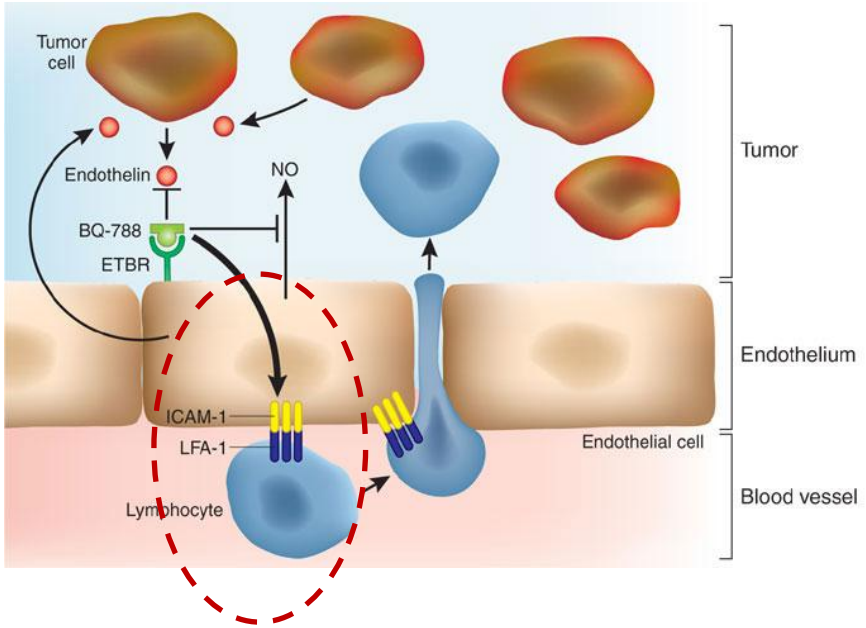
Contacts CPA-cellule T

3. PAIRS DE MOLÉCULES D'ADHÉSION

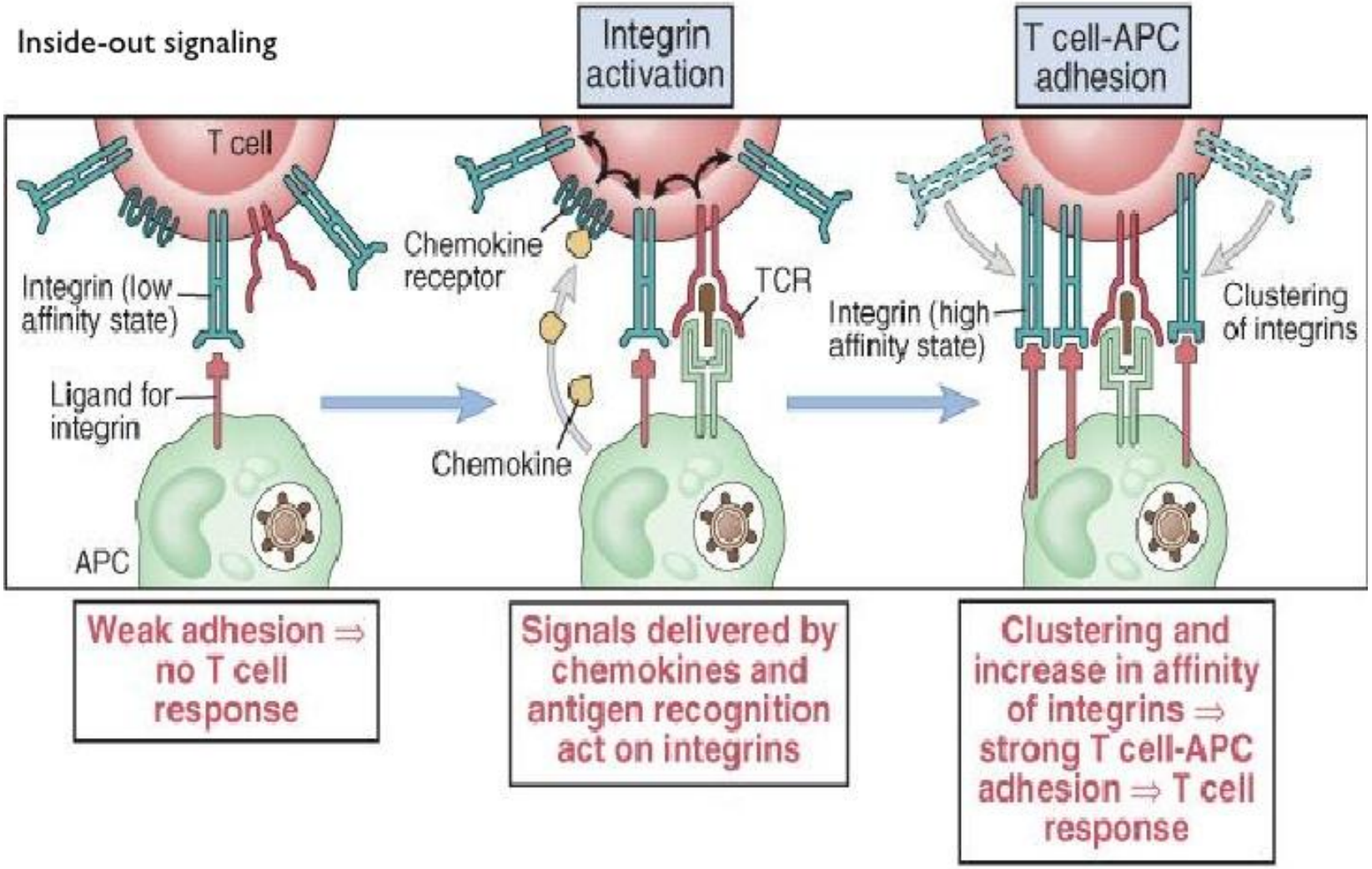
- LFA-1 (leukocyte function-associated antigen-1) (une integrine présente sur la T cell), se lie à

- ICAM-1 (intercellular adehesion molecule-1) (présente sur la CPA ou une autre cellule, telle que la cellule endothéliale).

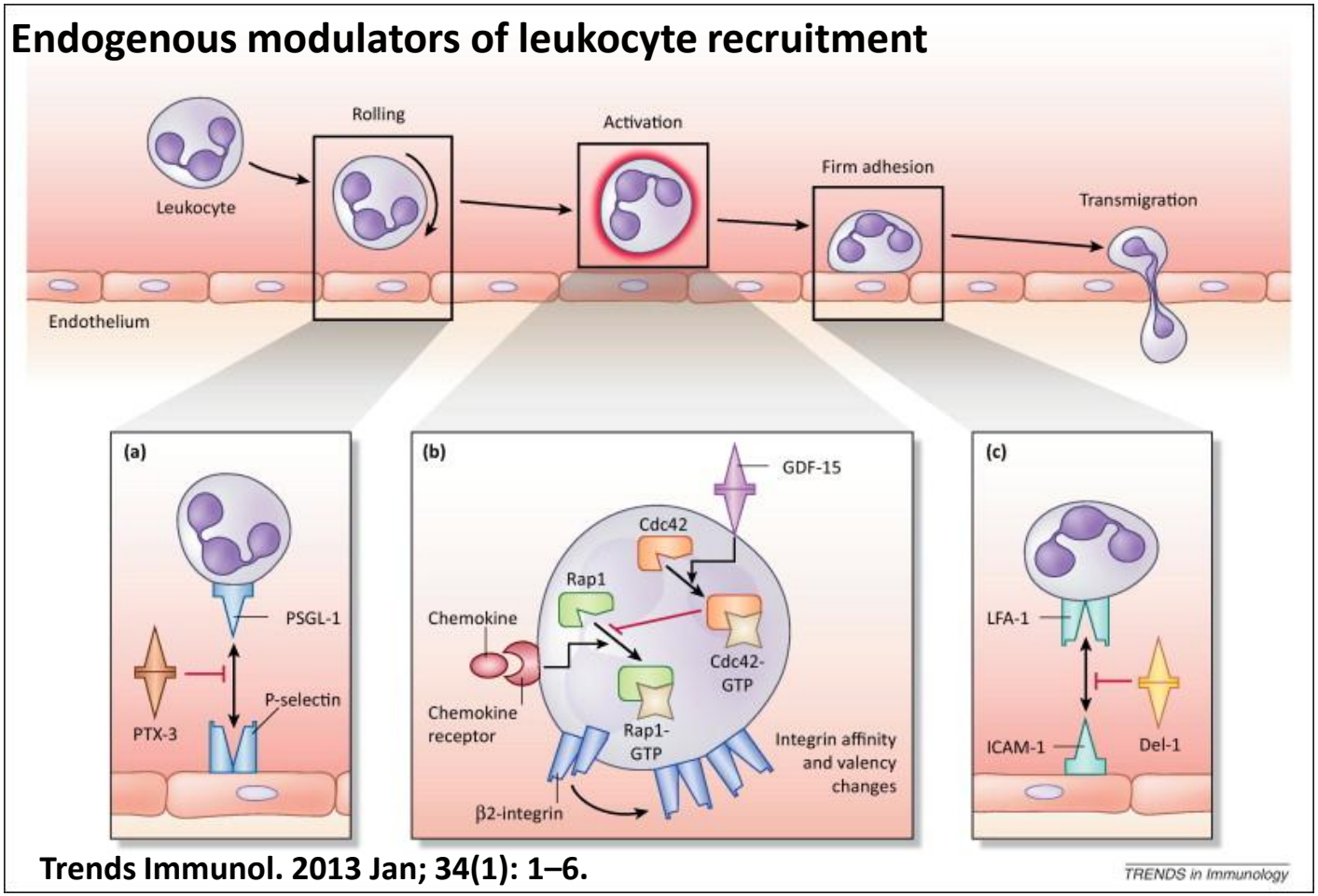




Inside-out signaling



Molécules d'adhésion – Rôle dans la réponse inflammatoire



Trends Immunol. 2013 Jan; 34(1): 1–6.

In addition to its role in leukocyte rolling, PSGL-1 binding to P-selectin mediates leukocyte-platelet interactions, which additionally involve the binding of leukocyte Mac-1 to JAM-C or glycoprotein Ib on platelets. The leukocyte-platelet interaction is involved in several inflammatory processes, including autoimmunity or acid-induced acute lung injury. Interestingly, blocking P-selectin-dependent neutrophil-platelet aggregation reverses lung injury. Therefore, PTX-3 may antagonize inflammatory reactions mediated by leukocyte-platelet aggregates. Taken together, PTX-3 is a specific inhibitor of the PSGL-1/P-selectin interaction and can thereby interfere with leukocyte rolling or other PSGL-1-dependent functions of leukocytes.

Contacts CPA-cellule T

4. PAIRS DE CYTOKINES ET RÉCEPTEURS DE CYTOKINES

Rôles des cytokines

- Régulation de l'immunité, l'inflammation et l'hématopoïèse.

- Ils régulent aussi un certain nombre de fonctions physiologiques et pathologiques.

Les récepteurs de cytokines se regroupent également dans la synapse où elles sont exposées à des cytokines sécrétées dans la synapse.

Synapses immunologiques: Le cytokines

Conséquences de la formation de synapses

La formation d'une synapse immunologique permet à la cellule T de:

1. devenir activée avec la mise en jeu de différentes voies de signalisation débouchant sur une nouvelle transcription génique;
2. libérer, par exocytose, le contenu de ses vésicules, à savoir les cytokines (par les Th CD4+) et molécules cytotoxiques (par les CTLs):
   - pour une cellule Th1: libération des cytokines comme l'IFN-γ et le TNF-β.
   - pour une cellule Th2: libération de cytokines qui stimulent les cellules B comme l'IL-4, l'IL-5, l'IL-10, and l'IL-13.
   - pour les CTLs: libération de molécules cytotoxiques comme les perforines et granzymes.

Les cytokines

What is cytokines?

- Polypeptides or glycoproteins produced in response to various antigens.
- Play role in innate and acquired immunity
- Produce from various kinds of cells, e.g.

  macrophages = monokines
  lymphocytes = lymphokines
- Cytokines that induce chemotaxis of leukocytes are referred to as **chemokines** (**chemotactic cytokines**)

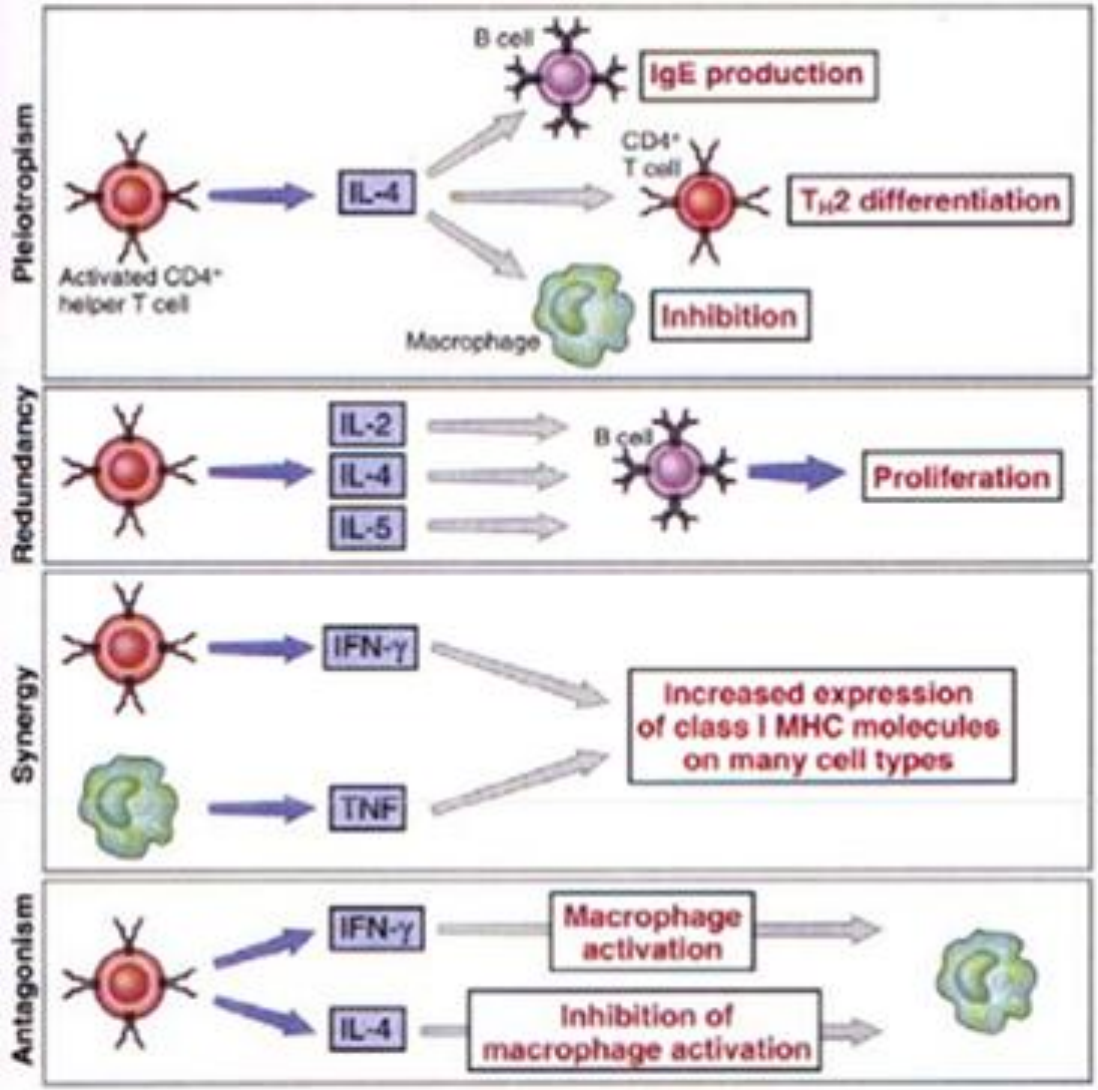
Properties of cytokines

Based on production:

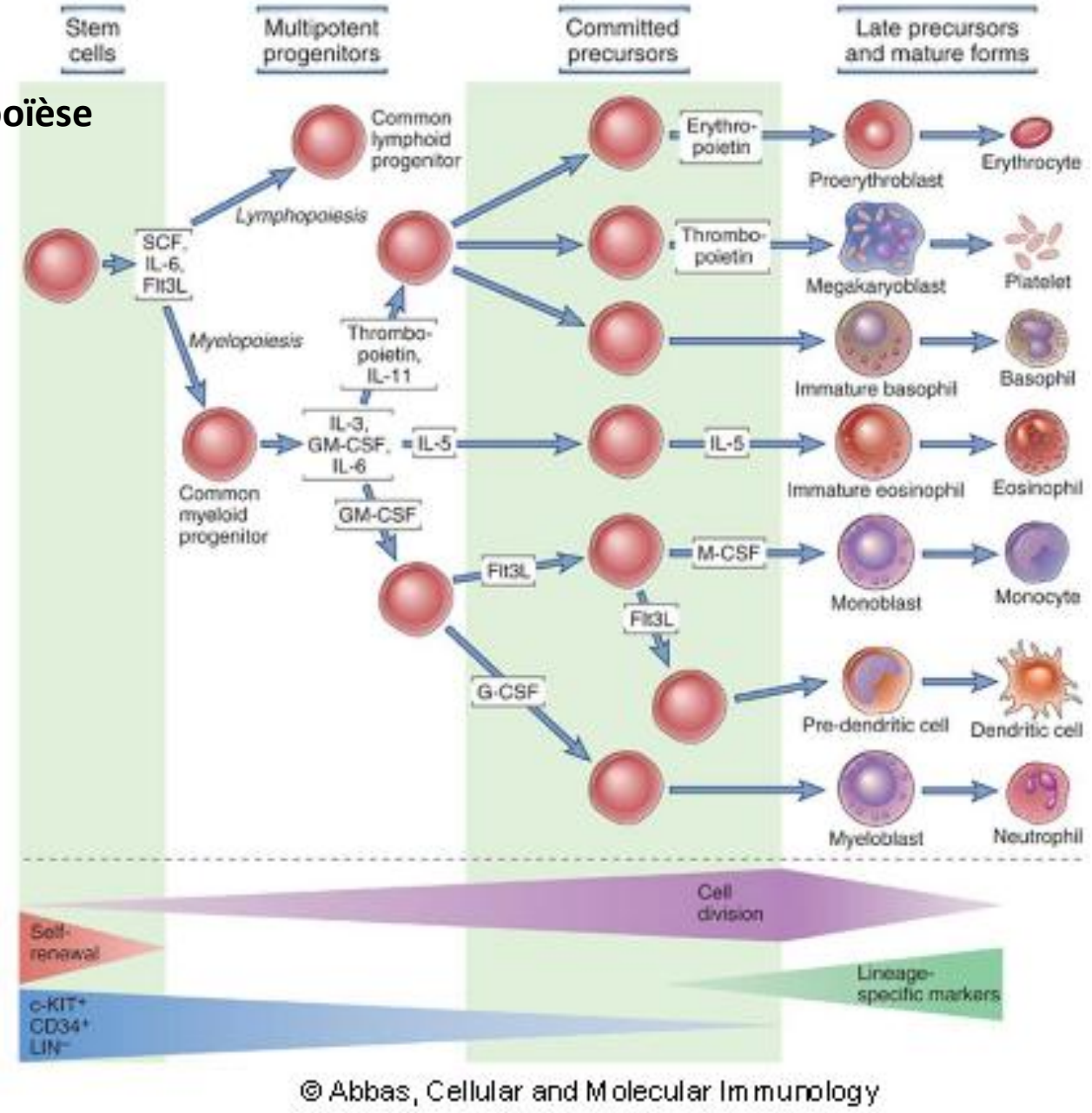
- Depends on Ag stimulation
- Short-time
- By more than one type of cells
- One cytokine can stimulate or inhibit the production of other cytokines

Based on function:

- Hormone-like action: *autocrine, paracrine, endocrine*
- One cytokine can affect more than one types of cells - **Pleiotropism**
- Different cytokines can perform some similar functions - **Redundancy**
- One cytokine can influence the function(s) and/or production of other cytokines
- **Interleukins** – cytokines interact between cells of the immune systems


Pleiotropism
B cell
IgE production
CD4+ T cell
T_H2 differentiation
IL-4
Activated CD4+ helper T cell
Macrophage
Inhibition
Redundancy
IL-2
IL-4
IL-5
B cell
Proliferation
Synergy
IFN-γ
TNF
Increased expression of class I MHC molecules on many cell types
Antagonism
IFN-γ
Macrophage activation
IL-4
Inhibition of macrophage activation

Rôle dans l'hématopoïèse



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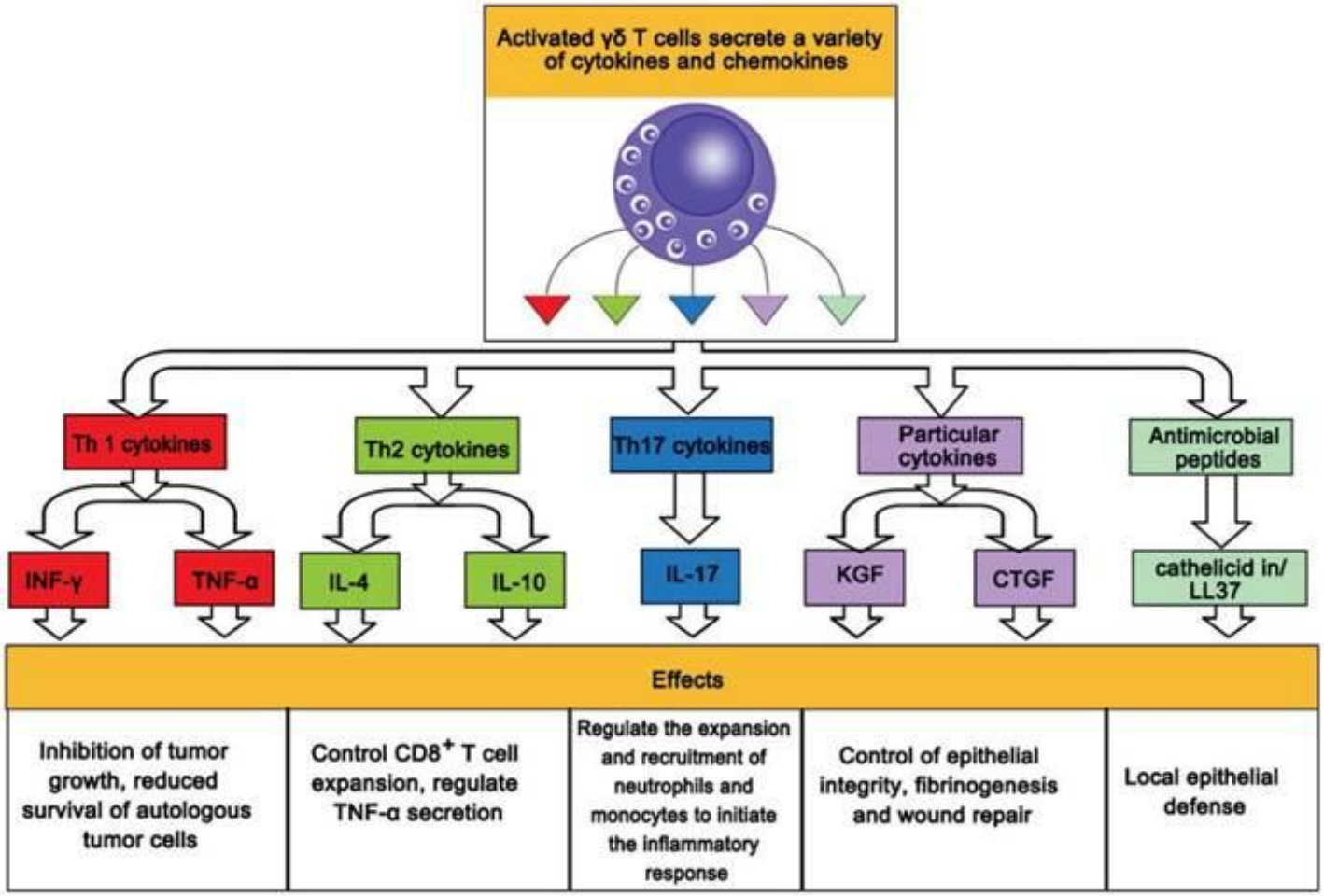
Les cytokines: réponses innée et adaptative

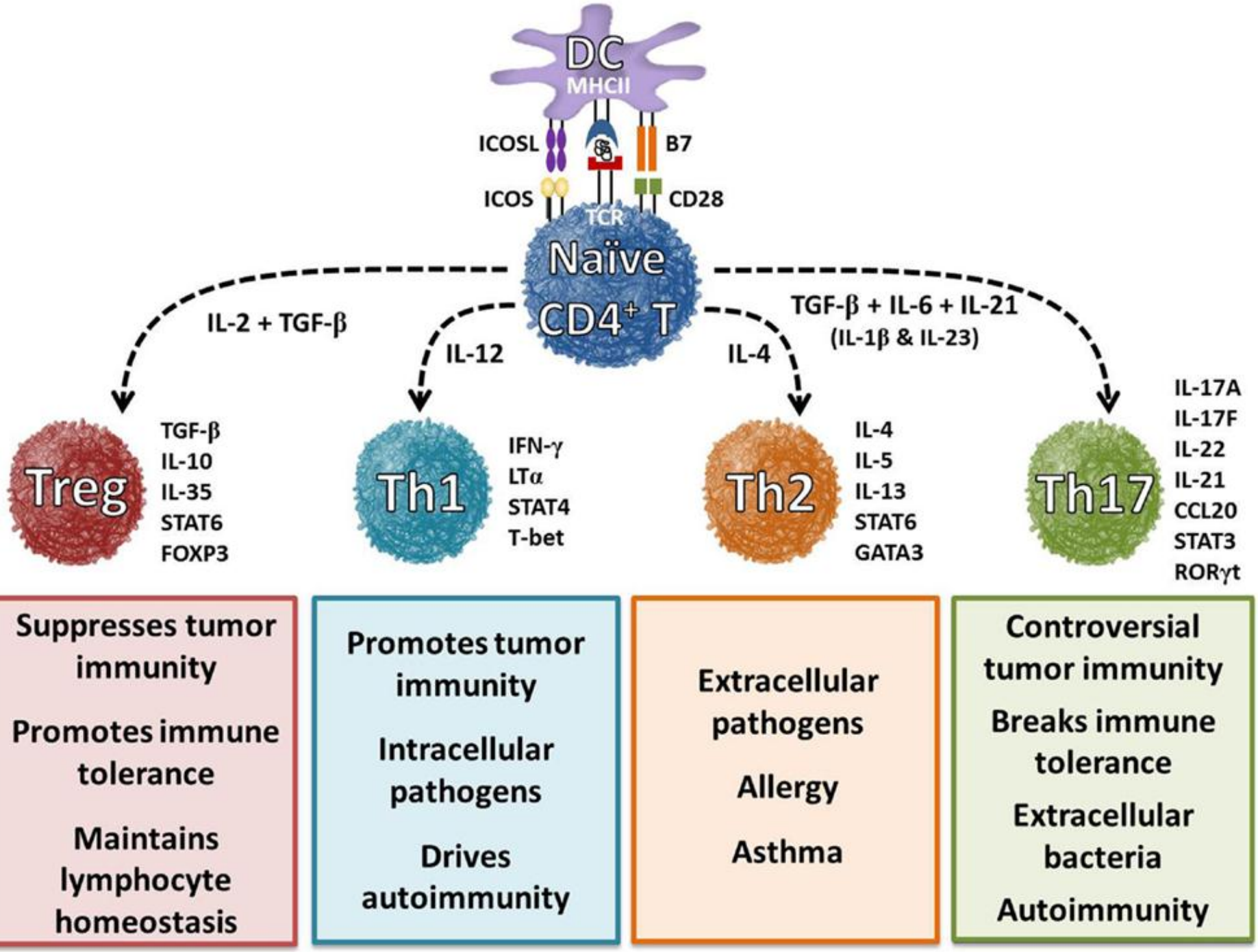
Les cytokines qui induisent et régulent l'immunité innée

- IFN de Type 1
- TNF-α
- IL-1
- IL-6
- IL-12
- Chemokines, etc.

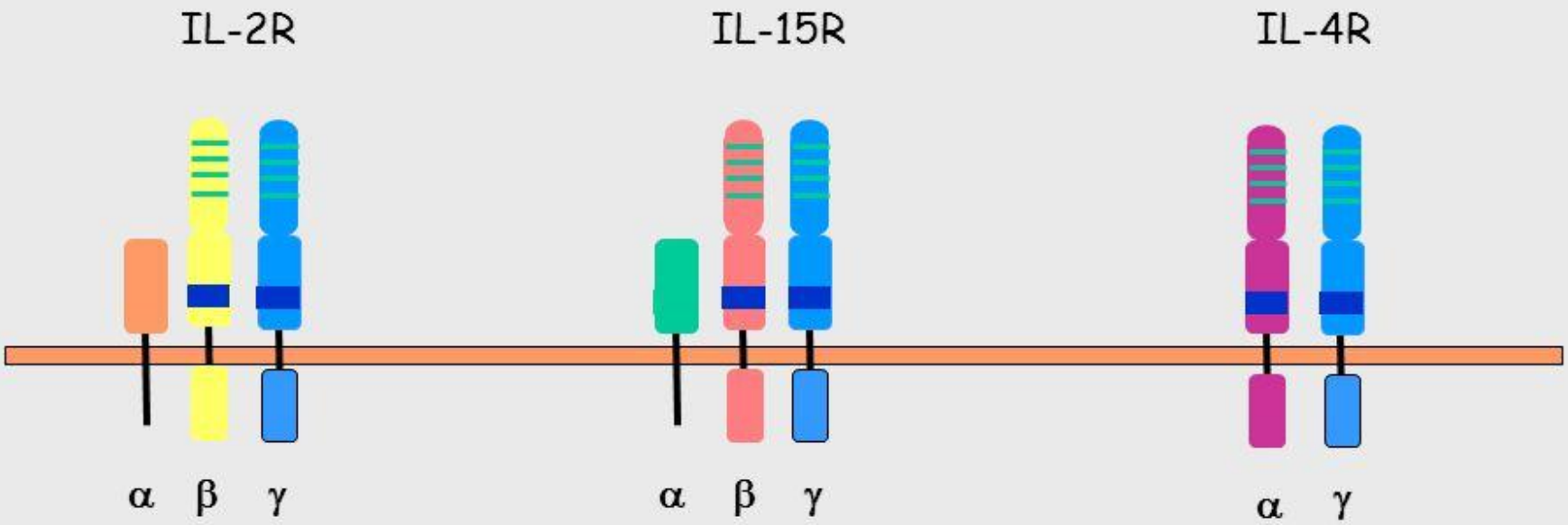
Les cytokines qui induisent et régulent l'immunité adaptative

- IL-2
- IL-4
- IL-5
- IL-13
- IL-16
- IL-17
- IFN-γ
- TGF-β
- Lymphotoxine


Activated γδ T cells secrete a variety of cytokines and chemokines
Th 1 cytokines
Th2 cytokines
Th17 cytokines
Particular cytokines
Antimicrobial peptides
INF-γ
TNF-α
IL-4
IL-10
IL-17
KGF
CTGF
cathelicid in/ LL37
Effects
Inhibition of tumor growth, reduced survival of autologous tumor cells
Control CD8+ T cell expansion, regulate TNF-α secretion
Regulate the expansion and recruitment of neutrophils and monocytes to initiate the inflammatory response
Control of epithelial integrity, fibrinogenesis and wound repair
Local epithelial defense


DC
MHCII
ICOSL
B7
ICOS
CD28
TCR
Naïve
CD4+ T
IL-2 + TGF-β
IL-12
IL-4
TGF-β + IL-6 + IL-21
(IL-1β & IL-23)
Treg
TGF-β
IL-10
IL-35
STAT6
FOXP3
Th1
IFN-γ
LTα
STAT4
T-bet
Th2
IL-4
IL-5
IL-13
STAT6
GATA3
Th17
IL-17A
IL-17F
IL-22
IL-21
CCL20
STAT3
RORγt
Suppresses tumor immunity
Promotes immune tolerance
Maintains lymphocyte homeostasis
Promotes tumor immunity
Intracellular pathogens
Drives autoimmunity
Extracellular pathogens
Allergy
Asthma
Controversial tumor immunity
Breaks immune tolerance
Extracellular bacteria
Autoimmunity

Les récepteurs de cytokines sont majoritairement multimériques.
ex: Récepteur de la famille IL-2



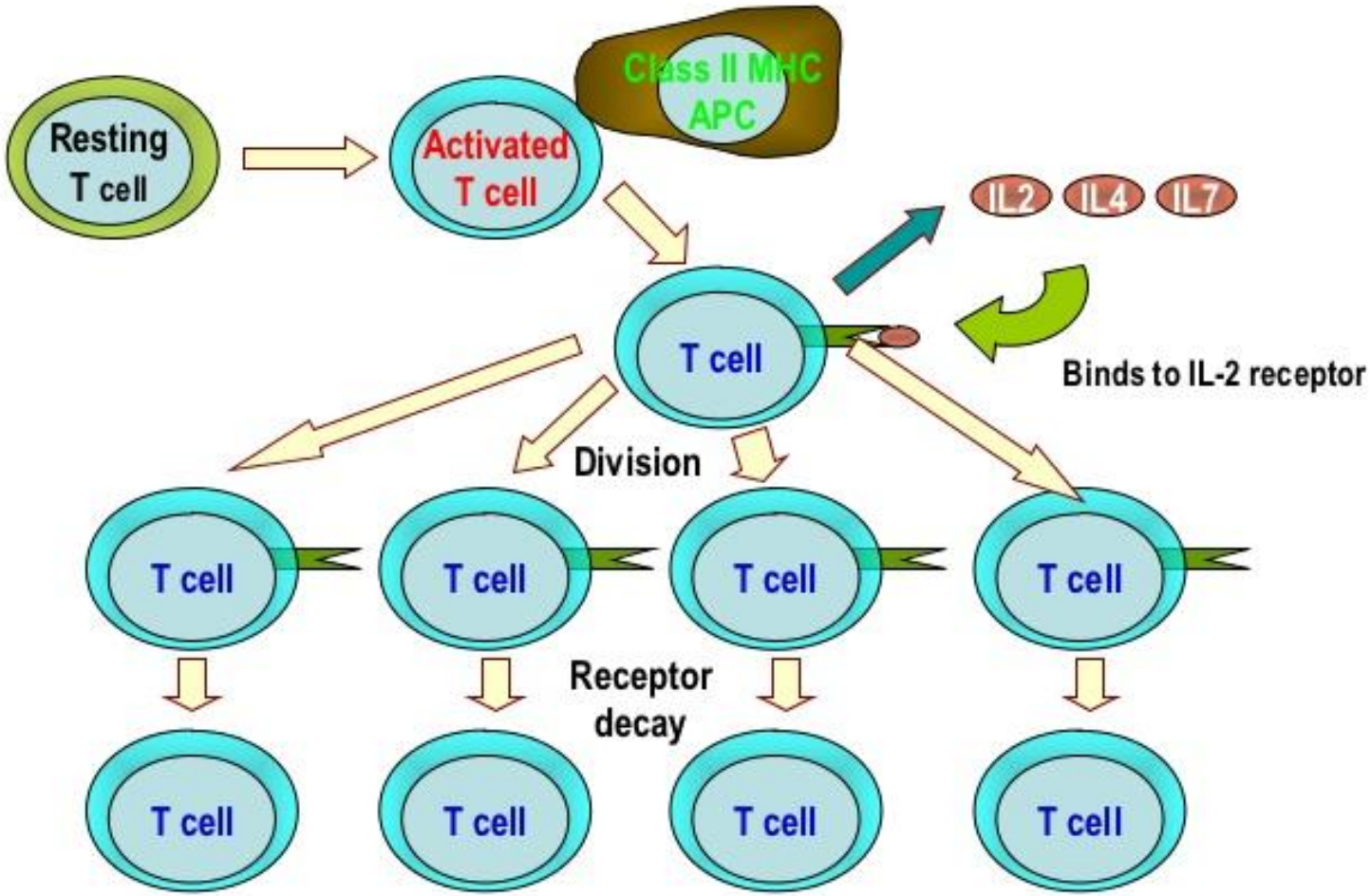
Chaîne α de l'IL2R = marqueur d'activation CD25

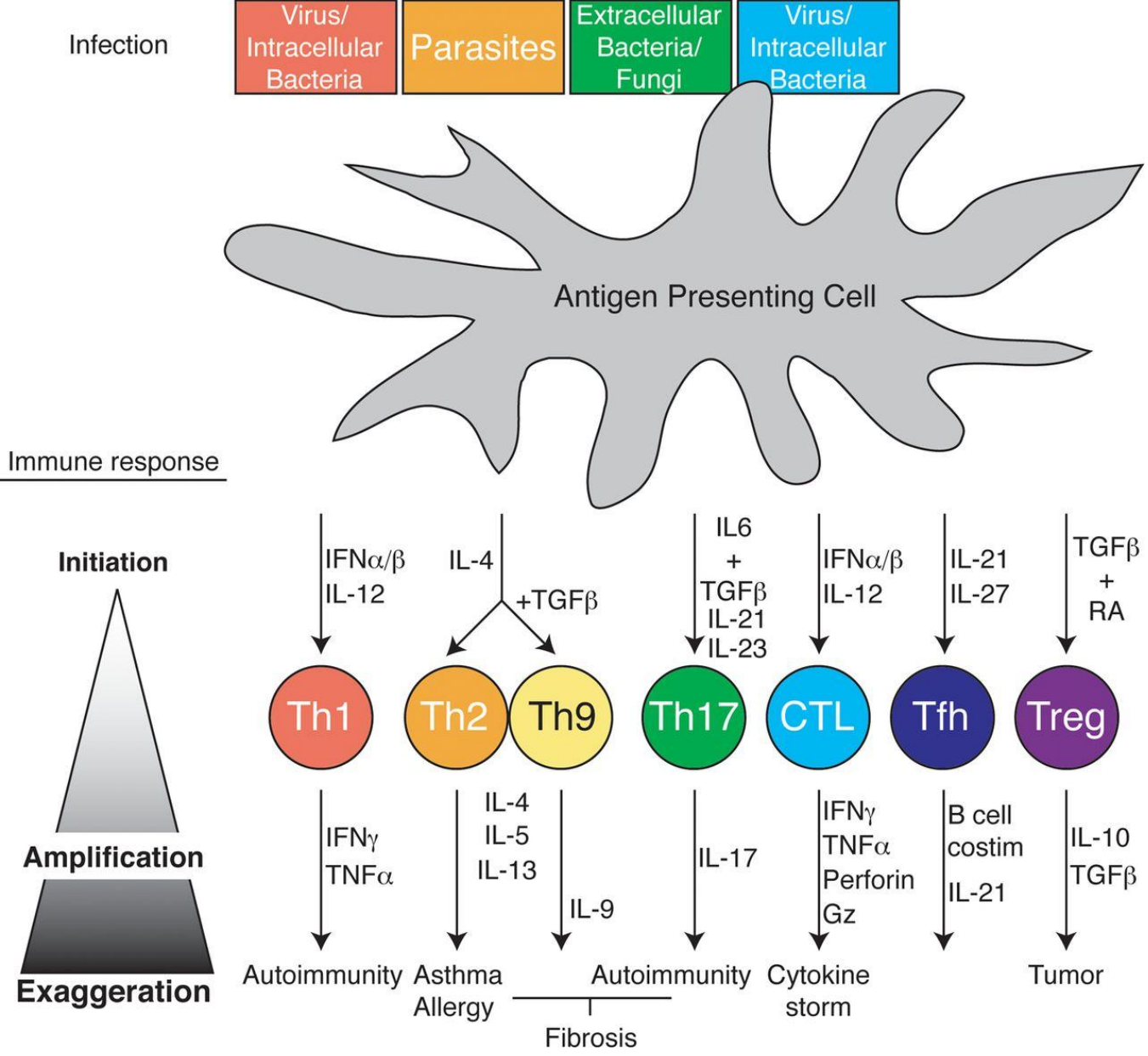
Chaîne γ commune avec IL-7, IL-9.

Si mutation => déficit combiné sévère lié au X (XSCID)

Récepteurs de la famille GM-CSF => chaîne β commune pour IL-3, IL-5, GM-CSF

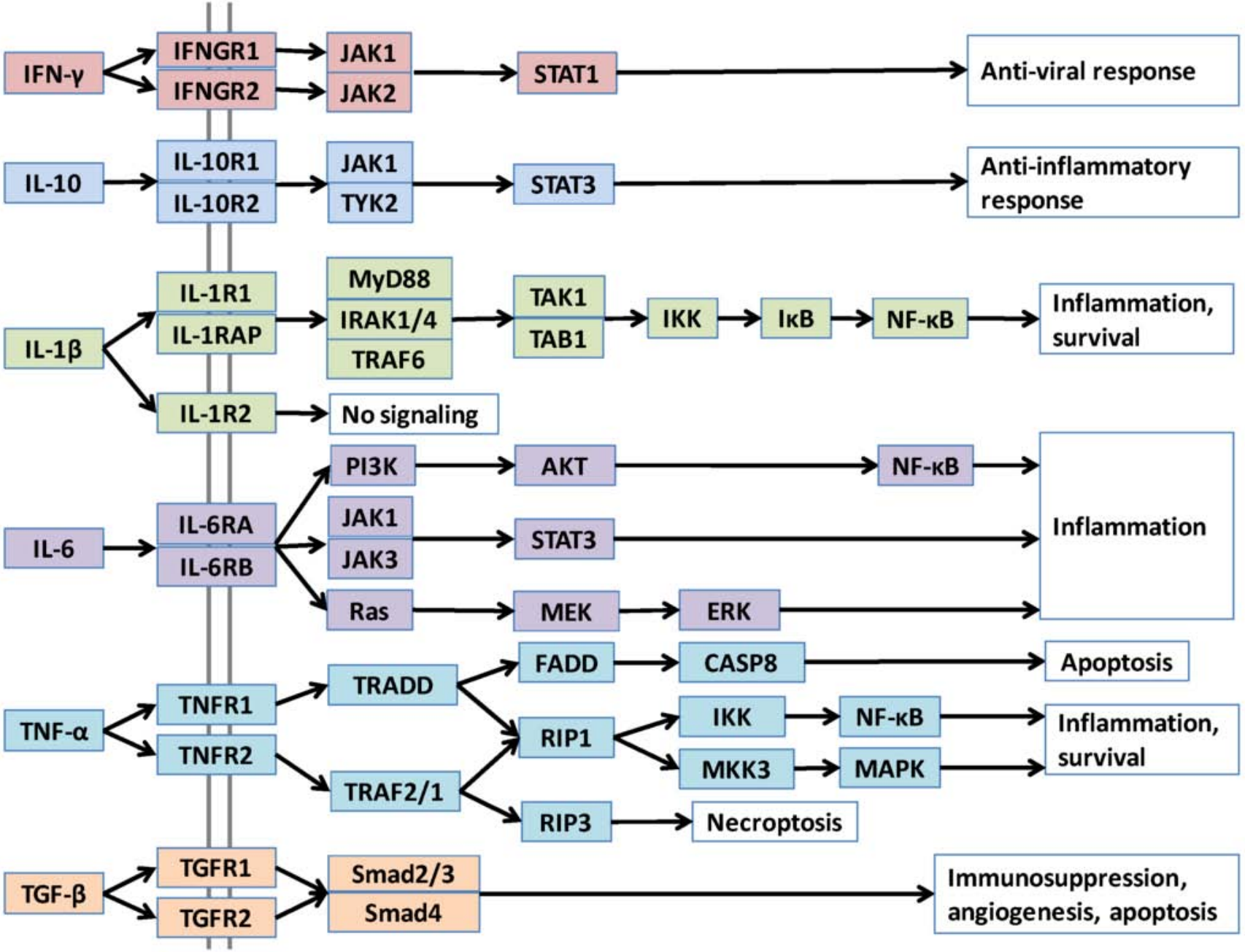
Autocrine Function of IL-2





T cell differentiation. Top: APC [i.e., dendritic cell (DC)] recognition of a spectrum of pathogens through various pathogen-associated molecular pattern receptors results in cytokine release from the APC. Along with TCR engagement, milieu cytokines initiate (top middle) differentiation to one of a variety T cell subsets programmed by transcription factors to specifically respond to the spectrum of the instigating pathogen [pathogen and T helper (Th) subset color coordinated]. Upon differentiation, T cells themselves produce cytokines, which feed back into the cellular milieu, amplifying and balancing the immune response to promote specific pathogen clearance (bottom middle) and host survival. Finally, sustained, ill-timed, or otherwise exaggerated T cell immune responses from any of the T cell subsets results in a range of immunopathologies from autoimmunity to allergy and cancer (bottom). IFN, interferon; TGF-β, transforming growth factor-β; Gz, granzyme.

Adv Physiol Educ. 2013 Dec;37(4):273-83.



Cancers 2014, 6(2), 663-683

Pathways of major cytokines, in node-and-edge representation.

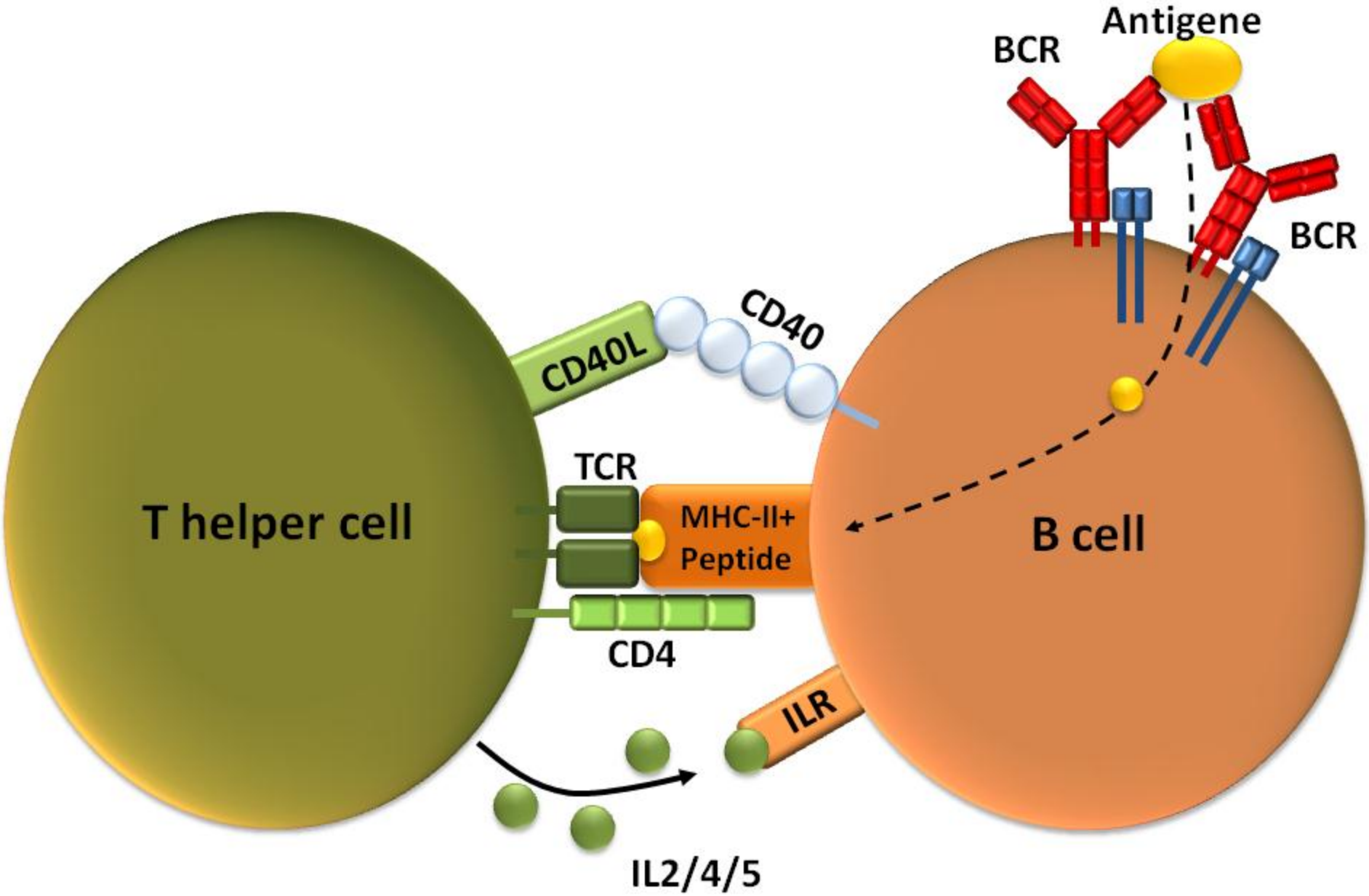
Table 1. Cytokines and their respective Janus kinase/STAT signaling pathways [1].

STATs associated with cytokine receptors	Activating cytokines	Prominent role in the mammary gland	Prominent role in breast cancer
STAT1	IFN-γ	Nulliparous gland, late involution	Potential role in tumor suppression in murine model [60]
STAT2	IFN type 1		
STAT3	IL-6, IL-11, IL-31, IL-10, IL-19, IL-20, granulocyte colony-stimulating factor, leptin, IL-21	On day of childbirth, first 6 days of involution	Activated in breast cancer and supports the development of proinflammatory tumor microenvironment
STAT4	IL-12, IL-23, IL-27		
STAT5A/5B	IL-3, IL-5, IL-2, IL-7, IL-9, IL-15, IL-21	During pregnancy after activation of STAT6	STAT5A along with the presence of STAT3 suggests more differentiated tumors and favorable prognostic characteristics STAT5B is associated with cell proliferation, survival, and oncogenic transformation [49]
STAT6	IL-4, IL-13	During adult mammary gland development, initial stage of pregnancy	STAT6-deficient mice are more resistant to tumor development from 4T1 cell lines

IFN: interferon; IL: interleukin; STAT: signal transducer and activator of transcription.

Les cellules B peuvent également former une synapse immunologique

Les cellules B peuvent se lier à des antigènes solubles à l'aide de leurs récepteurs d'antigène (BCR), ils peuvent également se lier à des antigènes liés à la surface des cellules dendritiques où ils forment une synapse immunologique semblable à celle du synapse B–T.



Interaction cellule B – Cellule Th

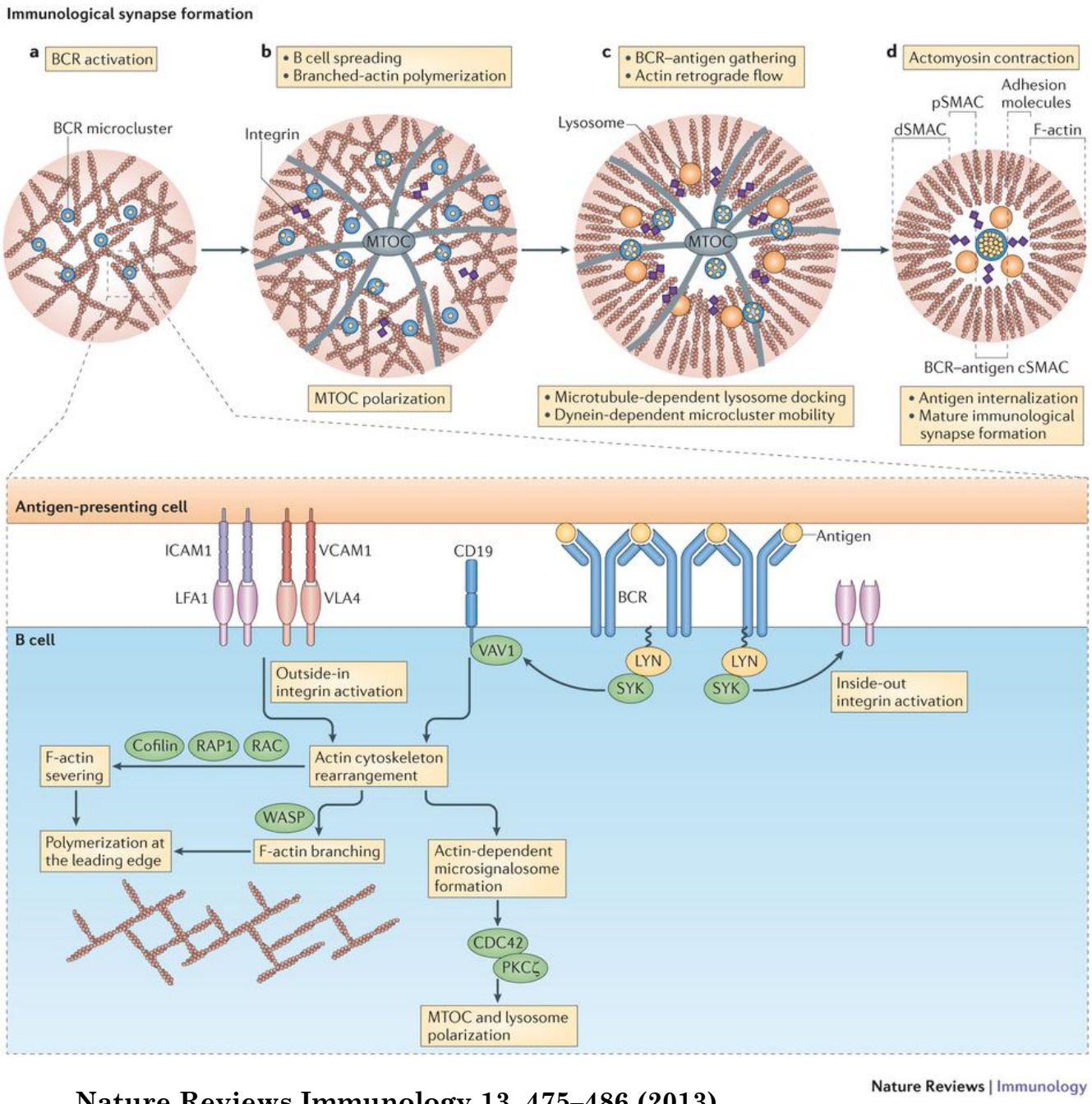


Figure 1: B cell immunological synapse formation. B cell receptor (BCR) engagement by an antigen triggers the formation of BCR oligomers, which converge to form signalling platforms called microclusters. CD19 is transiently recruited to these microclusters, leading to the activation of signalling molecules such as SYK, LYN and VAV1. The activation of the integrins lymphocyte function-associated antigen 1 (LFA1) and very late antigen 4 (VLA4) promotes B cell adhesion to the surface of the antigen-presenting cell, thereby lowering the threshold of activation (shown in inset). The downstream signalling events that these integrins initiate promote RAC-dependent actin polymerization as well as F-actin severing by the activation of RAP1 and cofilin. b | The actin cytoskeletal rearrangements are essential for the spreading and contraction response that is required for antigen gathering by B cells. Concomitantly, the microtubule network is organized towards the immunological synapse where the minus-end molecular motor dynein drives the concentration of microclusters at the synapse. c | The polarization of the microtubule-organizing centre (MTOC) is also essential for the local recruitment and secretion of MHC class II+ lysosomes that promote antigen extraction. d | Together, these dynamic events lead to the formation of a mature immunological synapse that is characterized by concentric regions: the central supramolecular activation cluster (cSMAC) in which BCRs that are engaged with antigens are concentrated, the peripheral SMAC (pSMAC), which contains adhesion molecules such as LFA1, and the distal SMAC (dSMAC) in which actin is enriched. This structure is essential for B cells to coordinate cell signalling with antigen extraction. CDC42, cell division control protein 42; ICAM1, intercellular adhesion molecule 1; PKCζ, protein kinase C ζ-type; VCAM1, vascular cell adhesion molecule 1; WASP, Wiskott–Aldrich syndrome protein.

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Intérêt de la formation de la synapse immunologique?

- Les molécules libérées par les cellules T effectrices sont des cytokines/perforines puissantes. Leur libération dans la synapse immunologique permet d'éviter leur action non spécifique contre des cellules innocentes.

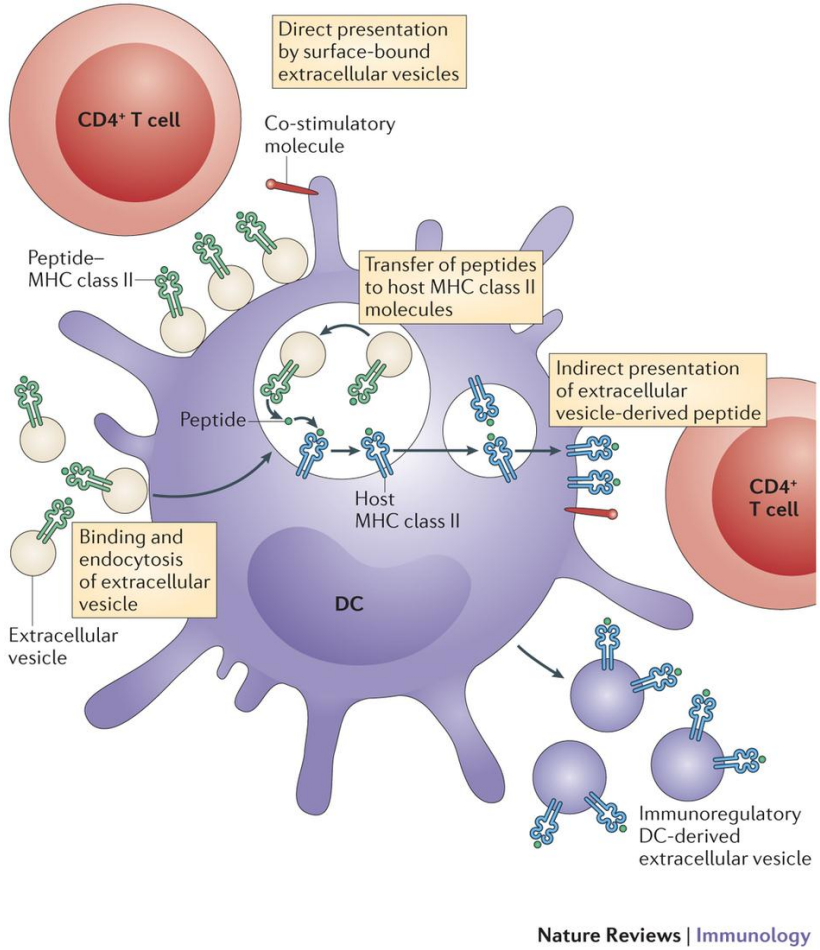
- En ce qui concerne les cellules B et les cellules dendritiques, la formation des synapses semble être un mécanisme destiné à regrouper les antigènes et le BCR dans une petite zone, ce qui augmente l'activation de la cellule B.

EXOSOMES

Les cellules présentant l'antigène comme les cellules dendritiques et les cellules B peuvent également présenter l'antigène aux lymphocytes T au moyen d'exosomes.

Les exosomes sont des vésicules membraneux minuscules libérées par la cellule. Leur surface est pourvues de complexes CMH-peptide.

La présentation de l'antigène par les exosomes peut, dans certains cas inhiber - plutôt que stimuler - une réponse immunitaire.



Extracellular vesicles, including exosomes, are small membrane vesicles derived from multivesicular bodies or from the plasma membrane. Most, if not all, cell types release extracellular vesicles, which then enter the bodily fluids. These vesicles contain a subset of proteins, lipids and nucleic acids that are derived from the parent cell. It is thought that extracellular vesicles have important roles in intercellular communication, both locally and systemically, as they transfer their contents, including proteins, lipids and RNAs, between cells. Extracellular vesicles are involved in numerous physiological processes, and vesicles from both non-immune and immune cells have important roles in immune regulation. Moreover, extracellular vesicle-based therapeutics are being developed and clinically tested for the treatment of inflammatory diseases, autoimmune disorders and cancer.

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Figure 2 | Role of extracellular vesicles in antigen presentation. Professional antigen-presenting cells (APCs) (that is, dendritic cells (DCs)) present peptide–MHC complexes that are derived from captured exosomes. Extracellular vesicles that are retained on the APC surface present their peptide–MHC complexes directly to T cells, although the co-stimulatory molecules are provided by the APC. Alternatively, internalized extracellular vesicles transfer their antigenic peptides to the MHC molecules of the host APCs. The host MHC molecules that are loaded with the exosome-derived peptide are then transported to the APC surface for presentation to T cells. The APCs also release extracellular vesicles that can regulate antigen-specific immune responses. Although only MHC class II complexes are shown, a similar process occurs for exosomal MHC class I molecules in the regulation of CD8+ T cells.

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What is the importance of the immunological synapse?

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The immunological synapse (IS) has proved to be a stimulating concept, particularly in provoking discussion on the similarity of intercellular communication controlling disparate biological processes. Recent studies have clarified some of the underlying molecular mechanisms and functions of the IS. For both T cells and natural killer (NK) cells, assembly of the IS can be described in stages with distinct cytoskeletal requirements. Functions of the IS vary with circumstance and include directing secretion and integrating positive and negative signals to determine the extent of response.

Although the immunological synapse (IS) was first described in terms of directed secretion of cytokines between T cells and antigen-presenting cells (APCs) [1,2], it was the landmark discovery of micrometer-sized segregated clusters of proteins at the T cell–APC intercellular contact [3–5] that led to the common use of the concept, and triggered an ongoing major research effort in imaging immune responses. Initial excitement stemmed from the strong correlation of specific patterns of protein clustering with T-cell proliferation. This suggested, but did not prove, a causal relationship. Observations of segregated protein clusters at contacts between targets and NK cells [6] or B cells [7] required that the concept of the IS was expanded to include other immunological effectors. Indeed, segregated clusters of proteins could form even when an effector response was not elicited, such as when ligation of inhibitory killer immunoglobulin-like receptors (KIRs) prevented NK-cell activation at a so-called inhibitory NK-cell IS ([6,8–11] and references therein). Variation in the organization of different types of IS is illustrated in Box 1 and Figure I). Most recently, a supramolecular structure resembling the IS was also observed during intercellular viral transmission [12,13]. Thus, assembly of an immune cell synapse can occur in different circumstances for a variety of functions (Table 1).

Stages in assembly of the IS

From early studies using T cells interacting with a supported lipid bilayer containing agonist peptide–MHC and intercellular cell adhesion molecule-1 (ICAM-1), T-cell receptor (TCR)–peptide–MHC interactions were first seen to accumulate in a ring surrounding a central cluster of leukocyte function-associated molecule-1 (LFA-1)–ICAM-1 interactions, creating an immature T-cell synapse, which later inverts such that a ring of integrin, the peripheral supramolecular activation cluster (p-SMAC) [3], surrounds a central cluster of TCR–peptide–MHC, the c-SMAC [3], at a mature IS [4,5]. The central accumulation of TCR–peptide–MHC is clearly dependent on actin cytoskeletal processes [14] and might be regulated in part by ERM protein phosphorylation controlling the link between membrane proteins and the cytoskeleton [15]. NK-cell cytotoxicity is controlled by a balance between activating and inhibitory signals [16–18] and uses the same cytolytic mechanisms as cytotoxic T lymphocytes (CTLs). Recent evidence that NK-cell synapses also form in stages is that, at an activating (i.e. cytolytic) NK-cell IS, CD2, f-actin, CD11a and CD11b rapidly accumulate in the peripheral ring of an NK-cell IS before recruitment of perforin towards the center of the synapse [19]. Importantly, colchicine, an inhibitor of microtubule formation, prevented accumulation of perforin but not CD2, CD11a or CD11b, at the IS.

NK cells and CTLs might use the IS differently to integrate signals. For example, polarization of the microtubule organizing center (MTOC), which occurred before accumulation of f-actin at the NK-cell IS, could also occur in some non-cytolytic NK–target conjugates [20]. Thus, an additional process after reorientation of the MTOC seems necessary for NK cell-mediated lysis. Strikingly, moderate interference of actin polymerization dramatically impaired NK-cell, but not CTL, cytotoxicity. Thus, NK cells might use cytoskeletal rearrangements as 'checkpoints' on the way to being committed to deliver a lytic hit, whereas CTLs commit to lysis more rapidly and robustly. These differences might relate to the strategy each cell uses for detection of disease: activation of CTLs is kept stringent by requiring TCR recognition of agonist peptide–MHC, whereas NK-cell cytotoxicity is regulated by a balance between activating and inhibitory signals triggered by invariant epitopes. Evidence that activating and inhibitory NK-cell signals integrate by control of cytoskeletal processes is that signaling by inhibitory KIR dephosphorylates Vav1, directly blocking actin cytoskeletal processes [21].

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Box 1. Variety in the structure of immunological synapses (ISs) for different types of immune cell interactions

Supramolecular organization of proteins has been observed in synapses involving various types of immune cells, including T cells, natural killer (NK) cells, B cells and dendritic cells. There is evidence that the underlying molecular mechanisms by which proteins are arranged at the IS involves both spontaneous processes and cytoskeleton-driven mechanisms. However, between different cell types, it has recently emerged that there are many intriguing differences in the specific organization of proteins at the IS. Thus, as is perhaps commonplace after an initial burst of research in a new area, complexities now suggest things are not quite as simple as perhaps once assumed.

In Figure I, two cells are shown to interact and below, one cell has been removed to reveal the organization of proteins at the intercellular contact for various types of immune cell interaction. In the helper T-cell IS, LFA-1 (leukocyte function-associated molecule-1) and talin initially cluster in the central supramolecular activation cluster (c-SMAC) with T-cell receptor (TCR) segregated into a peripheral ring. After some time, in the order of minutes, this arrangement inverts so that TCR clusters in the c-SMAC with LFA-1 and talin in the peripheral SMAC (p-SMAC) [3,5]. TCR clusters in small areas surrounded by LFA-1 in the early cytotoxic T-cell IS [25]. After some time, a peripheral ring of LFA-1 forms to enclose two segregated domains in the c-SMAC, one containing the TCR and the other containing the lytic granules [40,41]. Studies of rare genetic diseases have recently revealed some of the molecular determinants underlying the movement of lytic granules [42]. At the cytolytic NK-cell IS, SH2-domain-containing phosphotyrosine phosphatase-1 (SHP-1) initially clusters in small areas surrounded by LFA-1 [43]. At a later time, lytic granules cluster in the c-SMAC with LFA-1 in the p-SMAC [9,43,44]. MHC class I clustering in the target cell at the non-cytolytic NK-cell IS has several different patterns [8]. It has been speculated that this inhibitory NK-cell IS represents a stage towards assembly of a mature activating synapse from which progression has been prevented [11]. Although not depicted, γδ T cells also form an IS that is just beginning to be studied [45].

It is important to note that the data represented in Figure I derive from many researchers using a variety of particular systems and methodologies. For example, representation of the early cytotoxic T-cell IS derives from data examining the interaction of the cytotoxic T cell with a supported lipid bilayer containing intercellular adhesion molecule-1 (ICAM-1) and peptide–MHC and remains to be seen in cytotoxic T cell–target cell interactions. Also, it should be noted that the organization of proteins at an IS will probably vary with local concentrations of cytokines, chemokines and other environmental stimuli. A detailed tabulation of the location of molecules at the T-cell and NK-cell IS has been presented in Ref. [46], although it is rapidly emerging that many other proteins, such as potassium channel proteins [47] and even transcription factor signaling intermediates [48], can also be organized at the IS. As such, Figure I illustrates that the current data suggest that there are differences in the organization of types of IS but it remains a major challenge to the field to clarify the importance of these differences in different immune cell interactions.

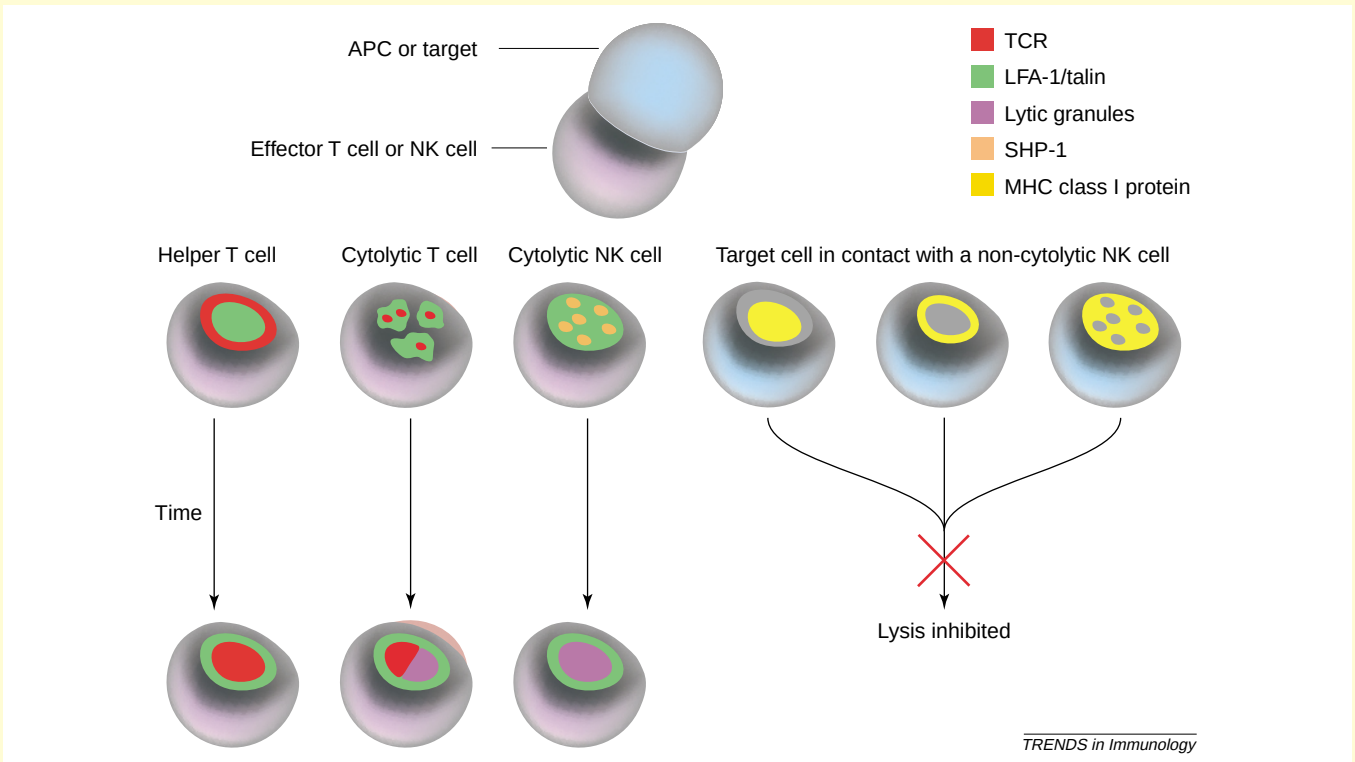

Figure I. Representation of the structure of the immunological synapse (IS) in different types of immune cell interactions. More research is needed to clarify if and how intercellular communication is influenced by such variations in protein organization at the IS. Abbreviations: APC, antigen-presenting cell; LFA-1, leukocyte function-associated molecule-1; NK, natural killer; SHP-1, SH2-domain-containing phosphotyrosine phosphatase-1; TCR, T-cell receptor.

Some of the checkpoints for a CTL might also be bypassed by activating signals associated with inflammation or stress, such as high expression of ICAM-1 or induction of NKG2D ligands. NKG2D is a costimulatory receptor for T cells and an activating receptor for NK cells [22,23] that recognizes stress-inducible MHC class I-related proteins expressed in specific membrane microdomains [24]. Recently, NKG2D, along with ICAM-1, was found to contribute to formation of an antigen-independent p-SMAC-like structure, at least at the IS formed between T cells and a supported lipid bilayer containing ICAM-1 and agonist peptide–MHC [25]. The presence of an antigen-independent p-SMAC-like structure might accelerate later stages of CTL-mediated killing

Table 1. The immunological synapse (IS) can have several functions, with varying importance for particular cell–cell interactions

Function	Comment	Refs
(I) Establishing checkpoints for lymphocyte activation	Stages in the assembly of the IS, having distinct cytoskeletal requirements, can provide a framework for establishing checkpoints for cellular activation. This might be particularly important for integrating positive and negative signals in natural killer (NK) cells in which an inhibitory NK-cell IS might represent a stage towards the assembly of an activating IS from which progression has been prevented. Spatio-temporal movements of CD45 can facilitate discrete stages in T-cell signaling.	[11,19,20,49]
(II) Enhancing signaling	By its very nature, the dense accumulation of protein at the IS can increase the rate of T-cell receptor (TCR) triggering, at least initially.	[32]
(III) Terminating signaling and/or effector function	Membrane-proximal signaling can be terminated by downregulation and degradation of TCR and also perhaps by removal of TCR ligation at the IS by intercellular transfer of peptide–MHC. Transfer of peptide–MHC from an antigen-presenting cell (APC) to a cytotoxic T lymphocyte (CTL) might also terminate effector functions by facilitating 'fratricide'. Intercellular transfer of proteins that commonly occurs at an IS might also be required for detachment of conjugated cells, although direct evidence for this is lacking.	[32,50,51]
(IV) Balancing signaling	Balancing the relative influence of enhancing (II) and terminating (III) signaling to maintain agonist-triggered signals in normal T cells. Overbalances toward terminating signaling in anergic T cells.	[32,33]
(V) Directing secretion	This was the first function proposed and there is now considerable evidence for this being a major function of the IS. Current research addresses exactly when secretion of cytokine or lytic granules necessitates a mature IS.	[41,52,53]

on detection of agonist peptide. Thus, the definitive nature of the peptide–MHC for target identification might enable CTLs to pass some checkpoints that are needed to promote specificity of NK cells.

Self-assembly of protein clusters at the IS

Specific patterns of MHC/KIR can assemble at an inhibitory NK-cell IS even in the presence of drugs that inhibit cytoskeletal or ATP-dependent processes [6,8]. Thus, supramolecular organization of some proteins can occur by mechanisms other than cytoskeletal or other ATP-dependent processes and perhaps micrometer-sized domains could be created by spontaneous segregation of receptors and ligands spanning similarly sized intercellular distances. The thermodynamics underlying this idea have been mathematically formulated by modelling the IS as consisting of apposing elastic membranes containing two differently sized receptor-ligand pairs [26,27]. It seems that the loss of entropy by segregation of proteins can be offset by the gain in energy from increased receptor–ligand interactions and minimising bending of the opposing membranes. Consistent with this idea, MHC protein was found to accumulate at an inhibitory NK-cell IS preferentially where the size of the synaptic cleft matched the size of the extracellular portions of KIR/MHC [10]. Indeed, there might be something fundamentally important about the relative size of the extracellular portions of MHC/KIR and MHC/TCR that allows ATP-independent self-assembly and exclusion of larger receptors and ligands, such as ICAM-1 or LFA-1 [28].

Functions of the IS

A mature T-cell IS forms only on recognition of agonist peptide–MHC [3,5], and direct comparison of the timing of protein phosphorylation with accumulation at the IS demonstrated that initial membrane proximal TCR signaling has largely abated before a mature IS forms [29]. Thus, a mature IS is not required to initiate T-cell activation [3,29] but appears to form as early TCR signaling is waning [29] as a consequence of initial signals. However, on ligation of T-cell NKG2D a ring of LFA-1 assembles [25], at least at the IS formed between T cells and a supported lipid bilayer, and thus supramolecular organization of some proteins might occur in the absence of agonist peptide–MHC.

For some time it has been known that T-cell cytotoxicity could be triggered by agonist peptide–MHC at a concentration too low to trigger other responses, such as IFN-γ secretion and internalization of CD3 [30]. Images of the CTL IS in the presence of low and high concentrations of agonist peptide–MHC, revealed that CTL cytotoxicity can be triggered without significant accumulation of CD2 or phosphotyrosine at the CTL–target interface [31]. Polarization of perforin and tubulin, however, was almost maximal even at a low concentration of agonist peptide–MHC. Thus, at least some aspects of a mature IS are unnecessary for cytotoxicity. A major next goal would be to uncover molecular mechanisms underlying different thresholds for the polarization of perforin and CD2.

Clustering of TCR in the central region of the IS might function to balance signaling. Recently, a Monte Carlo simulation of T-cell activation suggested that enhanced TCR signaling, caused by clustering in the IS, would be balanced by increased TCR downregulation [32]. In support, T cells lacking CD2AP are unable to downregulate TCR and are hypersensitive to antigen [32]. Also, phosphotyrosine was significantly increased in the central region of the IS formed by T cells lacking CD2AP, consistent with simulation of an IS in the absence of TCR downregulation. This collaboration of modeling, genetics and imaging allowed previously discussed concepts regarding adaptive signaling processes [33] to be integrated into the spatial framework of the IS with the conclusion that c-SMAC formation might have an important role in T-cell adaptation to different levels and strengths of antigen receptor signaling. Many biological systems display some degree of adaptive control in signaling processes, however, this might be a particularly crucial problem for the immune system given the diversity in amount and quality of antigenic structures, and

perhaps this lead to the evolution of a special structure, the c-SMAC, to assist this process. Signal-balancing properties of the synapse might dampen responses to the most abundant or high-quality peptide–MHC, allowing 'space' for other T cells to also proliferate. This might be important in allowing the immune system to establish a broad response to pathogens.

Counting of TCR-defining thresholds in the IS

The momentum of studies on the IS is fuelled by the development of innovative technology to meet the challenge of understanding the extreme sensitivity of T cells, able to detect even a single MHC–peptide complex [34]. Because T cells appear to have the ability of single molecule detection, there was clearly a need to adapt the emerging field of single molecule imaging to the study of T-cell activation. This was recently accomplished by tethering phycoerythin molecules to single MHC–peptide complexes on the surface of APCs [35]. The number of MHC–peptide complexes in the interface with a T cell could then be determined at the moment of T-cell interaction, and the consequences of this level of stimulation could be quantified through analysis of the Ca^{2+} signal. It was found that a single MHC–peptide complex triggers a transient Ca^{2+} signal and that 10 MHC–peptide interactions can trigger formation of a stable synapse. Similar ultrasensitive imaging methods will probably be required for understanding the early steps in signaling cascades, which rapidly drop below detectable levels despite the continued accumulation of downstream signaling intermediates [36]. It is not yet clear if or how the specific supramolecular structure of the IS has a role in defining thresholds at the IS, beyond the importance of clustering proteins at the intercellular contact.

There is still a lot to be learnt, just by watching

It is not the end of excitement about the IS, although the basis of the excitement has changed from the initial hype that these structures might explain T-cell activation decisions unilaterally. The picture is currently more complex in that protein clustering and localization can be observed in situations in which activation is blocked and activation can be observed in the absence of apparent supramolecular organization. However, the excitement remains because reactions at these interfaces are required for immune function and new tools to help understand the most fundamental aspects of this communication continue to move us forward rapidly. Particularly for understanding T-cell activation, we have identified the key receptors and ligands and the challenge now is to understand how they act in concert together to regulate T-cell responses. Similar challenges relate to understanding NK-cell activation, although some key receptor–ligand interactions, such as ligands for natural cytotoxicity receptors, also need to be identified.

As for so many biological processes, a lot can be learnt by simply imaging what goes where and seeing when known interactions happen. A caveat is that a deep understanding of the strengths and limitations of each imaging approach is necessary to attach reasonable interpretations to these images. New imaging methods, for example to probe lipid phases [37], will also be needed to advance our understanding of the IS. Another major technical challenge is to be able to observe molecular rearrangements facilitating intercellular communication *in vivo*. An inspiring recent observation is that nanotubular structures can allow intercellular transport of membrane vesicles [38]. About applying cutting edge microscopy to understand worm development in the mid-1970s, Nobel laureate John Sulston commented: 'Now to my amazement, I could watch the cells divide. Those Nomarski images of the worm are the most beautiful things imaginable… In one weekend I unraveled most of the postembryonic development of the ventral cord, just by watching' [39].

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T-CELL-ANTIGEN RECOGNITION AND THE IMMUNOLOGICAL SYNAPSE

Johannes B. Huppa and Mark M. Davis

Much excitement of the past five years in the area of T-cell-antigen recognition has centred around the immunological synapse — a complex cellular structure that forms at the interface of a T cell and a cell that expresses the appropriate peptide–MHC complexes. Thanks to new imaging technologies, we are now beginning to understand the role of cell-surface molecules and some of their attendant signalling modules in the context of cell-to-cell communication. Progress has been so rapid that T-cell-antigen recognition might be the first system in which the molecular basis of cell–cell recognition is understood.

T cells have a crucial role in orchestrating the body's adaptive immune response, and much effort has been put into defining the types of T cell that exist and their functions. Many of the cell-surface and cytoplasmic molecules that are involved in the various forms of T-cell recognition, activation and effector function have been identified and characterized. Although progress in defining the molecular steps that are involved in T-cell signalling and activation has been steady, it has remained less clear how the cell-surface molecules on T cells interact with each other and their ligands to mediate different stages of recognition. Neither classical biochemistry nor classical cell-biological approaches have been able to explain adequately how a T cell can scan a large number of other cells, find one with the appropriate antigen and carry out a complex activation process that can take many hours. Instead, T-cell biologists have taken advantage of new imaging technologies (BOX 1) to piece together a new approach to the problem of cell–cell recognition, one in which a biochemist's interest in the molecular underpinnings of a phenomenon can be integrated with a cell biologist's understanding of what is happening where at the whole cell level.

Much curiosity has centred around the discovery of what is now known as the immunological synapse — the area of cell contact. Schematically illustrated in FIG. 1, this synapse shows the highly complex and ordered distribution of molecules that was first observed between T helper (T_H) cells and B cells.

What is a synapse?

The word 'synapse' is derived from the Greek words meaning 'connection' or 'junction' between two similar entities (Oxford English Dictionary). It was first used to describe the junction between two chromosomes in the late 1800s and shortly afterwards was used for neuronal connections. The term immune synapse was first chosen by M. Norcross[1] to describe T-cell–antigen-presenting cell (APC) interactions and also by W. Paul and colleagues[2]. Here, we define it as any stable, flattened interface between a lymphocyte or natural killer (NK) cell and a cell that they are in the process of recognizing. Conceptually, this term puts the activation of these cells in the context of a highly organized and dynamic structure that can act as a platform for a bidirectional and cell-specific flow of information, and that might offer additional layers of modulation to a cell's response.

Here, we highlight the most recent findings in this area, and a summary of the model systems used to generate these data is given in BOX 2. Furthermore, we attempt to draw a more holistic sketch of antigen recognition by outlining examples that illustrate the interconnectivity between ligand binding, signal generation and signal integration. We conclude by discussing the possible significance of the immunological synapse for T-cell responses.

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Box 1 | Approaches to study the immunological synapse

Wide-field epi-illumination fluorescence microscopy
A common method of illumination in fluorescence microscopy, in which the illuminator is placed on the same side of the specimen as the objective lens, and the objective carries out a dual role as both a condensor and an objective.
Pros: fastest data acquisition, large field of view, wide range of excitation wavelengths
Cons: haziness of images due to out-of-focus light, image de-convolution required to achieve good image quality
Applications: live imaging of fast events such as the activation of effector T cells

Confocal laser scanning microscopy (CLSM)
A mode of light microscopy in which a focused laser beam scans the specimen in a raster and the emitted fluorescent light or reflected light signal, sensed by a photomultiplier tube, is displayed in pixels on a computer monitor. A variable pinhole aperture, located in a plane confocal with the specimen, rejects out-of-focus light and allows for optical sectioning.
Pros: excellent image quality
Cons: slower image acquisition, often limited choice of excitation wavelengths
Applications: ideal for slow or non-moving (for example, fixed) specimens

Spinning (Nipkow) disk confocal microscopy
Uses a Nipkow (spinning) disk that is impregnated with holes as a means to transfer an image onto a CHARGE COUPLED DEVICE (CCD). The scanning disk contains multiple, symmetrically placed spirals of pinhole apertures through which light is passed and split into multiple 'minibeams'. When spun, the light scans the sample in a raster pattern. Sample emission is detected to form an image on the CCD.
Pros: good image quality, fast image acquisition (video rate for smaller images), reduced photobleaching
Cons: reduced flexibility in the choice of excitation wavelengths
Applications: live-cell imaging

Two-photon and multi-photon laser scanning microscopy
Uses an infrared laser beam, the energy density of which allows the doubling or tripling of frequency at the point of beam focus in the specimen, for fluorochrome excitation. Molecules that simultaneously absorb two (or three) photons at 900 nm emit the same fluorescence as if they were excited by a single higher energy photon of 450 nm (or 300 nm). The method allows deep tissue penetration (~350 microns). Photobleaching and phototoxicity is minimized because fluorescence emission is contained within a single focal plane.
Pros: highest tissue penetration (up to 350 microns compared with 80 microns for conventional CLSM), least phototoxicity/photobleaching for most fluorophores (excitation wavelength >400 nm)
Cons: expensive, slow image acquisition
Applications: best for *in vivo* microscopy of tissues (such as lymph nodes and fetal thymic organ cultures)

Fluorescence lifetime imaging microscopy (FLIM)
FLIM takes advantage of the decrease in the lifetime of donor fluorescence when FLUORSCENCE RESONANCE ENERGY TRANSFER (FRET) occurs between a pair of fluorophores. Because only the lifetime of the donor fluorophore is measured, spectral bleed-through does not add to the background.
Pros: best way of FRET detection, especially when using communicating fluorophores with overlapping emission spectra (for example, cyan fluorescent protein, CFP and yellow fluorescent protein, YFP)
Cons: not yet commercially available

The immunological synapse takes shape

The first evidence for cross-talk between receptor-mediated signalling, cytoskeletal reorganization and directed transport of cell-surface receptors came from studies that used soluble antibodies to crosslink T-cell receptors (TCRs) and other cell-surface molecules for lymphocyte stimulation. Such treatment resulted in a phenomenon described as 'capping', in which cell-surface receptors, filamentous actin and lipids such as gangliosides congregate towards one end of the cell[3]. Immunofluorescence studies on fixed T-cell–APC conjugates by Kupfer *et al.*[4,5] indicated a marked polarization of the T cell towards the B cell, in particular the movement of the microtubule organizing centre (MTOC) from the far side of the T cell to a location underneath the synapse. Of note, this cytoskeletal rearrangement is not just a consequence of T-cell stimulation, but seems to be required for sustained TCR-mediated signalling, as the actin-depolymerizing drug cytochalasin D interferes with calcium influx — a prominent feature of T-cell activation after TCR ligation[6]. Together, these studies provided the first evidence for both the complexity of the immune synapse as well as its significance for T-cell activation, although the latter remains controversial (see later).

Through 'optical sectioning' of a T-cell–APC conjugate, Kupfer and co-workers[7] subsequently visualized these cells for the first time in three dimensions. Importantly, it was shown that key molecules such as the TCR and the adhesion integrin leukocyte function-associated antigen 1 (LFA1) were not only capped at the interface, but also were organized in distinct areas within the interface (FIG. 1a). These areas were termed supra-molecular activation complexes (SMACs) (FIG. 1b). The central region of the SMAC (cSMAC) is enriched in TCRs and one of its downstream signalling effectors, protein kinase C-θ (PKC-θ)[7]. Cell adhesion seems to predominate in a peripheral ring that surrounds the cSMAC — the pSMAC — which shows a high surface density of LFA1, as well as the cytoskeletal linker talin[7]. Large and bulky molecules such as CD43 and CD45 were localized in a region distal to the synapse outside the pSMAC — known as dSMAC.

What are the mechanisms that enable the site-specific transport of molecules to create such orderly assemblies, and what is the significance of macroscopic segregation for antigen recognition? One logical way to address these questions is to study the dynamics of synapse formation. This was greatly facilitated by new advances in live-cell-imaging technologies. Early efforts using video microscopy together with calcium imaging provided the first insight into the morphological changes that occur after the activation of T_H cells[8,9]. Another advance came with the addition of molecular tags to key cell-surface molecules that enabled the observation of the rapid clustering of intercellular adhesion molecule 1 (ICAM1) linked to green fluorescent protein (GFP) in the context of T_H-cell activation[10]. In a technical tour de force, Dustin and colleagues[11,12] exposed T_H cells to labelled MHC molecules and other molecules in artificial model membranes, and used confocal microscopy to monitor the formation of a synapse. These latter studies also illustrate the power of being able to follow a single T cell from the initiation of activation to stable synapse formation. This enables better correlations between a given stimulus and its consequences to be established.

CHARGE COUPLED DEVICE (CCD). A slab of silicon semiconductor that is divided into an array of pixels that function as photodiodes and a light-sensitive photodetector.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET). An imaging technique to analyse intermolecular distances based on the transfer of energy from a donor molecule to an acceptor molecule without the emission of a photon.

Ligand threshold for synapse formation

T cells are highly sensitive to antigen, with estimates ranging from 1–400 specific peptide–MHC complexes on a presenting cell required to activate fully at least some T cells in a population[13–15]. There are major uncertainties with these studies however: they rely on average values of peptide loading on a given MHC molecule and there is no way of measuring just how much surface area is 'surveyed' by a given T cell and how far it gets in the activation process. To illustrate the limitations of this bulk-cell population approach (as opposed to a single-cell approach), suppose you had detected full activation in a few T cells at an average of 100 peptides per APC. Not only will you have outliers in the APCs that might have 300 peptides per cell (and which might be responsible for most of the activation events when peptide is limiting), but it also matters a great deal whether the T cell surveys 1% of the APC surface or 50%.

An important recent advance has been the development of a new approach that provides more precise information about T-cell sensitivity and the nature of T-cell responsiveness to different numbers of ligands[16]. Here, the peptide extends out of the peptide-binding groove and is labelled on an amino-terminal biotin with a streptavidin–phycoerythrin conjugate. Phycoerythrin is a large (240 kDa) multimeric protein that contains ~34 chromophores[17]. Because of its brightness, it is possible to visualize single phycoerythrin molecules with a standard cooled charge coupled device (CCD) camera[18]. In this way, it is possible to count the exact number of ligands that a T cell encounters on another cell, and then monitor the consequences of that interaction with respect to the increase of intracellular calcium concentration and the behaviour of different GFP-labelled proteins. In this way, it was shown that at least some $CD4^+$ T-cell blasts can respond to even a single ligand, by stopping and fluxing calcium weakly (FIG. 2a). Two ligands in the interface produce a more sustained rise in calcium and ten or more ligands produce a maximal response and promote the formation of a stable synapse. Parallel experiments using two different cytotoxic T-cell models give a similar dose-response curve (M. Purbhoo *et al.*, unpublished observations). So, in at least four T-cell model systems, T cells can detect just one ligand, but all require about ten to increase and maintain calcium levels fully (FIG. 2b). Increased calcium levels are pivotal to T-cell activation, as this induces the nuclear localization of nuclear factor of activated T cells (NFAT) — a transcription factor that is responsible for many of the gene-expression changes associated with activated T_H cells[19].

Importantly, 25–30 peptide–MHC complexes were required at the interface to induce T cells to stop and flux calcium when CD4 was blocked (FIG. 2b). These data indicate that CD4 is intimately involved in detecting antigens at low surface densities, which prompted

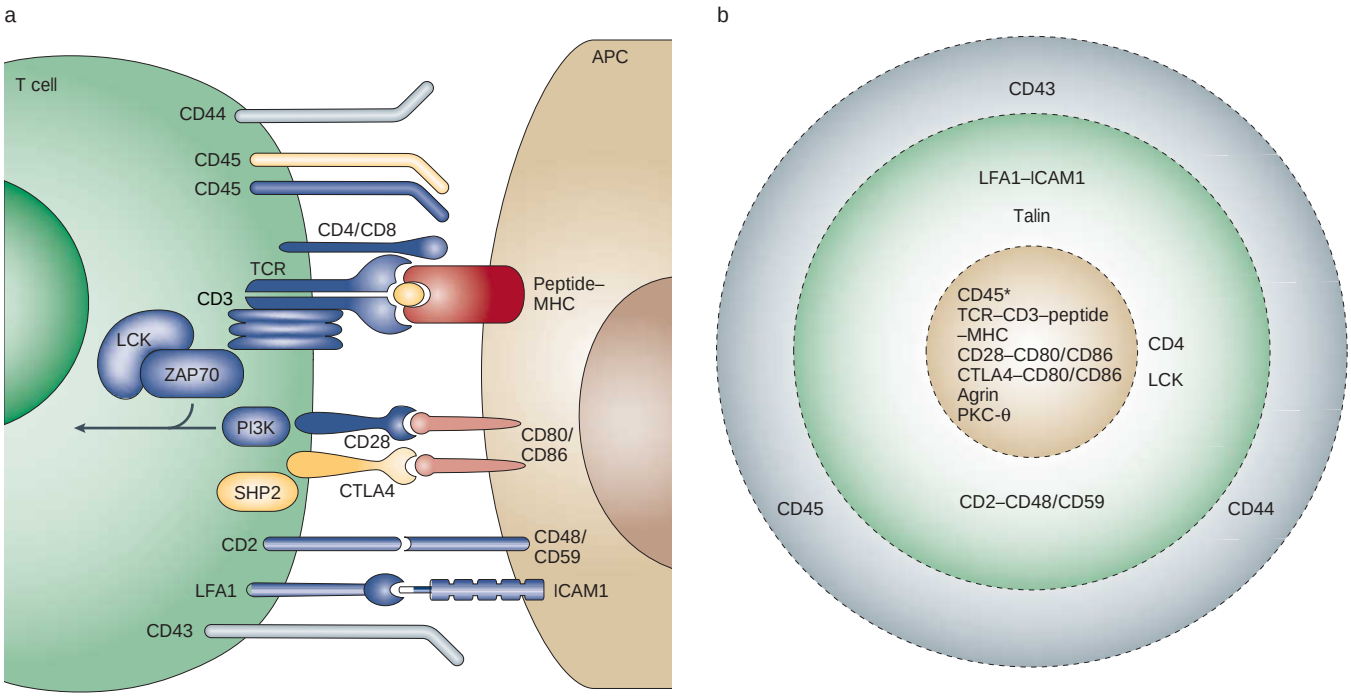

Figure 1 | **Overview of a mature T-cell synapse. a** | A profile view showing a selection of the key ligand pairs and signalling molecules that are involved in T-cell recognition. The stimulatory peptide–MHC molecule is shown in red, activating/co-stimulatory molecules are blue, inhibitory molecules are yellow and molecules that are not contributing to signalling are grey. The arrow indicates converging signals that lead to T-cell activation. **b** | The face on view of the synapse with the characteristic 'bulls-eye' zone pattern, including the central region of the supra-molecular activation complex (cSMAC) (yellow), the peripheral ring surrounding the cSMAC (pSMAC, green) and the region distal to the synapse outside the pSMAC (dSMAC, grey) as well the molecules/ligand pairs that are found enriched within. APC, antigen-presenting cell; CTLA4, cytotoxic T lymphocyte antigen 4; ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; PI3K, phosphatidylinositol 3-kinase; SHP2, SRC homology 2-domain-containing protein tyrosine phosphatase 2; TCR, T-cell receptor; ZAP70, ζ-chain-associated protein 70. *CD45 enters the cSMAC at later stages.

Box 2 | T-cell–APC model systems: pros and cons

Jurkat T cells and Raji B cells plus superantigen
Easy handling and somatic mutant lines are available for many genes, however Jurkat cells are deficient for phosphatase and tensin homologue (PTEN) and so maintain high levels of 3′ phosphatidylinositides, even, when not activated. In addition, these cells lack several adhesion and accessory molecules that are typical of normal cells, rendering them a 'non-physiological' model. Activation has to occur in the presence of superantigen as the nominal antigen is unknown.

T-cell hybridomas
Antigens are well defined, but cells often show non-physiological behaviour, possibly as a result of cell fusion.

Stable T-cell clones and T-cell lines
Antigens are well defined and most clones/lines are susceptible to either gene transfection or retroviral expression. However, there is often a marked degree of physiological variability (sensitivity, kinetics of activation, expression level of cell-surface receptors, for example).

Primary (*in vitro* stimulated) T cells
A fair approximation to the physiological scenario. With the appearance of retroviral gene transduction, ectopic expression of GFP-fusion proteins is now feasible.

***In vivo* imaging**
The best approximation of T-cell physiology. Spatial resolution of molecular distribution is reduced due to lower signal intensity.

Planar lipid bilayers and coverslip-immobilized surface proteins/antibodies
Having a structure such as an immunological synapse reduced from three to two dimensions enables high-image resolution and the use of interference-reflection microscopy (IRM) and TOTAL INTERNAL REFLECTION MICROSCOPY (TIRFM). The ability to define these lipid bilayers allows for a 'purify and reconstitute' approach, but only on the antigen-presenting cell side of the interface.

Irvine *et al.*[16] to suggest a 'pseudodimer' model, in which CD4 crosslinks two TCRs, one which binds to an agonist peptide–MHC complex and a second that binds weakly to an endogenous peptide–MHC complex, a large number of which (~20%) seem to be compatible with TCR binding and are recruited into the synapse . Alternatively, it might be that ligation of only one ligand, together with the delivery of the tyrosine kinase LCK to the site by CD4 or CD8 is sufficient to initiate a signalling cascade. Most studies on the initiation of T-cell activation by soluble peptide–MHC class II complexes, however, show a requirement for dimers or higher order multimers[20,21]. Interestingly, monomeric stimulatory MHC class I ligands seem to stimulate $CD8^+$ cytotoxic T lymphocytes (CTLs) quite efficiently, however only when these cells were made adherent in an integrin-dependent manner (to fibronection or CD18/CD11-specific-antibody-coated cover slips)[22,23] and not when left in suspension[24].

Formation of a mature synapse

Ligand recognition causes the T cell to stop migrating and to form an increasingly stable cell contact with the corresponding APC. As schematically shown in FIG. 3, this involves the reorientation of the MTOC (as well as its associated vesicles), and the recruitment of receptors and signalling molecules to the nascent immunological synapse.

Molecular recruitment: role of co-stimulation. Early thinking about how the TCR and other molecules accumulated at the synapse centred around the idea of random diffusion of membrane molecules and trapping by ligand ligation[25].

Recent work, however, has indicated that this is an active (that is, energy dependent) process in which cell-surface molecules from all over the T cell are transported to the synapse through cytoskeletal linkage and molecular motors. This was first shown through the use of beads bound to T-cell membranes[26]. Both TCR signals and CD28- or LFA1-mediated co-stimulation was required for bead movement. A similar transport phenomenon involving LIPID RAFTS was also observed[27]. More recent work could directly link this type of transport to the accumulation of MHC molecules and synapse formation[28]. So, one unexpected role of the 'second signal' in T-cell activation, is the transport of membranes, and probably a subset of their associated proteins, to the immunological synapse, and this is probably an important factor in its formation.

Lessons from artificial APCs. To account for mechanisms that might explain molecular segregation within the synapse, imaging efforts needed to be extended to visualizing the dynamics of specific proteins of known function. As mentioned earlier, one important approach to this issue has come from the use of artificial lipid bilayers that contain fluorescent-labelled T-cell ligands to activate T_H cells. T cells form ordered synapses with such membranes, and proliferate in an antigen-dependent manner as long as co-stimulation is provided for example, through ICAM1 (REF. 12). ICAM1 — the ligand of the T-cell integrin LFA1 — begins to cluster in the centre of the synapse and moves rapidly to a peripheral location. By contrast, peptide–MHC complexes are first found in a peripheral ring, but accumulate within minutes in the centre of the synapse[12]. Of note, the density of peptide–MHC complexes in these central structures was found to be proportional to the half-life of monomeric TCR binding in solution, which usually correlates with the signal strength of the interaction. FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP) experiments showed lateral immobility of these peptide–MHC complexes, indicating a continuous engagement by TCRs. A simple model for the correlation between TCR half-life and ligand density in the synapse would be that it is a function of direct binding and efficient (or inefficient) dragging into the cSMAC, but recent work has shown that a strong agonist can trigger the surprisingly robust recruitment of poor TCR ligands to the site of T-cell contact, as long as stimulatory ligands are also present[28]. This is not consistent with the model described earlier. We have, therefore, proposed a reostat model, in which a small quantity of the best ligands available determines the speed of TCR transport[28].

Does molecular size matter? Signalling events are tied to active cytoskeleton-dependent recruitment mechanisms (and vice versa), but do they alone account for the

TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY (TIRFM). TIRFM is used to observe molecule fluorescence that is restricted to surfaces and interfaces and, therefore, is increasingly used to investigate the interaction of molecules with surfaces.

LIPID RAFTS
Specialized membrane domains in the plasma membrane that are enriched in a subset of glycolipids, cholesterol and certain proteins containing either a particular transmembrane domain or which are post-translationally modified with saturated acyl chains. Their exact properties (size, existence of sub species) and function in living cells is debated.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP). The diffusion coefficient and the mobile fraction of the detected species can be determined by FRAP where a small region is irreversibly bleached once with a short, intense laser pulse and the subsequent kinetics of the fluorescence recovery in the same bleached volume is recorded.

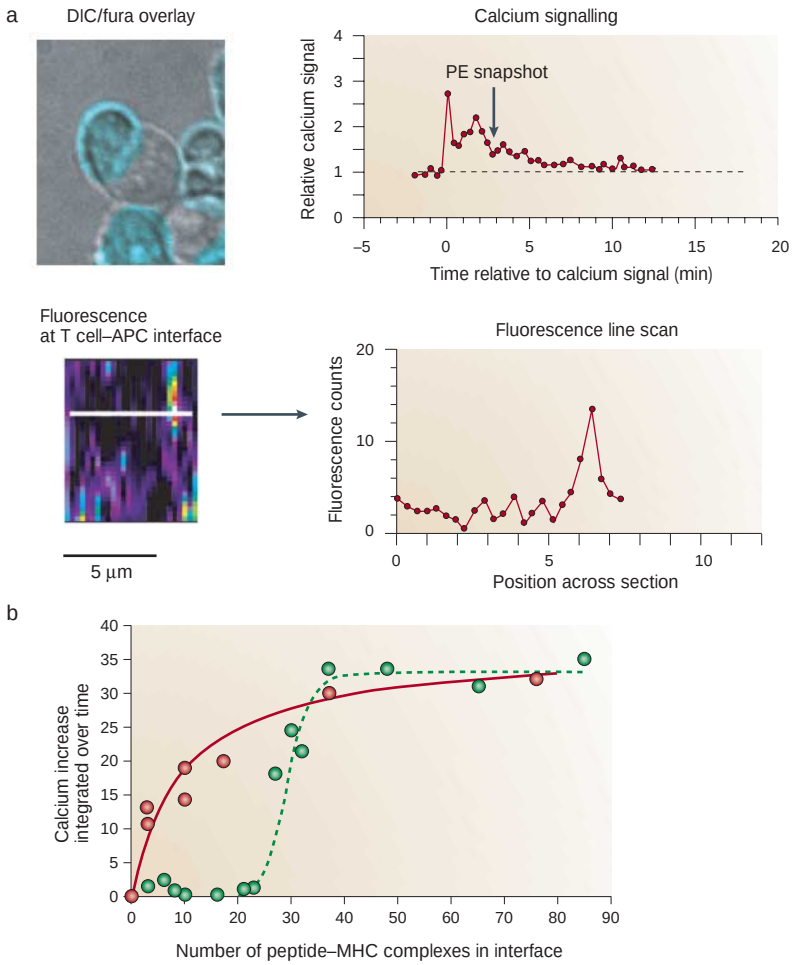

Figure 2 | **T-cell sensitivity and synapse initiation. a** | T cells respond to a single ligand (upper left panel: fura-2-loaded T cell in contact with an antigen-presenting cell (APC) presenting one stimulatory peptide; upper right panel: ratiometric measurement of the calcium signal at indicated time points). Individual stimulatory peptides bound to cell-surface MHC class II ($I\text{–}E^k$) molecules are visualized through their linkage to streptavidin–phycoerythrin (PE). The number of ligands a T cell contacts can be precisely counted (lower left panel: face on projection of the interface with a single stimulatory peptide–MHC class II molecule as determined by the PE fluorescence signal, lower right panel: line scan of the PE fluorescence counts). The calcium response in the T cell can be recorded before and after the 'PE snapshot' and correlated to the number of ligands that contact the T cell. Image reproduced with permission from REF. 16 © (2003) Macmillan Magazines Ltd. **b** | The increased calcium concentration in the T cell plateaus at about ten ligands, but only when CD4 is involved. In a dose-response curve, the total calcium increase (here, integrated over time) as induced by a given number of stimulatory peptide–MHC ligands is plotted for T-cell receptor (TCR) transgenic $CD4^+$ T-cell blasts (red curve). More than 30 stimulatory ligands are required for a full calcium response when CD4 is blocked (dotted, green curve). DIC, differential interference contrast.

macroscopic segregation of synaptic polypeptides? One parameter that might contribute to this behaviour is the size of the extracellular domains of various membrane proteins and that of their ligands. Proponents of this hypothesis argue that smaller, but stable, binding pairs might exclude larger proteins from their immediate vicinity[29,30]. Vice versa, smaller membrane proteins might be prevented from interacting with their cognate receptors when surrounded by larger molecules, especially if space is limited in the cleft of a synapse[29,30]. Adjacent ligand pairs that were different in size would impose a curvature on the membranes in which they are embedded, which is thermodynamically less favourable. Function could be generated by co-segregation of similarly sized molecules, which then act together. Indeed, increasing the length of the co-stimulatory molecule CD48, which acts together with peptide–MHC–TCR binding by interacting with the accessory molecule CD2, has an inhibitory effect on T-cell activation — a finding that is consistent with a 'size-matters' hypothesis[31].

Mathematical models have been developed to 'predict in hindsight' the peripheral location of the large ICAM1–LFA1 ligand pair (42 nm) and the central location of the much smaller peptide–MHC–TCR pair (15 nm) on the basis of large-scale self-organization[32,33]. A case for such a similar-size-driven scenario had been proposed for the cross-talk between the peptide–MHC–TCR pair and the larger CD45 polypeptide. CD45 phosphatase activity maintains T-cell-antigen sensitivity by rendering the TCR-proximal kinase LCK active, yet CD45 has also been suggested to simultaneously have an inhibitory effect on T-cell activation through direct dephosphorylation of activated CD3 subunits[30]. CD45 should be segregated from activated TCR–CD3 complexes for reasons of size — a state that would also facilitate T-cell activation. In a study using artificial bilayers for T-cell stimulation, CD45 was indeed excluded at an early stage from the central TCR zone and was found to be enriched in the most peripheral dSMAC[34,35], however, it clustered with the TCR in the pSMAC at later stages[34]. Immunolocalization studies on fixed cell conjugates showed a transient recruitment of CD45 into the pSMAC, and fluorescence resonance energy transfer (FRET) indicated a molecular association between CD45 and TCR in the cSMAC[36]. In summary, for CD45 and the TCR, a causal relation between molecular size, synapse location and function could not be established.

Similarly, CD43 — a heavily glycosylated and bulky molecule of unknown function — was found to be excluded from the synapse[37]. But CD43 interacts through members of the EZRIN–RADIXIN–MOESIN (ERM) FAMILY with the actin cytoskeleton. Deletion of the ERM region resulted in an even synaptic distribution of CD43 with no obvious effect on the segregation of smaller receptor–ligand pairs nor on T-cell activation[38–41].

Therefore, although the hypothesis that molecular size affects cross-talk within the immunological synapse and helps to localize particular molecules is an attractive one, there is not as yet any compelling evidence that it has such a role.

Agrin and MGAT5. *Agrin* and *MGAT5* (mannosyl α1,6-glycoprotein acetyl β1,6-N-glucosaminyltransferase 5) were recently identified as genes that influence the degree of molecular segregation. The secreted glycoprotein agrin — originally isolated from the neuromuscular junction in which it helps to control the clustering of the acetylcholine receptor — has also been discovered in T cells in which it influences the clustering of various cell-surface receptors[42]. Whereas a heavily glycosylated form of agrin was found to be evenly distributed over the plasma membrane of resting T cells, a less glycosylated form that is predominant

EZRIN–RADIXIN–MOESIN (ERM) FAMILY
The ERM family consists of three closely related proteins ezrin, radixin and moesin, which provide a regulated linkage between the cortical actin cytoskeleton and certain transmembrane proteins in the plasma membrane. There is increasing evidence that ERM family proteins participate in signal transduction.

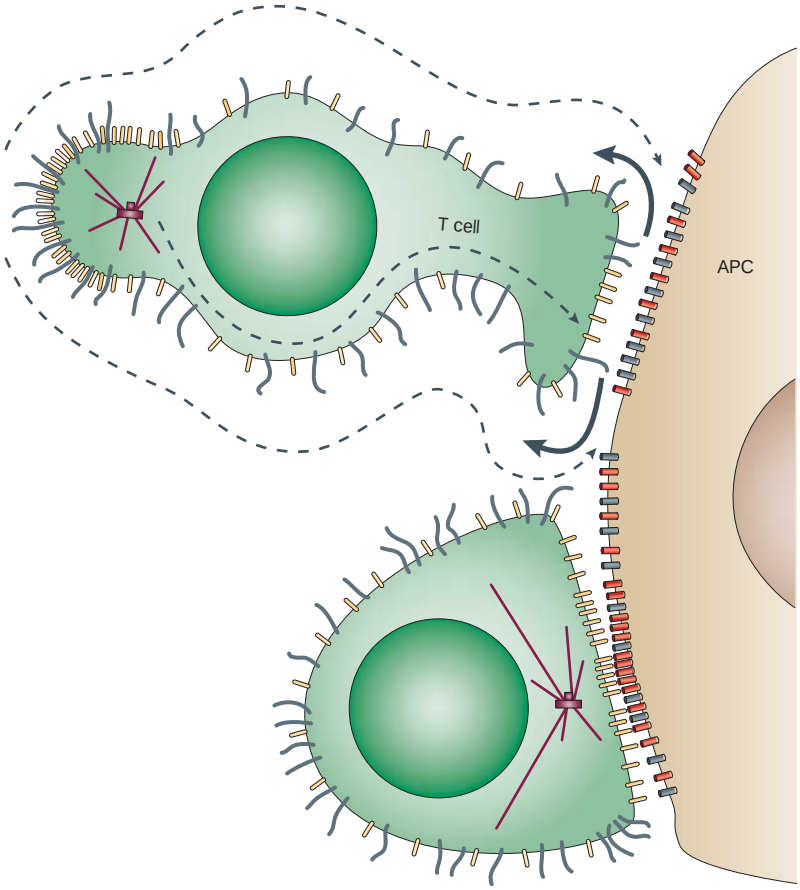

Figure 3 | **Morphological and cytoskeletal changes in the initial engagement and formation of a stable T-helper-cell synapse.** After initial engagement of the T-cell receptor (TCR) with its cognate peptide–MHC complex a T cell stops migrating and the microtubule organizing centre (MTOC) is reoriented from the uropod to beneath the immunological synapse. TCR molecules (yellow) are recruited into the synapse and other cell-surface molecules (for example, CD43) are excluded. Stimulatory (red) and non-stimulatory (grey) peptide–MHC complexes are present in the synapse as indicated.

on activated T cells co-localizes with clustered TCRs in the cSMAC. The addition of agrin purified from activated T cells in picomolar concentrations induced the polarization of TCRs, CD28 and membrane lipid rafts on resting T cells. The precise role of agrin in T-cell activation and how it mediates its effects on receptor clustering and raft aggregation remain to be identified. However, future studies involving agrin-deficient T cells will be required to determine the importance of agrin for T-cell function *in vivo*.

An important regulatory role for N-glycan synthesis in T-cell activation has recently been shown by Dennis and colleagues[43]. Deficiency in the Golgi-resident glucosaminyltransferase MGAT5 — an enzyme involved in the N-glycosylation pathway — lowers T-cell activation thresholds by directly enhancing TCR clustering. MGAT5 function is required for the biosynthesis of tetra-antennary glycan structures that end with N-acetylactosamine — a dissaccharide that binds to galectins with an affinity that is comparable to that of the peptide–MHC–TCR interaction (0.1 mM). When added exogenously, galectin-3 associates with subunits of the TCR–CD3 complex on the cell surface and also with several other cell-surface proteins. A galectin–glycoprotein network has been proposed, which might restrict the mobility of cell-surface receptors including the TCR, therefore, raising the threshold for TCR signalling. Interestingly, transcription of the *MGAT5* gene was increased 48 hours after T-cell stimulation, indicating that MGAT5 enzyme activity is limiting in resting T cells and its activation-induced upregulation could function to negatively influence T-cell sensitivity to antigen at later stages.

Signalling within the immunological synapse

As T-cell activation changes the makeup of the synapse, communication between the conjugated cells is, in many cases, likely to be dynamic and mutual, rather than static and unidirectional. As it is intimately linked to the formation, maintenance and termination of the immunological synapse, T-cell-antigen recognition must be iterative and subjected to circumstantial parameters, which include the quality and quantity of the antigen, the type and developmental stage of the T cell and APC, the microenvironment in which these cells meet and perhaps even previous 'experience' of both the T cell and APC. Translating these parameters into molecular and cellular behaviour has long been a daunting task, which is only now becoming accessible by using new imaging approaches. These include visualizing activated signalling proteins in fixed conjugates, real-time recording of the recruitment behaviour of signalling molecules at sites of receptor ligation as well as the localized production of secondary messengers.

TCR and CD4: not always acting together. A three dimensional 'optical sectioning' approach together with live-cell imaging, calcium monitoring and GFP–gene fusions showed a surprising divergence in the behaviour of the TCR–CD3 complex and CD4 (REF. 44). Whereas both TCR-associated CD3ζ–GFP and CD4–GFP accumulated rapidly after contact with an APC in dense patches in the contact area, only CD3ζ–GFP accumulated in a cSMAC at later times. By contrast, CD4–GFP redistributed to the periphery in the course of several minutes. In a study carried out using a T-cell-hybridoma model system, which measured the physical interaction between TCR and CD4 by FRET, CD4 was diffusely enriched over the entire contact area[45]. Therefore, these studies indicate a particular role for CD4 in signal initiation.

Recruitment dynamics of signalling molecules. Live-cell studies on the dynamics of TCR-proximal signalling were pioneered in readily transfectable tumour lines. Activation-induced recruitment of ζ-chain-associated protein 70 (ZAP70)–GFP from the cytoplasm to the plasma membrane was first shown in HeLa cells[46,47]. Real-time ligation of TCR has been recently related to the buildup of signalling assemblies in Jurkat T cells stimulated with plate-bound antibody[48]. Using FRAP, the authors showed that recruitment of ZAP70 to the TCR–CD3 complex is by no means static, but involves rapid cycles of binding and release.

Retroviral and lentiviral gene-transfer methods now make it feasible to image signalling proteins fused to GFP in primary T cells. Studies on both GFP-labelled[49] and antibody-stained fixed T cells show that LCK first localizes to the cSMAC and then redistributes to the periphery[36]. In T-cell blasts (but not in naive T cells), a large proportion of LCK was found in internal vesicles associated with the MTOC, which translocated to the interface within the first ten minutes after cell contact[49]. Whether this pool of LCK contributes to T-cell signalling remains to be determined.

Early peak in TCR-proximal signalling. The kinase activity of LCK and ZAP70 requires phosphorylation of specific tyrosine residues (Tyr394 of LCK, and Tyr492 and Tyr318 of ZAP70), and phospho-specific antibodies have been generated to track the location of active kinases in fixed conjugates by immunofluorescence.

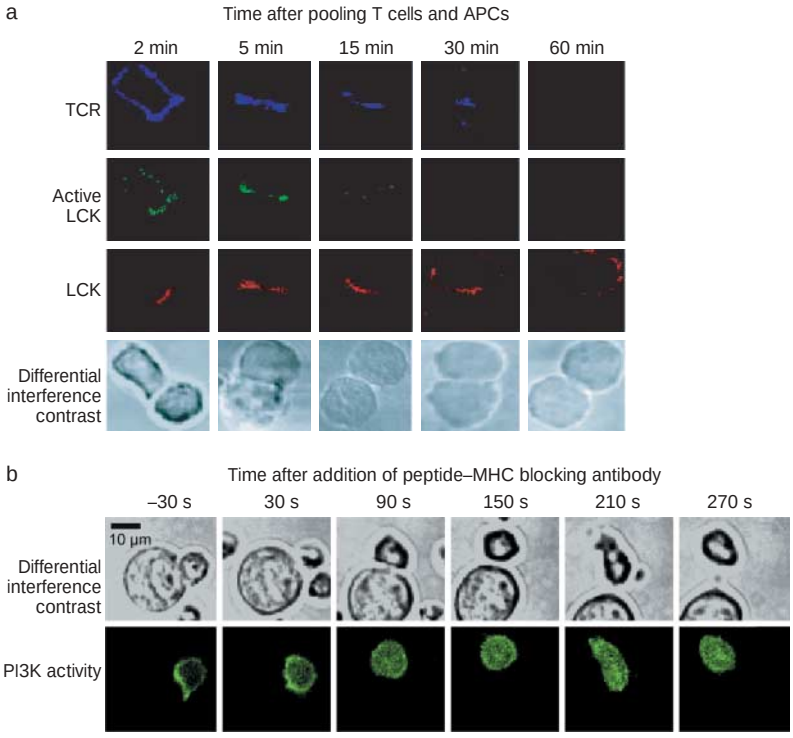

Figure 4 | **Signalling in the immunological synapse. a** | T-cell receptor (TCR) signalling precedes the formation of the immunological synapse[50]. The tyrosine kinase LCK is activated at the periphery of the immunological synapse. TCR-transgenic splenic T cells were conjugated with splenic antigen-presenting cells (APCs) loaded with antigenic peptide. At the indicated time points, cells were fixed, permeabilized and stained with antibodies specific for the TCR (blue), LCK (red) and phosphorylated active LCK (green). The position of the cells is shown in the bottom row of images by differential interference contrast (DIC). Images reproduced from REF. 50 with permission from Science © 2003. **b** | Continuous TCR signalling that is required for synapse maintenance and full effector potential[52]. DIC shows that addition of antibodies specific for antigenic peptide–MHC class II complexes interferes with the T-cell–APC conjugate leading to the dissolution of the immunological synapse and T-cell detachment from the APC. Phosphatidylinositol 3-kinase (PI3K) activity gives an indication of signalling at the immunological synapse and can be assessed by the amount of PH(AKT)–YFP (pleckstrin homology domain of AKT, also known as protein kinase B (PKB), fused to yellow fluorescence protein) that is bound to the plasma membrane. The data in the lower panel of images shows the response of a 5-hour-old T-cell–APC conjugate to antibody treatment — PI3K signalling becomes less polarized over time, indicating that the synapse has dissolved. Numbers above the images refer to the time (seconds) after antibody addition. Image reproduced with permission from REF. 52 © (2003) Macmillan Magazines Ltd.

Within 45 seconds after contact between $CD4^+$ T-cell blasts and B cells, activated ZAP70 was found in clusters that were evenly distributed over the entire interface[36]. Surprisingly, activated ZAP70 disappeared three minutes after APC contact, but reappeared seven minutes after contact. The authors propose a 'staging and resetting' model that differentiates two phases of spatial–temporal activation, one before and one after SMAC buildup within the synapse. In conjugates of naive T cells and dendritic cells (DCs), active LCK and ZAP70 predominated in peripheral areas (high in CD4 and low in TCR)[50]. Consistent with biochemical studies, Shaw and co-workers[50] detected active kinases only in the first 15 to 30 minutes after initial cell contact — that is, before the immunological synapse had acquired a mature phenotype (FIG. 4a). Clearly, TCR-proximal signalling events peak before the formation of a mature synapse and this renders models of synapse function in which its sole function is to facilitate signalling over a long period of time obsolete (see later). A recent study by Shaw, Chakraborty and co-workers[51] aims to reconcile the rapid attenuation of TCR-proximal signalling after initial cell contact with a physiological function of a 'mature' immunological synapse. Here, the authors combine classical *in vitro* experimentation (biochemistry and immunohistochemistry) on the T-cell phenotype of Cd2-associated protein (Cd2ap)-deficient mice with a computational approach. Cd2ap-deficient T cells fail to efficiently degrade activated internalized TCR–CD3 complexes, maintain TCR-proximal signalling over an extended period of time, do not form cSMACs/pSMACs within the immunological synapse and are hypersensitive to challenge with antigen. By simulating the dynamics of receptor–ligand binding, signal transduction and protein movement with a Monte-Carlo algorithm, the authors are able to account for most of the main characteristics of the Cd2ap-deficient T cells. The authors conclude that the immunological synapse acts as a servo-controller that both boosts receptor triggering and attenuates strong signals[51].

Synapse maintenance through TCR signals. T_H-cell–APC contacts are often sustained for more than ten hours. If not for the verification of antigenicity, what then might be the function of a highly ordered synapse and what are the forces that keep it in shape? Some answers came from a recent study that focused on the kinetics of the requirement of TCR-mediated signals for the activation of effector T_H cells[52]. The generation of two secondary messengers downstream of TCR ligation — the calcium signal and the induction of phosphatidylinositol 3-kinase (PI3K) activity — were recorded to boost the detection of TCR-derived signals. Despite the rapid removal of activated TCRs from the site of APC contact, $CD4^+$ effector T cells continued to produce both messengers throughout the lifetime of the synapse (FIG. 4b). These signals depended on TCR ligation at all times. Enforced termination of signalling resulted in the immediate dissipation of an ordered synaptic architecture, often leading to the breakup of the conjugates. The production of interleukin-2 (IL-2)

and T-cell proliferation — two hallmarks of T-cell activation — were markedly impaired when antigen recognition was prematurely abrogated. A similar scenario holds up for naive CD4$^+$ T cells, as shown by Schrum and Turka[53], in that prolonged, yet not necessarily uninterrupted, TCR stimulation had a cumulative effect on their proliferative capacity, resulting in up to nine cell divisions[53]. By reversibly inhibiting the activity of LCK, Valitutti and colleagues[54] could show that TCR signalling maintains the synapse structure and that T cells combine signals received through the synapse even when signalling is temporarily interrupted. In conclusion, at least in the case of T_H cells, continuous TCR signalling is required for synapse maintenance and prolonged signalling for full effector and proliferative potential.

The litmus test: in vivo imaging. The standard tissue-culture assays that have been used in the experiments described so far are only a crude approximation of the complex environment that lymphocytes are exposed to *in vivo*. To what extent is the synaptic organization that is observed *ex vivo* relevant? Are such synapses formed *in vivo*?

The first *in vivo* evidence for the immunological synapse obtained by immunohistology of fixed lymph nodes showed that the TCRs on antigen-specific naive CD4$^+$ T cells redistributed to the APC contact side only in response to antigen[55]. Antigen-specific CD43–GFP-expressing T cells in intact explanted lymph nodes were visualized, and immunological synapses between these cells and DCs, from which CD43 was excluded, were observed. This had been shown before by the same group using a standard tissue-culture system[56].

When cultured in a three-dimensional collagen matrix cell-culture system, naive T cells had only transient interactions with APCs, yet still proliferated at levels that were achieved in static cultures, indicating that a stable synapse is not the only way to activate T cells[57]. Two studies in lymph-node cultures give conflicting results with respect to this issue. In one study, T cells moved rapidly and formed few stable contacts in tissue perfused with 100% oxygen[58], whereas in another system providing atmospheric (that is, 20%) oxygen, T cells formed stable contacts[56]. Together, these studies confirm in principle the existence of the immunological synapse *in vivo*. However, the duration of such cell contacts will probably be best determined in the intact living organism. A recent study on the migration behaviour of CD4$^+$ T cells in inguinal lymph nodes of anaesthetized mice confirmed an overall high motility[59]. T cells cycled between states of low and high mobility about every two minutes with an average velocity of 11 micrometer per minute, achieving peak velocities >25 micrometer per minute. There are, without doubt, marked differences in both the makeup and the lifetime of immune synapses, reflecting the *in vivo* situation and physiological state in which they are formed.

A plethora of synapses

We have defined a synapse as any flat interface established between T cells or their precursors and APCs, in which the APCs could be as diverse as thymic stroma, so-called professional APCs or target cells in the periphery. The exchange of antigenic information as conveyed through the interaction between TCR and peptide–MHC complexes is the common denominator of all synapses. However, as the meaning of this information depends on location in an individual and cell context, it is probably not surprising that both the composition of the immune synapse as well as the duration of T-cell–APC interaction can vary markedly. And although most of the pioneering studies used model systems comprised of primary T_H cells, T-cell lines and Jurkat T cells on the one hand and B-cell lines on the other hand, the field is now moving towards more specialized scenarios, and considerable 'deviations from the rule' are becoming increasingly evident.

Effector cells: T helper cells and CTLs. T_H cells usually remain conjugated with APCs for many hours (10–24 hours) and show continuously increased levels of intracellular calcium[52,60] with ensuing changes in gene transcription that is detectable within several hours after initial APC contact[19] (FIG. 5). The secretion of translated IL-2 and other cytokines is directed towards the immune synapse where they can act most specifically on the recognized cell. T-cell proliferation begins about 24–48 hours after synapse formation and continues for several days, leading to a total of three to four cell divisions. As synapse maintenance requires continuous TCR-medated signals[52], we consider it probable that synapse termination results from their cessation. Despite an early peak in TCR-proximal tyrosine phosphorylation, which is followed by a rapid decline to undetectable levels, TCR signalling levels off completely after more than ten hours of cell contact. What attenuation mechanisms could be at work? TCR–CD3 complexes are rapidly removed from the cell surface when activated. However, they are subsequently replaced by newly synthesized receptors, and TCR cell-surface expression is fully restored after 24 hours[52]. So, although receptor internalization could have a decisive role in attenuating TCR-signalling events at an early stage, it seems less important for their complete termination at later stages when TCR cell-surface expression is restored. Negative regulation of TCR signalling through cytotoxic T lymphocyte antigen 4 (CTLA4) could provide the missing piece in this puzzle. CTLA4 competes with CD28 for binding CD80/CD86, and when bound to its ligand, it inhibits TCR-proximal signalling through the recruiment of the intracellular phosphatase SHP2 (SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase 2), which dephosphorylates activated CD3 subunits[61,62]. Although absent from naive T cells, it is found in endosomal compartments in primed T cells yet becomes increasingly enriched within the synapse of conjugated T_H cells[63], either through direct transport of intracellular CTLA4 to the plasma membrane or by reduced internalization of newly synthesized CTLA4 molecules when T cells are activated (or by both mechanisms).

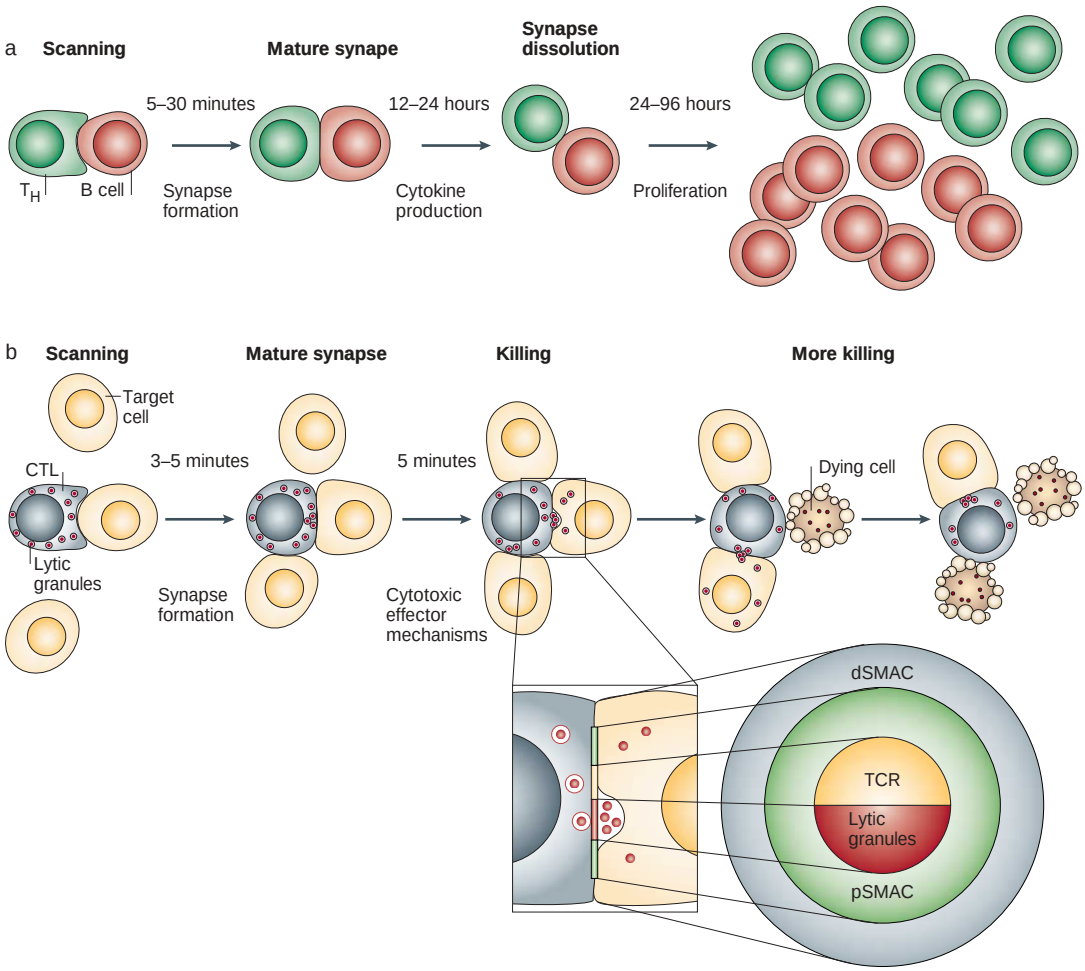

Figure 5 | **Comparison between synapse formation in CD4⁺ T helper cells and cytotoxic T lymphocytes (CTLs).** **a** | $CD4^+$ effector T cells form one mature synapse with B cells within 5–30 minutes after initial cell contact. This synapse lasts many hours during which cytokine production and release occurs and requires continuous T-cell receptor (TCR) signals for maintenance. Eventually conjugates break apart and cells undergo several divisions. **b** | CTLs form a rather transient synapse with their target cells and deliver their lethal hit within a few minutes. Lytic granules are rapidly transported to a region within the central region of the supra-molecular activation complex (cSMAC), which is devoid of TCRs. Whereas granule release takes place within minutes, target-cell blebbing occurs within 20–30 minutes, during which cells can still be loosely attached to one another.

A different scenario seems to occur for T-cell-mediated cytotoxicity, in which many of the events described earlier occur on an accelerated timescale and in which CD28-mediated co-stimulation seems to be less crucial. Whereas the contribution of the CTL compartment to the adaptive cytokine response is increasingly evident[64], the goal of the CTL–target cell synapse still seems to consist of the delivery of a lethal hit through the targeted release of cytolytic granules. These granules are rapidly focused underneath the synapse where they are in close proximity to the MTOC and then are shunted (probably on microtubule rails) through a specialized region within the cSMAC, which is devoid of TCRs[65]. CTLs flux calcium for a comparatively short period of 2–20 minutes and cell detachment can occur shortly after initial cell contact. Primed CTLs are, similar to their $T_H$cell counterparts, highly sensitive to antigen, requiring only a few stimulatory peptide–MHC complexes for killing (M. Purbhoo *et al.*, unpublished observations). Although target-cell death ensues in the order of hours, its irreversible initiation seems to require only minutes of CTL contact. In contrast to T_H cells, CTLs can form many synapses with several target cells at once[66], which causes an oscillating movement of the T cell MTOC between the target cell contact sites[67].

Thymic selection. Bousso *et al.*[68] were the first to use two-photon microscopy to monitor thymocyte behaviour in fetal organ cultures[68]. This approach supported the visualization of individual cells, however, it does not yet provide a high enough resolution to image the spatial–temporal redistribution of cell-surface molecules in the area of contact. The specialized microenvironment of the thymus can now be mimicked *ex vivo* by pooling thymic stromal cells with thymocytes under conditions that support the growth of a multicellular 'reaggregate' entity in which positive and negative selection occurs readily and on an appropriate timescale. Considerably thinner than intact thymic lobes or fetal organ cultures, this system has

finally put the microscopic imaging of synapses between thymocytes and stromal cells within reach. Using this approach, Richie *et al.*[69] showed that conjugates formed rapidly when antigenic (negatively selecting) peptide was added; however, retrovirus-introduced CD3ζ–GFP became enriched in the periphery and did not accumulate in the centre of the synapse as is the case when mature T_H cells interact with professional APCs. Although a lack of co-stimulation might in part explain this behaviour, this result illustrates the unique nature by which immature T cells process TCR-dependent stimuli. Further analysis of the contacts that occur between thymocytes and stromal cells is now being carried out and will help to elucidate the complex cell-surface biochemistry that ultimately results in negative as well as positive selection[70].

T cell–DC synapses. DCs are pivotal to adaptive immunity in that they either prime or anergize naive T cells in primary lymphatic organs, leading to either immunity or peripheral tolerance[71]. In response to various stimuli[72], DCs upregulate their ability to process extracellular antigen and to provide co-stimulation[73,74], making them the most potent APCs that are known.

Mature DCs themselves become stimulated when recognized by naive T cells that are reactive to the antigen they present[75]. In contrast to most other 'passive' APCs mentioned earlier, DCs undergo occasional fluxes in intracellular calcium and target intracellular MHC class II molecules along newly formed microtubules specifically to the synapse of the contacting antigen-specific T cell. How this cross-talk is achieved remains an open question. Signalling through ligated peptide–MHC complexes has been observed in B cells[76], and it is tempting to speculate that such a mechanism provides T-cell specificity to antigen-presenting mature DCs[77,78].

Conclusions and outlook

What is the function of the immune synapse? The initial reports of synapse structure and dynamics indicated that its function was to enhance and sustain signalling[7,11,12]. Doubts about this interpretation were first raised by van der Merwe and Davis[79], who argued that it was equally plausible that the synapse structure functioned as a conduit for effector molecules, such as cytokines or cytotoxic agents, that needed to be targeted specifically at the cell that is being recognized[79]. This view gained some support from the work of Gunzer *et al.*[57] who saw only transient interactions between naive T cells and DCs in a collagen matrix system, and yet nearly full activation and proliferation[57]. As TCR-proximal signalling is at its highest before a mature synapse with its typical 'bull's eye' appearance has formed, Shaw and co-workers[50] favour the idea that the immunological synapse is involved in TCR down-regulation and endocytosis[50], and propose, together with Chakraborty and colleagues[51], that the synapse balances TCR signalling and degradation.

In light of our more recent findings[52], we would suggest a similar, yet possibly more holistic, view in which although mature T-cell synapses are not strictly required for proliferation, they help to regulate the process[52]. We envision it as a platform that provides sufficient architectural complexity to accommodate regulatory mechanisms that are required to guide T-cell activity in accordance with its developmental stage, its range of functions, the nature of the APC involved, as well as both the quality and quantity of TCR ligands to be recognized. The fact that some synapses are long-lived, but others are of short duration, supports this notion.

A brave new imaging world. We live in exciting times in which continuous improvements in imaging technologies have led to amazing pictures of T cells at work and the beginning of a true integration of molecular and cellular dynamics following the behaviour of individual molecules. This might prove fruitful for the determination of the kinetics of receptor interactions as they occur between two cells. Sophisticated and computer-assisted electron microscopy might provide the missing link between conventional light microscopy and protein biochemistry. *In vivo* imaging could provide insights into pathogenic processes, such as the generation of autoimmunity, the inability to clear pathogen and tolerance towards cancer. And although much has been learnt in the field of T-cell-antigen recognition in the past five years through the use of imaging technologies, more can be expected from the next few years.

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T cell responses: naïve to memory and everything in between

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THE MAMMALIAN IMMUNE SYSTEM can be broadly divided into two main arms: innate and adaptive immunity. As its name implies, the cells and receptors of the innate immune system are critical for the rapid recognition of the infectious agent and initiating a proinflammatory response. While the inflammation generated by innate immune cells [neutrophils, macrophages, monocytes, natural killer (NK) cells, dendritic cells (DCs), etc.] is important in the initial containment of the infection, it also informs and directs the expansion and differentiation of adaptive immune cells. Responding to the inflammatory environment created by the innate response, cells of the adaptive arm of the immune response (B cells, αβ T cells, and γδ T cells) are stimulated to expand in number (proliferate) and to differentiate into cells with a range of functions appropriate for the immunological challenge. Upon elimination of the invading pathogen, the majority of adaptive cells die and leave behind an (evergrowing) array of memory cell subsets. These memory cells offer a diversity of migratory properties and functions, collectively mediating a rapid and protective immune response upon reinfection. Thus, the major advantages of an adaptive response to the host are twofold. First, it allows the host to form an immune response that is specifically tailored to the invading pathogen. Second, it forms a pool of memory cells from these specific effectors that can last for many years, capable of protecting the host against reinfection by their rapid response. This combination of specificity and memory are the mechanistic underpinnings for the clinical success of vaccination.

Critical to almost all functions of the adaptive immune response is the activation and programming of T cells from their naïve/resting state. Although there is much more to be learned, we now have a good basic understanding of the signals and cell types involved in the various stages of the T cell response initiated within the secondary lymphoid organs (SLOs). To provide a comprehensive overview, this review will summarize the T cell response broken down into three major stages: activation, differentiation, and memory formation. We will then assemble these components into a description of the anatomy of an immune response and its relationship to productive immune protection.

T Cell Activation

The primary mediator of T cell activation is the T cell receptor (TCR). Generated by recombination of genomic DNA sequences during T cell development in the thymus, each TCR is essentially unique and is responsible for the specificity of each T cell (26, 79). Successful recombination of a functional TCR and emergence from the thymus results in a resting, "naïve" T cell capable mainly of migrating through the secondary lymphoid tissues (lymph nodes and spleen) and peripheral circulation but as yet incapable of producing any kind of response that could protect against infectious challenge. Producing a T cell that is capable of mediating immune protection first requires "activation" of the naïve T cell. This involves coordinated interactions between a number of molecules on the T cell and an antigen-presenting cell (APC), a cell that bears an antigenic peptide derived from the infectious agent noncovalently bound to a major histocompatibility complex (MHC) class I or class II molecule (Fig. 1*A*). The TCR is composed of two chains (α and β), which recognize the peptide antigen only when it is bound in the context of an appropriate class I or class II MHC. On the T cell, the TCR associates with a complex of membrane proteins collectively known as CD3 (composed of γ-, δ-, ε-, and ζ-subunits), and it is the cytosolic region of this complex that is responsible for propagating an intracellular signal subsequent to TCR ligation. Each TCR also associates with either a CD4 or CD8 coreceptor, depending on the type of T cell. These two molecules bind to MHC (class I for CD8 and class II for CD4), further stabilizing the interaction between the T cell and APC (25).

Subsequent to recognition of the cognate peptide and MHC by a specific TCR, the T cell and APC undergo actin-mediated membrane reorganization, facilitating the grouping of these TCRs on the cell surface and the formation of the immunological synapse (1, 6). Besides the TCR, other relevant molecules (costimulatory and/or adhesion) are also recruited to the site of the TCR-MHC interaction, forming a large multimolecular structure known the supramolecular activation complex (SMAC; Fig. 1*B*). This complex consists of a focal point of signaling molecules (cSMAC) surrounded by a ring of adhesion molecules (pSMAC) (62). This arrangement promotes both prolonged and stronger intracellular interactions and the appropriate spatial ordering of all the different TCR/coreceptor/costimulatory molecules (23). The grouping of TCR/peptide/MHC within the cSMAC results in the phosphorylation of CD3 components by Src family kinases Lck and Fyn (Fig. 1*A*) (2, 13, 14, 27, 76). This phosphorylation recruits and activates ζ-chain-associated protein kinase 70 (ZAP-70) (11), which, in turn, phosphorylates linker for activation of T cells (LAT) (93), leading to the creation of the LAT signalosome, a multiprotein complex responsible for the remainder of the downstream signaling after TCR ligation (21). The culmination of TCR and concomitant costimulatory signals (as discussed below) collectively induces a transcriptional program resulting in robust IL-2 production/secretion, an autocrine and paracrine factor that stimulates T cells to proliferate.

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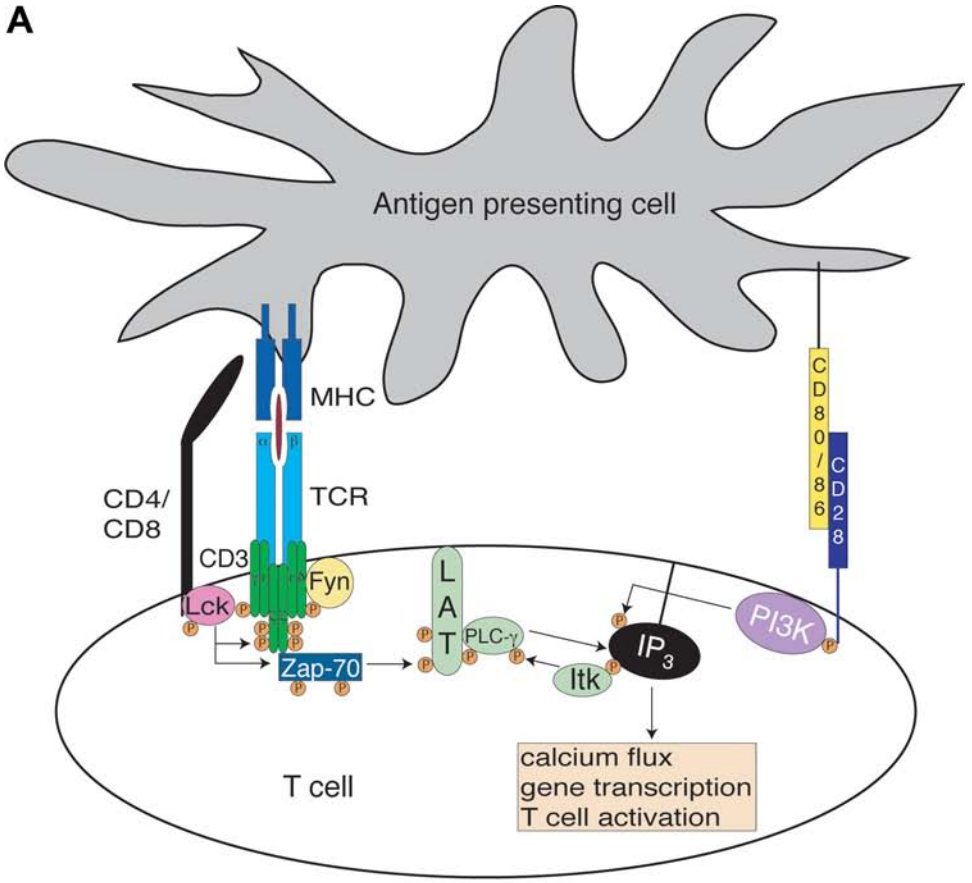

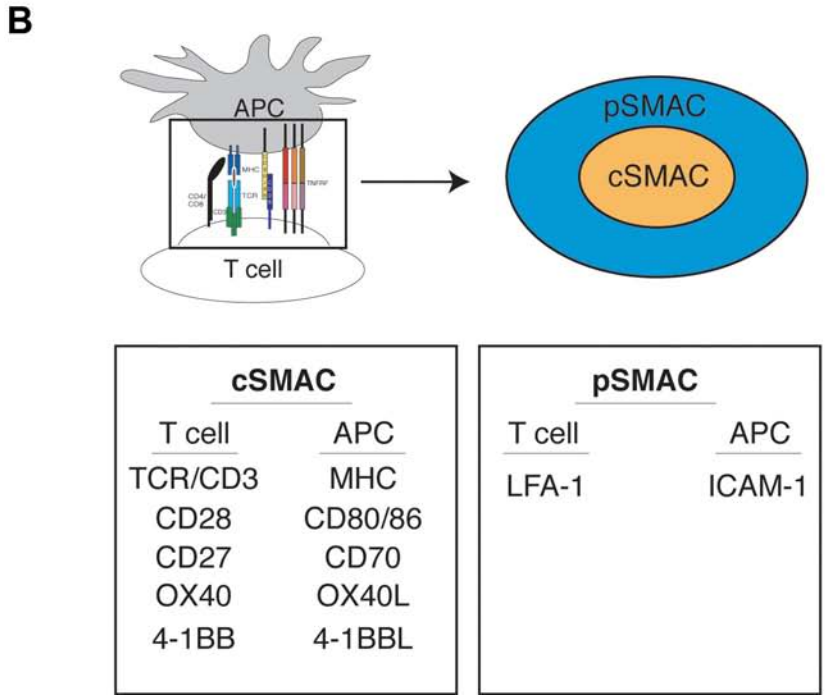

cSMAC	
T cell	APC
TCR/CD3	MHC
CD28	CD80/86
CD27	CD70
OX40	OX40L
4-1BB	4-1BBL

pSMAC	
T cell	APC
LFA-1	ICAM-1

Fig. 1. T cell activation. *A*: simplified model of early events in T cell signaling. In the absence of CD28 costimulation, canonical T cell signaling is mostly stalled at the linker for activation of T cells (LAT) signalosome stage, where phospholipase C (PLC)-γ remains unactivated. MHC, major histocompatibility complex; TCR, T cell receptor; P, phosphorylation; ZAP-70, ζ-chain-associated protein kinase 70; IP_3, inositol 1,4,5-trisphosphate; PI3K, phosphatidylinositol 3-kinase; *B*: sustained interaction between a T cell and an antigen-presenting cell (APC) results in the formation of an immunological synapse. Membrane reorganization creates a greater apposition of surface areas into which signaling and adhesion molecules are ordered into a "bulls-eye" arrangement. The relevant molecules are listed according to location. SMAC, supramolecular activation complex; L, ligand; LFA-1, lymphocyte function-associated antigen 1.

It has long been known that simply stimulating a T cell with its cognate antigen alone does not lead to activation but instead results in a T cell refractory to further stimulus (42, 81). The discovery of this hyporesponsive state, generally known as anergy, led to the hypothesis that T cell activation requires additional input to become fully activated. These data led to a search for surface receptors that might be responsible for preventing the induction of anergy. Using monoclonal antibodies to disrupt normal functioning, experimenters determined that blockade of CD28 (or its ligands CD80 and CD86 on APCs) during T cell-APC interactions resulted in an anergic T cell phenotype (31). More extensive studies have revealed a number of aspects of the intracellular signaling that are responsible for rescuing T cells from the anergic state, generally flowing through phosphatidylinositol 3-kinase and phospholipase C-γ and by the generation of a Ca^{2+} flux (3, 69, 78, 86).

The identification of CD28 as the primary costimulatory pathway for T cell activation confirmed the "two-signal" model of T cell activation. However, numerous lines of evidence have suggested that, although CD28 ligation is a necessary second signal, other membrane-bound and/or membrane-soluble inflammatory signals are necessary to achieve complete T cell activation, paving the way for "three-signal" and "four-signal" models as well (see below). Collectively, the data seem to indicate a role for inflammatory cytokine mediators in directing the differentiation of the stimulated T cell into an effector appropriate for the immunological insult being addressed (17, 50). Likewise, the data reflect a general role for members of the TNF receptor superfamily (CD27, OX-40, 41BB, and CD30) when interacting with their appropriate ligand on APCs (CD70, OX-40L, 41BBL, and CD30L, respectively) to promote the survival of proliferating cells through their differentiation process and on into memory (59, 77).

The identification of these costimulatory signals has also provided mechanistic insights as to the connection between the innate and adaptive arms of immunity. Few (and sometimes none) of the costimulatory ligands described above are found on the surface of resting, immature APCs, i.e., an APC unstimulated by microbes or by any proinflammatory mediators typically made by innate immune cells responding to infectious challenge (32). Thus, in the steady state, T cell interactions with a specific antigen on these resting APCs results in anergy and immune tolerance, a process that appears to be responsible for eliminating self-reactive T cells to antigens expressed only in the periphery and thereby preventing autoimmunity (7). However, when an APC becomes activated by sensing pathogens or inflammation through one or more cytokine and/or innate pattern recognition receptors, the various costimulatory ligands are expressed, allowing T cell activation, proliferation, and differentiation (40). Thus, the production of innate inflammatory signals and mediators is a necessary prelude to the effective transition to an adaptive response.

Finally, T cells also express an array of inhibitory receptors, helping to fine tune the eventual response of the T cell to fit the inflammatory milieu where it was stimulated. These inhibitory receptors can act to both limit costimulatory signaling as well as costimulatory molecule ligation. A good example is cytotoxic T lymphocyte antigen (CTLA)-4, an inhibitory molecule expressed on activated T cells that both produces intracellular phosphatase activity that dampens downstream signaling of TCRs and CD28 and also acts as a competing receptor for CD80 and CD86 (indeed, CTLA-4 actually has higher affinity for CD86 binding than does CD28) (53). As a result, depending on its level of cell surface expression, CTLA-4 can directly interfere with CD28 associating with CD80/CD86. A number of other inhibitory receptors have been identified (programmed cell death-1, lymphocyte activation gene 3, and V-domain Ig suppressor of T cell activation), and blockade of their function using monoclonal antibodies is being successfully exploited clinically for the purposes of augmenting immunity against various cancers (66).

T Cell Differentiation

T cell support of immune responses comes in two broad categories: generation of "helper" T cells and generation of "cytotoxic" T cells. A broad generalization segregates helper function to CD4 T cells and cytotoxic functionality to CD8 T cells. Other less prominent, although not necessarily less important, T cell subsets exist (γδ T cells and NK T cells) but will not be specifically addressed in this review. However, many of the principles of T cell differentiation and cytokine production described below can also apply to these other subsets, and other reviews have been directed toward their function and importance (8, 28). Helper CD4 T cell responses support the immune response by the robust generation of cytokines and chemokines that either activate neighboring cells to perform specific functions (cytokines) or recruit (chemokines) new immune cell subsets to sites of pathogen encounter. While CD8 T cells also are capable of a diverse array of cytokine production, their function appears to be largely focused on the elimination of pathogen-infected host cells by cytotoxic means. This is most commonly accomplished by the delivery of cytotoxic granules into the cytosol of the infected cell (recognized by TCR binding to peptide/MHC on the target cell) by a CD8 T cell. It is important to note that while these are the canonical functions of CD4 and CD8 T cells (helper cytokine production and cytotoxic activity, respectively), numerous exceptions to these rules have been documented, and, in any setting, the potential of cytokine-producing helper CD8 T cells and/or cytotoxic CD4 T cells must be considered.

In the face of the diverse spectrum of pathogens encountered by the host (viruses, bacteria, and parasites), the host produces a spectrum of specialized T cell responses uniquely suited to the invading pathogen (Fig. 2). Interactions between pathogens and pattern recognition receptors on cells of the innate immune system results in the production of various inflammatory cytokines. Naïve T cells retain their specificity by expression of their unique TCR but remain uncommitted to their helper fate until engagement of their TCR is accompanied by the integration of molecular signals downstream of their cytokine receptors. In response to the specific cytokine environment (cytokine milieu), antigen-stimulated T cells will be genetically programmed into a variety of potential subsets that possess effector mechanisms appropriate for eliminating the pathogen. Helper T cell responses are thus classified into T helper (Th) subsets, with the major ones (although not all) designated as Th1, Th2, Th17, Th9, Tfh, and Tregs. The Th1 and Th2 subsets were so named because they were the first two subsets discovered (64). In recent years, a convention has emerged of naming the T cell subset based on its cytokine production profile (Th17 and Th9) or biological significance [folicular helper (Tfh) and

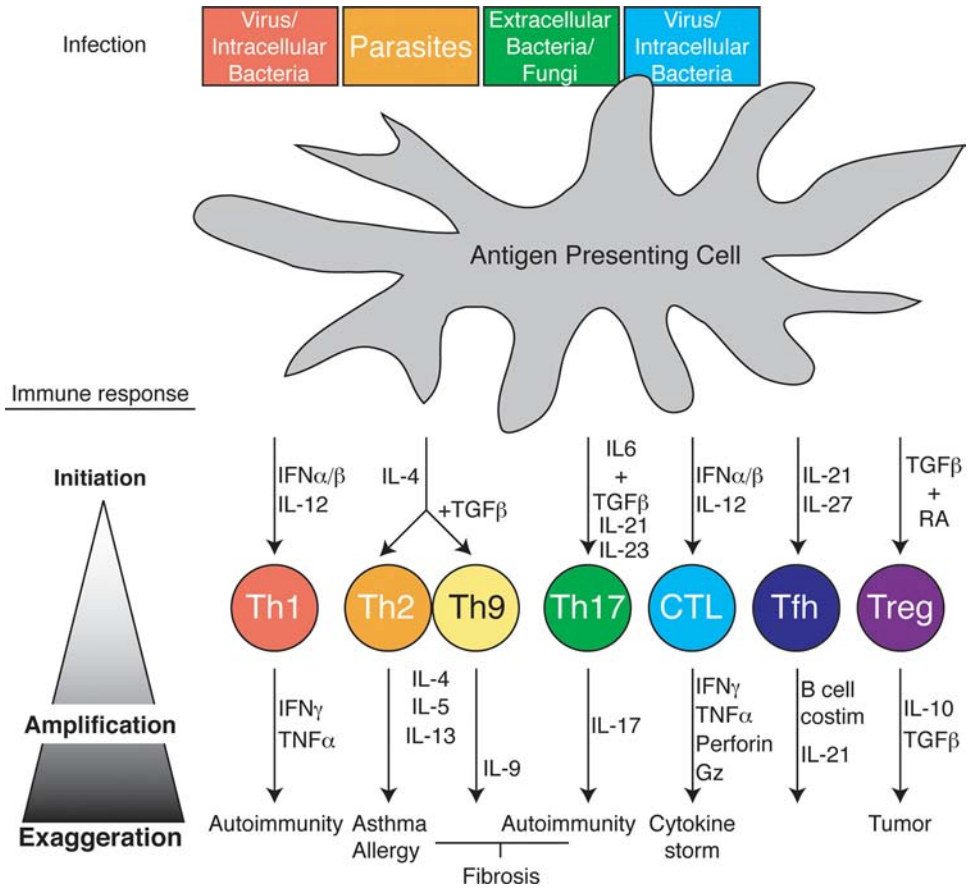

Fig. 2. T cell differentiation. *Top*: APC [i.e., dendritic cell (DC)] recognition of a spectrum of pathogens through various pathogen-associated molecular pattern receptors results in cytokine release from the APC. Along with TCR engagement, milieu cytokines initiate (*top middle*) differentiation to one of a variety T cell subsets programmed by transcription factors to specifically respond to the spectrum of the instigating pathogen [pathogen and T helper (Th) subset color coordinated]. Upon differentiation, T cells themselves produce cytokines, which feed back into the cellular milieu, amplifying and balancing the immune response to promote specific pathogen clearance (*bottom middle*) and host survival. Finally, sustained, ill-timed, or otherwise exaggerated T cell immune responses from any of the T cell subsets results in a range of immunopathologies from autoimmunity to allergy and cancer (*bottom*). IFN, interferon; TGF-β, transforming growth factor-β; Gz, granzyme.

regulatory (Treg)], which is preferred since it carries with it relevant functional, rather than historical, information about the subset in question. Figure 2 shows a visual summary for each subset described in further detail below.

The generation of interferon (IFN)-α/β and IL-12 cytokines in response to an intracellular pathogen (i.e., viruses and mycobacterium tuberculosis) stimulates responding T cells to induce the expression of the transcription factor T-bet (65). As a transcription factor, T-bet then docks in the promoter regions of genes, promoting T cells to differentiate into the Th1 subset (82). The Th1 subset is characterized by its generation of large and persistent amounts of IFN-γ and TNF-α. These cytokines then feed back into the general cytokine milieu, activating neighboring cells like macrophages to elevate their phagocytic and antigen-presenting properties. Additionally, genes turned on in neighboring cells stimulated with either type of IFN will shut down host proteins commonly hijacked by viruses, thereby restricting viral replication and quarantining the virus.

When naïve T cells are activated in the presence of IL-4, produced by a variety of innate cell types in response to parasites (84), it induces T cell differentiation into the Th2 subset (20). Th2 cells generate large amounts of IL-4, IL-5, and IL-13. These cytokines disseminate, activating neighboring eosinophils, mast cells, and basophils, which specialize in the elimination of parasites (83). Additionally, Th2-generated cytokines promote B cells to produce IgE and IgA isoforms of antigen-specific antibody, which circulate to mucosal surfaces and neutralize future threats of parasitic encounter. Interactions with other parasites that generate milieus rich in IL-4 and transforming growth factor (TGF)-β result in the generation of a similar but developmentally distinct Th subset, Th9 (18, 87). Like Th2 cells, Th9 cells thwart parasites through the production of IL-4 and IL-13 but also produce, as their name implies, IL-9 (29). IL-9 supports CD4 T cell expansion and survival but additionally has potent effects on mast cells, promoting their activation and expansion. Both Th2 and Th9 subsets achieve their unique helper characteristics through the upregulation of genes activated by the transcription factor GATA-3 (94). GATA-3, in turn, is upregulated by IL-4 driven from the innate response.

In response to extracellular bacteria and fungi (34), innate immune cells generate large amounts of both TGF-β and IL-6 (39). When naïve T cells receive these signals with additional and sustained IL-21 and IL-23 stimulation, they become "Th17" helper T cells (56) under the differential control of a transcription factor called RORγT (91). As the name suggests, this T cell subset is characterized by its ability to produce the cytokine IL-17. IL-17 potently activates neutrophils and, along with IL-8 and other chemokines generated by Th17s, strongly recruits neutrophils to the site of fungal and bacterial invasion. With their release of potent oxidative chemical species, neutrophils are able to directly kill many bacteria and fungi. The importance of Th17s is highlighted in individuals with genetic alteration resulting in diminished IL-17. These individuals suffer from recurrent, severe bacterial and fungal infections (73).

While the inflammatory environment is heavily influenced by the specific nature of the invading pathogen, not all T cell

differentiation is pathogenic specific. Some T cell differentiation occurs to support immune functions common to all infection responses. The cytokines IL-21 and IL-27 are generated in response to a variety of pathogens and serve to polarize naïve T cells, via the induction of transcription factor Bcl-6 (54), to specifically home to B cell follicles in SLOs (i.e., the spleen and tonsils). Once there, these Tfh cells express a variety of cytokines and costimulatory molecules to assist in the germinal center reaction of B cells, promoting the robust generation of high-affinity antibodies (9). As a testament to the breadth of Tfh influence, mice genetically deficient in molecules necessary for Tfh differentiation fail to produce germinal centers or high-affinity antibodies, a condition that makes them more susceptible to a broad spectrum of infectious agents (16).

Unchecked, persistent, or overexuberant immune responses carry with them the danger of immunopathology. To avert this disaster, the immune system produces Tregs, a regulatory subset of T cells that puts the brakes on a variety of inflammatory processes (51, 75). Unlike other T cell subtypes, Tregs can be produced directly from thymic selection [natural Tregs (nTregs)] as well as differentiated [induced Tregs (iTregs)] under the influence of environmental factors such as TGF-β and retinoic acid (12). In either type of Treg, suppressive activity is mediated by active expression of the transcription factor FOXP3 (22, 33). The hallmark effects of Tregs are the impairment of T cell proliferation and cytokine production from other T cell subsets despite their engagement of their antigen-specific TCR. Tregs use a diverse repertoire of mechanisms to achieve these means, such as production of the suppressive cytokines IL-10 (63), TGF-β, or IL-35. These cytokines appear to be instrumental in reestablishing immune quiescence at the elimination of the invading pathogen as well as maintaining immune tolerance to self. In the genetic absence of *FOXP3*, and thus the absence of regulatory T cells, both people and mice exhibit broad multiorgan autoimmunity (4, 10).

Given their capacity for cytolytic activity, the other major subset of T cells, CD8 T cells, specialize in the eradication of intracellular pathogens and even cancer. When CD8 T cells recognize their antigens in the presence of IFN-α/β and IL-12 cytokines, they differentiate into cytotoxic T cells. Like Th1 cells, cytotoxic T cells generate robust amounts of IFN-γ and TNF-α. In addition, these activated and polarized CD8 T cells generate large amounts of secretory vesicles that, when released in close contact to other cells, directly lyse neighboring cells. This activity is mediated through the perforin and granzyme protein families contained within the vesicles. Since cytotoxic CD8 T cells recognize antigen presented on the more ubiquitously expressed MHC class I molecule, CD8 T cells can interact with virtually every cell in the body. Cells presenting antigen in the form of peptide/MHC class I on their surface are identified by the T cell and directly lysed by interactions of their pathogen peptide/MHC with the TCR of CD8 T cells. TCR engagement directs lytic vesicles to the region of interaction, releasing the molecule into the synapse between the cells, thereby lysing the neighboring cell. In this way, CD8 T cells recognizing tumor-associated antigens can lyse cancerous cells upon a competent encounter. Like CD4 Th1 cells, cytotoxic CD8 T cells are programmed through the transcription factor T-bet, but their differentiation is also supported through the transcription factor Eomesodermin (70). It bears repeating that while cytotoxic function seems to be a common feature of CD8 T cells regardless of the cytokine milieu, the inflammatory environment can also influence their cytokine production profile in a similar fashion as CD4 T cells, producing such documented subsets referred to as Tc2s and Tc17s (35).

Finally, it is important to note that immunological disease occurs when any of these processes occur in an unyielding or overly robust manner or in the absence of a traditional immunological/pathogenic trigger (Fig. 2). Distinct roles have been elucidated for both Th1 and Th17s in multiple autoimmune conditions in humans [type 1 diabetes (47) and multiple sclerosis (48, 58)]. Additionally, robust Th2/Th9 responses have been clearly linked to asthma (52, 55) and allergy (60). Unresolved Th2 and Th17 inflammation results in tissue fibrosis and loss of functional organ architecture (i.e., pulmonary fibrosis) (73, 89). Cytotoxic and Th1 responses have lethal consequences when the natural host response or therapeutic intervention goes too far and elicits a massive bolus of inflammatory cytokines (i.e., cytokine storm), sending the host into pyrogenic shock. Highlighting the exquisite balance of the immune system, too much regulatory T cell activity is also detrimental to the host as it impairs host tumor immune surveillance, permitting the persistence of oncogenic cells (57, 95). Thus, appropriate immune homeostasis for the host depends on the coordinated temporal regulation of immune activation and immune suppression.

T Cell Memory

As discussed above, T cells have an amazing capacity to proliferate and adopt functional roles aimed at clearing a host of an infectious agent. Just as remarkably, although less understood, is the drastic decline in the T cell population once the primary response is over and the infection is terminated. What remains afterward is a population of T cells with a "memory" for the pathogen they had just taken part in controlling. These remaining T cells, after the collapse of the primary response, are altered in their functional abilities. Compared with their naïve counterparts, these memory T cells have less stringent requirements for subsequent activation via antigenic and costimulatory receptors, an increased proliferative potential, and a more rapid effector response. In addition, memory cells can traffic through both SLOs and peripheral tissues, giving them access to tissues poorly accessed by naïve (peripheral tissues) or effector (SLO) T cells. Collectively, these functions produce an in situ response to reinfection in a fraction of the time taken by the primary response.

A T cell response typically peaks ~7–15 days after initial antigen stimulation. For a productive response, this peak corresponds roughly to the eradication of the pathogen. Over the next few days, 90–95% of antigen-specific T cells then die off, leaving behind a pool of memory cells with a range of phenotypes and functionalities. For both CD4 and CD8 T cells, there are two main subclasses of memory cells: central-memory (T_{CM}) and effector-memory (T_{EM}) T cells. T_{CM} cells are commonly defined phenotypically as expressing high levels of the IL-7 receptor (CD127), high levels of adhesion markers like CD44 and CD62L, low levels of the surface marker killer cell lectin-like receptor subfamily G member 1 (KLRG-1), and high levels of the chemokine/homing receptor C-C chemokine receptor type 7 (CCR7). Furthermore, T_{CM} cells are functionally characterized by their increased potential for proliferation

after antigen reencounter. T_{EM} cells phenotypically contrast with T_{CM} cells in that they generally express low levels of CD62L, low levels of CD127, high levels of KLRG-1, and deficiency in CCR7. As their name implies, T_{EM} cells display rapid effector function (granzyme B and IFN-γ production) but a limited proliferative potential. The high expression of CD62L and CCR7 by T_{CM} cells allow for preferential homing to SLOs (which constitutively produce the CCR7 ligands CCL19 and CCL21), where they are well situated to protect from a systemic infection and seed the peripheral tissues with new effector cells after stimulation. In contrast, their lack of CCR7 and CD62L expression results in preferential T_{EM} cell trafficking through nonlymphoid tissues. This trafficking pattern, in conjunction with their increased cytolytic capacity, marks them as "first responders" at the peripheral site where reinfection could occur. Taken together, these phenotypic and functional characteristics favor a model where T_{EM} cells control the initial exposure to a pathogen at the site of infection, affording T_{CM} cells the time required to proliferate and create a new round of effectors, ultimately promoting the final elimination of the pathogen. That said, it is safe to say that T_{CM} and T_{EM} cells occupy opposite ends of an everdiversifying spectrum of T cell memory subsets. Many more surface and functional markers than those described above have been identified, painting a much more nuanced view of memory T cell subsets than the simplified T_{CM}/T_{EM} dichotomy described above. For example, the cell surface marker KLRG-1 highly correlates with effector and T_{EM} cell types, yet a specific function for this molecule has yet to be defined. Furthermore, populations of high-KLRG1, high-CD127 cells can be found, the function of which may be more related to T_{CM} than T_{EM} cells by virtue of their responsiveness to IL-7. Thus, the very act of specifically naming individual T cell memory subsets to some extent ignores the plasticity that T cells have for interchanging or blending functionalities and developmental fates. For a review of this concept and the evidence to support it, see Ref. 41. Figure 3 shows a simplified model of T cell fates dependent on the environmental cues it receives as well as cell-intrinsic factors.

Once formed, subsets of memory cells can survive for decades [the half-life of memory T cells is ~8–15 yr (30)], providing protection for the better part of a lifetime. That said, different T cell subsets have different life expectancies, and the cell fate decisions between these subsets are heavily influenced and guided by the inflammatory environment of the T cell (Fig. 3). This can be (very) roughly broken down into variations in the duration and magnitude of antigen and inflammation. It is ironic that insufficient and overexuberant antigen exposure both result in compromised immune memory, for reasons of under- and overdifferentiation, respectively (Fig. 3). Multiphoton microscopy has allowed for the characterization of APC-T cell contact, showing that there is a minimum interaction time for proper activation of T cells (36, 61). Similarly, *Listeria monocytogenes*-infected mice given antibiotics to abruptly end an infection demonstrated decreases in both antigen exposure and inflammation. This decrease causes defective CD4 T cell expansion and, in the CD8 T cell compartment, appropriately expanded primary cells that fail to proliferate to a secondary challenge (43, 90). At the opposite end of the spectrum, continual stimulation of T cells (such as in chronic viral infection) can lead to "clonal exhaustion" where the unrelenting antigen stimulation overdifferentiates all the viral-specific T cells into effectors.

With regard to inflammation, IL-2, IL-12, and IFN-α/β can increase the differentiation of cells to effectors. In CD8 T cells, the effector molecules granzyme B, perforin, and IFN-γ are all upregulated by IL-2, increasing cytolytic capacity (85). However, at high concentrations of IL-2, the increased push toward an effector state comes at the expense of memory T cell development. The opposite case emerges in common γ-chain (a shared subunit of IL-2, IL-7, IL-15, and IL-21) and IL-2 receptor-α deficiencies, where phenotypically memory cells are promoted at the expense of the primary effector response (5, 67, 68). However, these memory T cells have a defective secondary proliferative capacity. Another common γ-chain cytokine, IL-21, now appears to be a natural counterweight to IL-2 and inflammatory signals. IL-21 is produced primarily by

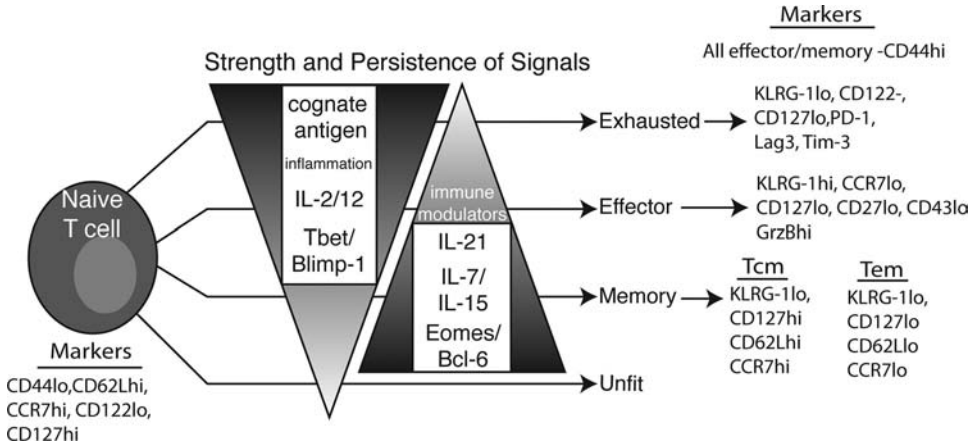

Fig. 3. T cell memory. T cells may assume many phenotypes in response to stimulation. The eventual fate of a T cell depends on many environmental queues, including, but not limited to, cytokines, inflammatory and immune-modulatory products, and tissue-specific factors. These signals, in turn, influence the transcriptional profile of the T cell, leading to developmental choices. Generally, IL-7/15/21 and Eomes/Bcl-6 are considered to tip the scale toward memory. In contrast, IL-2/12, inflammatory products, and T-bet/Blimp-1 weigh toward terminal differentiation and effectors. Exhaustion can result when T cells experience these factors too intensely or for too long. A proper balancing of all these factors will lead to long-lived, protective memory. Markers commonly used to identify cells within a particular functional grouping are given on the *right*. KLRG-1, killer cell lectin-like receptor subfamily G member 1; PD-1, programmed cell death-1; Lag3, lymphocyte activation gene 3; Tim-3, T cell Ig mucin-3.

activated CD4 T cells and acts on CD8 T cells to increase proliferative potential upon secondary stimulation and survival, likely due to its ability to suppress terminal differentiation. Evidence for this is that IL-21 receptor-$\alpha^{-/-}$ mice are unable to control chronic viral infection and have reduced long-term memory, and their responding cells exhibit an exhausted phenotype (19, 24, 92). Furthermore, a recent report (49) has identified primary immunodeficiency patients with defective IL-21 alleles who fail to mount productive T cell responses. Taken together, these findings suggest a balancing of inflammatory and immune-modulating signals, where both are required for proper effector and memory functions, but overcommitment to either can lead to dysfunctional T cells.

It is important to note that there is a certain intrinsic ability of effector T cells to form a memory population. CD8 T cells treated with a high concentration of IL-2 before transfer into a congenic host have an increased effector phenotype and poor maintenance compared with low-concentration IL-2-treated cells yet will form a functional memory population (72). Furthermore, some intrinsic ability to form memory combined with heterogeneity of IL-2 receptor-α expression of naïve T cells could allow for memory generation to always occur regardless of inflammatory and IL-2 levels.

As with the different Th subsets, the control exerted over T cells by antigen, cytokines, and the inflammatory milieu are due to their influence on many of the transcription factors described above (Fig. 3). The T-box transcription factors T-bet and Eomes are critical regulators for all T cells, promoting effector and memory development, respectively (46). T-bet is upregulated by IL-2 and IL-12 and induces the IL-12 receptor, making it necessary for effector development (80, 82). Overexpression of T-bet is associated with terminal differentiation and a reduction in memory formation (43). Eomes, while also induced by signals similar to those that induce T-bet expression, is more associated with the development of memory (70). However, both T-box factors are required for memory and for proper cytotoxic activity of CD8 T cells. Thus far, the most likely model to emerge is that they both transcriptionally regulate overlapping and nonoverlapping gene sets. These two factors are also capable of antagonizing a subset of each other's genes. Therefore, it is likely that the ratio of T-bet and Eomes expression dictates the effector-memory balance.

One of the more interesting developments in T cell memory differentiation is the relatively recent discovery of the importance of the transcription factors Blimp-1 (PR domain zinc finger protein 1) and Bcl-6 (15). These factors were originally described in B cells, where they are critical and opposing regulators of plasma cell differentiation (Blimp-1) and memory formation (Bcl-6). In recent years, however, they have been found to be central mediators of analogous fates in T cells. Blimp-1 is primarily needed for the proper induction of effector functions (45, 74), including migration and cytotoxic activity, whereas Bcl-6 is required specifically for T_{CM} cells (37, 38). Each of these factors have been noted to directly repress the transcription of the other, indicating that the sum of the signals that a T cell receives will allow an increase of one factor and repression of the other. This balance would allow the T cell to then assume the fate ascribed by that factor. Collectively, an (over)simplified model of T cell differentiation emerges, with IL-2, IL-12, T-bet, and Blimp-1 driving the production of effector cells during the early phases of the immune response and IL-21, Eomes, and Bcl-6 favoring the development of longer-lived memory cells. An overabundance of signals in either direction could negatively affect the T cell population, leading to exhausted effectors or dysfunctional memory.

T Cell Response Overview

We can now assemble the stages of T cell activation described above into a larger, "30,000-ft" overview of the T cell response to a pathogen. Pathogen invasion is followed by the replication of the pathogen accompanied by tissue damage. The combination of these two components serve to activate numerous pathogen- and damage-associated molecular pattern receptors present in the local tissue as well as tissue-localized innate immune cells such as macrophages and DCs. The resulting production of inflammatory chemokines and cytokines serves to draw in a host of innate cells (macrophages and neutrophils), which can provide some local support for the immediate containment of the infection. This inflammatory process further induces the activation of local APCs, such as DCs, to not only take up cellular/pathogen debris but to also increase their expression of the chemokine receptor CCR7, which induces their migration into the T cell zones of the local SLO (Fig. 4*A*).

During the course of migration, antigen acquired within the inflamed tissue is processed and presented in both class I and class II MHCs on the cell surface for presentation to T cells. In conjunction with antigen processing and presentation, the DC further matures in its expression of the various costimulatory surface molecules and cytokines described above (Fig. 4*B*). The combination of increased surface antigen/MHC and costimulatory molecules facilitates the effective stimulation of antigen-specific CD4 and CD8 T cells, whose presence has increased due to local inflammation increasing T cell trafficking in, and restricting trafficking out, of the SLO. The resulting stimulation of antigen-specific CD4 T cells results in their migration to the boundary between the T cell zones and the B cell follicle (88). There they have the opportunity to interact with antigen-specific B cells that have responded to pathogen antigens that have either been dragged in by migrating DCs or have drained through the lymphatics. Effective communication between the CD4 T cell and B cell results in their migration into, and formation of, a secondary follicle, where B cells undergo somatic hypermutation and class switch recombination to form higher-affinity pathogen-specific antibodies. A subset of B cells also differentiate into antibody-secreting cells and migrate into the bone marrow and other niches (96), where they begin to produce antibody targeting the infectious agent.

Concomitantly, antigen-specific CD4 and CD8 T cells clonally expand and rapidly begin migrating out of the SLO via the efferent lymphatics, accessing the circulation through the thoracic duct (Fig. 4*C*). As a result of their activation, they now express the appropriate adhesion molecules and chemokine receptors that allow them to migrate through the inflamed vasculature and enter the infection site. CD4 T cells encounter pathogen-specific antigen presented on local class II APCs (DCs, macrophages, and monocytes) and produce their effector cytokines appropriate for the infection. Tissue cells infected with intracellular pathogens present pathogen-specific antigens in their class I MHC to CD8 T cells, which lyse the infected

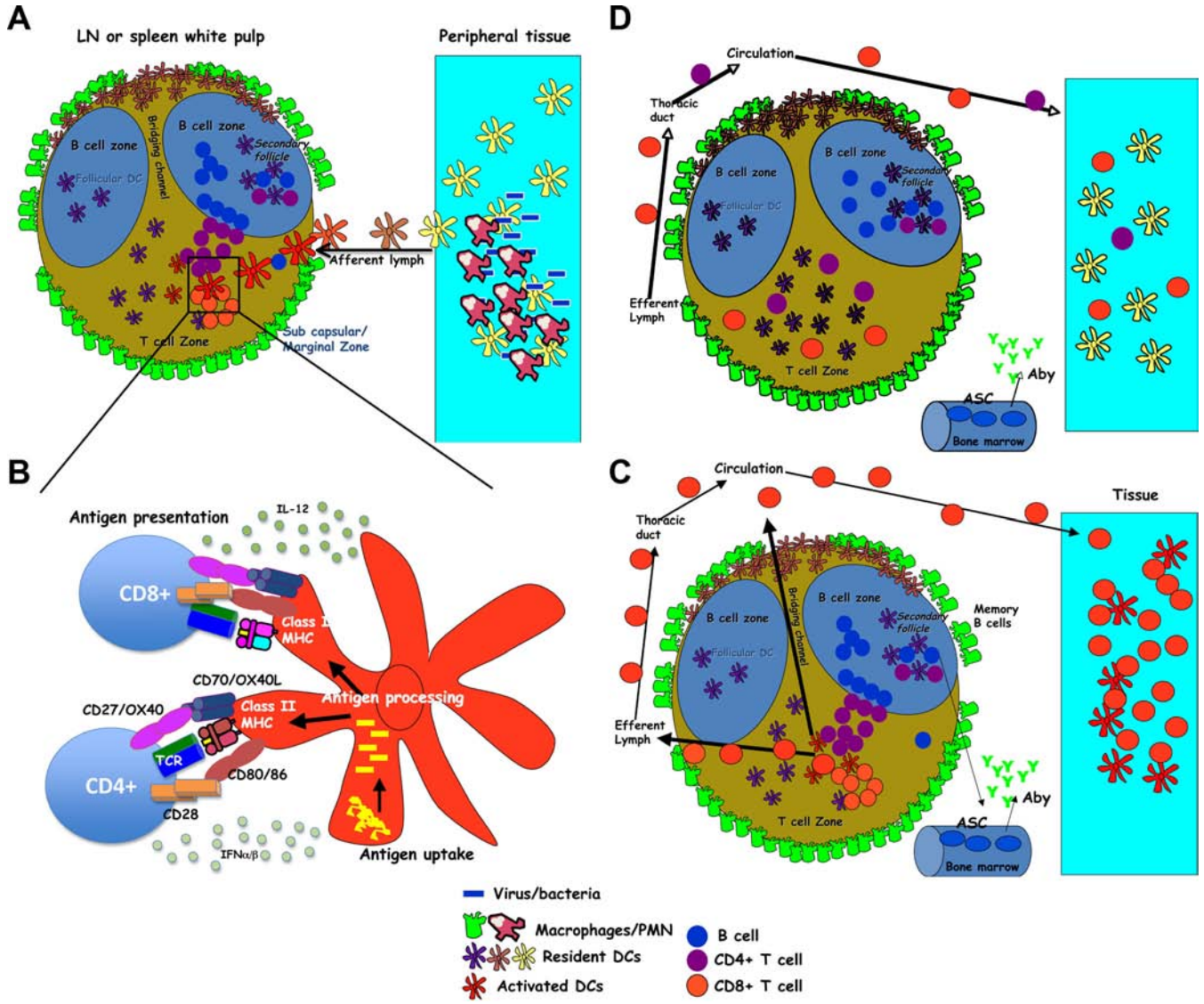

Fig. 4. The anatomy of a T cell response. *A*: microbe invasion and proliferation at the site of infection, leading to the initial recruitment of phagocytes and containment of the infection. Tissue-resident APCs acquire antigen and migrate into the local secondary lymphoid organ (SLO) after being activated by the local inflammatory processes. LN, lymph node. *B*: during transit, APCs process and present pathogen-derived antigens in the context of class I and class II MHC. APCs also upregulate various cell surface molecules and cytokines important in providing the necessary costimulation to T cells within the SLO. *C*: antigen-stimulated CD4 T cells collaborate with B cells to promote antibody production, class switching, and memory B cell formation. Both CD4 and CD8 T cells clonally expand and migrate out of the SLO and into the infection site, where their effector functions facilitates the elimination of the pathogen. Aby, antibody; ASC, Antibody secreting cell; *D*: most T cells die off, leaving a memory pool with precursor frequency, antigen sensitivity, and trafficking capacity optimized for initiating rapid secondary responses in situ. PMN, polymorphonuclear leukocytes.

cells as well as produce additional effector cytokines. The collective effect of T cell and antibody activity at the site of infection results in the elimination of the infectious agent. The majority of antigen-specific T cells die off as the inflammatory processes that mediated pathogen eradication are replaced by an increasing amount of immunoregulatory cells and cytokines that restore normal tissue homeostasis.

The resolution of the response produces a pool of memory T cells that possess phenotypes and functions tailored to respond to reinfection. Regardless of their specific phenotype (i.e., T_{CM}, T_{EM}, etc.), the pool of memory cells differs from naïve cells in three important parameters (Fig. 4*D*). First, despite the 90–95% die off of antigen-specific T cells, the frequency of cells that remain is still 10–100 fold higher than the precursor frequency of naïve cells that were present before the pathogen encounter. Much like putting more cops on the beat, the increased precursor frequency makes for enhanced patrolling and surveillance of the host. Second, memory cells have the capacity to access both SLO and peripheral tissues, even under conditions of homeostasis. Contrary to the primary response, where the trafficking patterns of the naïve T cells forces them to wait in the draining SLO until antigen and/or migrating APCs present them with antigen days after the initial infection, the presence of memory cells in situ enables their immediate response to a pathogen reencounter. This contributes to the dramatically shorter response time of the secondary response. An interesting aspect of the immediate response by memory cells is that their production of effector chemokines and cyto-

kines serve to draw in many innate immune cells as effectors. Thus, while the quality and quantity of innate immune activation facilitates the primary adaptive response, it is the adaptive response that facilitates the recruitment and activation of innate cells during the secondary response. Third, memory cells are more sensitive to antigen stimulation through their TCR and are somewhat less dependent on costimulatory molecules to enable their productive response. As a result, memory cells are able to respond to the presentation of minimal amounts of antigen during the early hours after the initial reinfection.

Finally, it is worth noting that what is described in this review is fully idealized and does not incorporate all of the possible permutations that can and do exist in T cell responses generated in the real world. That is, the response described above is what can happen when everything is working exactly as it should, culminating in effective, long-lived memory against an infectious pathogen. While idealized responses do occur in nature, the most interesting and productive ends of the T cell research spectrum focus on what happens when things go wrong and why. Examining these extremes of immune dysfunction, within the framework of an idealized response, promises to be a fruitful future source of investigation with both basic and clinical relevance.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

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Cell-cell cooperation at the T helper cell/mast cell immunological synapse

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It has been suggested that mast cells might serve, under certain circumstances, as antigen-presenting cells (APCs) for T cells. However, whether cognate interactions between mast cells and class II–restricted CD4$^+$ T cells actually occur is still an open question. We addressed this question by using peritoneal cell–derived mast cells (PCMCs) and freshly isolated peritoneal mast cells as APC models. Our results show that in vitro treatment of PCMCs with interferon-γ and interleukin-4 induced surface expression of mature major histocompatibility complex class II molecules and CD86. When interferon-γ/interleukin-4–primed PCMCs were used as APCs for CD4$^+$ T cells, they induced activation of effector T cells but not of their naive counterparts as evidenced by CD69 up-regulation, proliferation, and cytokine production. Confocal laser scanning microscopy showed that CD4$^+$ T cells formed immunological synapses and polarized their secretory machinery toward both antigen-loaded PCMCs and freshly isolated peritoneal mast cells. Finally, on cognate interaction with CD4$^+$ T cells, mast cells lowered their threshold of activation via FcεRI. Our results show that mast cells can establish cognate interactions with class II–restricted helper T cells, implying that they can actually serve as resident APCs in inflamed tissues. (Blood. 2009;114: 4979-4988)

Introduction

Mast cells are known to play a pivotal role in allergic hypersensitivity reaction and, more generally, in inflammation.[1,2] By virtue of their strategic and widespread location in tissues, namely at host-environment interface, and of their functional characteristics, mast cells behave as sentinels of the immune system.[3] Once activated, mast cells release several preformed and de novo–synthesized mediators (including histamine, proteases, leukotrienes, prostaglandins, and various cytokines and chemokines), resulting in the recruitment and activation of other immune cells.[3]

Several lines of evidence highlight an emerging role of mast cells in numerous steps of innate and adaptive immune responses, indicating that their contribution to immunity goes far beyond their well-known role in allergy.[3-8] Functional interplay between mast cells and T cells has been suggested by studies that document colocalization of activated mast cells and T cells in inflamed tissues[9,10] or involvement of mast cells in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.[11-13] Additional lines of evidence show that mast cells can contribute to the development of different T cell–associated responses by influencing the activation, the proliferation, the differentiation, and the recruitment of T cells.[7,14,15] Recent in vitro studies showed that IgE-activated mast cells can enhance T-cell proliferation by a mechanism involving tumor necrosis factor α (TNF-α) secretion, cell contact, and mast cell expression of OX40L.[6,7] In turn, it has been shown that activated T cells can induce in vitro histamine, TNF-α, matrix metalloproteinase-9 secretion, and interleukin 4 (IL-4) mRNA expression in mast cell subsets.[16-18] Finally, mast cells have been reported to promote in vivo T-cell migration to inflammatory sites by secreting chemotactic factors, such as lymphotactin and IL-16[19] and to orient Th differentiation via the production of IL-4 and histamine.[20,21] Taken together these studies highlight the existence of functionally important mast cell/T-cell crosstalk and raise the question of whether mast cell/T-cell cognate interactions might occur in the course of immune responses.

Immunological synapses (ISs) are the morphologic manifestation of the cognate interactions occurring between T cells and other cells of the immune system serving as antigen-presenting cells (APCs). These specialized areas of signal transduction, formed at the T cell/APC contact site, are characterized by large scale clustering and segregation of surface molecules and intracellular signaling components.[22-24] Among the different molecular rearrangements occurring at the IS, the polarization of T-cell Golgi apparatus toward the APCs for polarized secretion is a distinctive, rapid, and efficient T-cell response, occurring within minutes after T cell/APC encounter both in resting and activated CD4$^+$ and CD8$^+$ T cells.[25-27] It is, therefore, considered, together with T-cell receptor (TCR) enrichment into the IS, a morphologic hallmark of specific TCR engagement.

To investigate whether cognate interactions could be established at the contact site between CD4$^+$ T cells and mast cells, resulting in the formation of ISs and in T-cell polarization toward the APCs, we used, as potential APCs for ovalbumin (OVA)–specific CD4$^+$ (OT-II) T cells, (1) a recently described cellular model (peritoneal cell–derived mast cells, PCMCs) based on the expansion in vitro, for several weeks, of murine peritoneal mast cells,[28] and (2) freshly isolated peritoneal mast cells.

Our results show that, after priming with a combination of interferon-γ (IFN-γ) and IL-4, a fraction of PCMCs express mature major histocompatibility complex (MHC) class II molecules,

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present antigenic determinants to OT-II T cells, and elicit functional responses in effector T cells but not in their naive counterparts. We also show the formation of ISs and T-cell polarization at the contact site between T cells and mast cells (both PCMCs and freshly isolated peritoneal mast cells), thus unambiguously illustrating the formation of cognate interaction between these cells. Our results show that, although less efficiently than professional APCs, mast cells can indeed present antigens to T cells, resulting in antigen-dependent cell-cell cooperation.

Methods

Abs and cell-surface immunofluorescence

The following Abs were purchased from eBioscience: fluorescein isothiocyanate (FITC)–anti-FcεRI (MAR-1), FITC–anti-CD40 (HM40-3), PE–anti-CD44 (IM7), FITC–anti-CD117 (ACK2), FITC–anti-CD69 (H1.2F3), FITC–anti-CD80 (16-10A1), phycoerythrin (PE)–anti-CD86 (GL-1), PE–anti-ICOSL (HK53), PE–anti-OX40L (RM134L), FITC–anti-IA^b (M5/114), PE–anti-Vα2 (B20.1), anti–4-1BBL (TKS-1), FITC–anti-rat IgG, and isotype-matched control Ig. Biotinylated anti-IA^b mAb (KH74) was purchased from BioLegend. In direct immunofluorescence experiments, cells were blocked with 2.4G2 hybridoma supernatant for 15 minutes at 4°C (gift from Dr Joost van Meerwijk, Inserm U563); otherwise, cells were blocked with phosphate-buffered saline (PBS) containing 10% normal goat serum and 10% normal mouse serum. Antibodies were added in PBS containing 1% bovine serum albumin (BSA) and incubated for 30 minutes at 4°C. Flow cytometric data were acquired on a BD FACScan cytometer with the use of BD CellQuest software (Version 4.0) and were analyzed with the use of FlowJo software (Version 7.2.5; TreeStar Inc).

Mice

C57BL/6 male C57BL/6 OT-II mice aged 6 to 12 weeks old and synthetic peptide corresponding to amino acid residues 323 to 339 (ISQAVHAAHAEINEAGR) of ovalbumin (OVAp) were kindly provided by Dr S. Guerder (Inserm U563). All mice used in this study were raised and housed under specific pathogen–free conditions and handled according to protocols approved by the Inserm Ethics Committee on Animal Experimentation in compliance with European Union guidelines.

Generation of PCMCs

Peritoneal cells from C57BL/6 mice were collected by peritoneal washing with 4 mL of RPMI 1640. Cells were washed and seeded in a 24-well culture plate at 10^6/mL in Opti-MEM supplemented with 10% FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin (Invitrogen), 3% supernatant of Chinese hamster ovary transfectants secreting murine stem cell factor (a gift from Dr P. Dubreuil, Inserm U891). Twenty-four hours later, nonadherent cells were removed, and fresh culture medium was added to adherent cells. Three days later, nonadherent cells and adherent cells harvested by flushing were pelleted and resuspended (3×10^5/mL) in fresh culture medium. The same procedure was repeated twice a week. Cells were used for experiments between week 4 and week 10. PCMCs were characterized by histochemistry: cell suspensions were cytocentrifuged onto glass slides, air-dried, and stained with toluidine blue or Alcian blue followed by Safranin.[29]

Ex vivo peritoneal mast cells

Peritoneal cells from 20 C57BL/6 mice were collected by peritoneal washing. Nonadherent cells were washed in magnetic-activated cell sorting buffer, and $CD117^+$ cells were positively selected by using CD117 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. Mast cell–enriched cell population was immediately primed with 50 ng/mL IFN-γ and 10 ng/mL IL-4 for 72 hours in complete Opti-MEM and used as APCs.

Degranulation assay

Mast cell degranulation was determined by measuring the release of β-hexosaminidase. PCMCs were incubated in culture medium with or without anti–dinitrophenol (DNP) IgE (0.5 μg/mL SPE-7; Sigma-Aldrich) overnight at 37°C. The cells were then washed and distributed in 96-well flat-bottom plates at a density of 10^5 cells in 50 μL of Tyrode buffer. The cells were adapted to 37°C for 20 minutes and then treated with prewarmed 50 μL of the mentioned stimuli diluted in Tyrode buffer for 45 minutes at 37°C. β-Hexosaminidase release in the supernatants was measured according to Bachelet et al.[30]

Confocal microscopy

Effector $CD4^+$ OT-II T cells (10^5) were plated with APCs (2×10^5) either unpulsed or pulsed with 10 μg/mL OVAp or 400 μg/mL ovalbumin in 96-well U-bottom plates and spun to 500 rpm to allow conjugates formation. The cells were transferred onto poly-L-lysine–coated slides and then fixed with 4% paraformaldehyde in PBS. Cells were first permeabilized and blocked in 10% normal goat serum/10% normal mouse serum in PBS containing 0.5% saponin. Cells were stained with the following primary antibodies in PBS containing 1% BSA, 0.5% saponin 45 minutes at room temperature: anti–IFN-γ (clone AN-18), anti-CD3ε (clone 17A2), anti-$p56^{lck}$ (clone 28/lck) from BD PharMingen; polyclonal anti–protein kinase C (PKC) θ (Santa Cruz Biotechnology Inc); and anti–α-tubulin (clone DM1A; Sigma-Aldrich). In some experiments, 2 μg/mL avidin-sulforhodamine 101 (highly cationic glycoprotein that selectively stains mast cell granules[31,32]; Sigma-Aldrich) was added to unambiguously identify mast cells. After washing, secondary antibodies (Alexa Fluor–conjugated; Invitrogen) were applied in PBS containing 1% BSA, 0.5% saponin. In some experiments, PCMCs were labeled with anti–mast cell tryptase polyclonal rabbit Ab (Santa Cruz Biotechnology Inc). The samples were mounted and examined with the use of a Zeiss LSM 510 or a Zeiss LSM 710 confocal microscope with a 63× Plan-Apochromat objective (1.4 oil), electronic zoom 3, as described.[27] Scoring of the slides was performed in a blinded fashion by evaluating for each condition at least 100 conjugates in randomly selected fields from at least 3 independent experiments.

Results

Phenotypic and functional characterization of murine PCMCs

Cells obtained from peritoneal washing of C57BL/6 mice were cultured in the presence of stem cell factor to generate PCMCs as described by Malbec et al.[28] After 1 month in culture, more than 98% of the cells expressed on their surface characteristic markers of mast cells, such as FcεRI and CD117 (Figure 1A), and stained positive for mast cell tryptase (Figure 1B) as previously described for PCMCs.[28] None of these cells were positive for B220 or CD11c (data not shown).

PCMCs were further characterized by toluidine blue staining and Alcian blue/safranin staining, a procedure commonly used to discriminate mast cells of the mucosal type (Alcian blue high, safranin low) from connective tissue—type mast cells (CTMCs; Alcian blue low, safranin high[29,33]). Toluidine blue staining for metachromatic granules was positive, with approximately 40% of the cells showing a stronger staining compared with the remaining cells (Figure 1C). Alcian blue/safranin staining showed that all PCMCs counterstained red with safranin, whereas they were either negative or faintly positive for Alcian blue, indicating that these cells were mostly of the CTMC-like phenotype (Figure 1D). Interestingly, similarly to toluidine blue staining, also safranin staining was heterogeneous: approximately 40% of the PCMCs exhibited a stronger safranin staining than did the remaining cells (Figure 1D). Taken together toluidine blue and safranin staining suggested that, among the PCMCs, approximately 40% of cells had

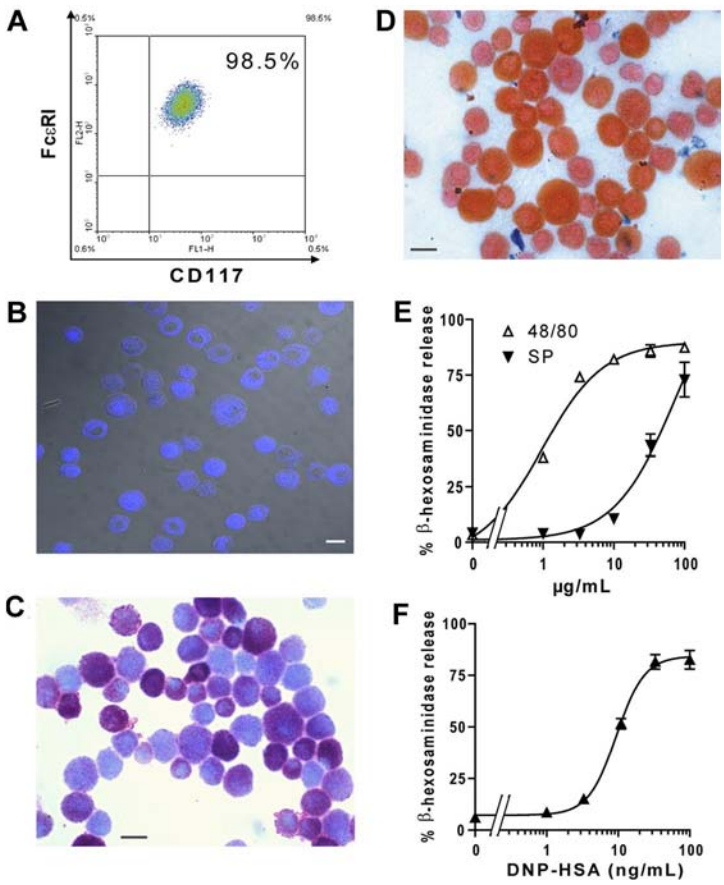

Figure 1. Phenotypic and functional characterization of PCMCs. (A) CD117 and FcεRI expression on PCMC surface. The number in the top right indicates the percentage of double-positive cells. (B) Mast cell tryptase staining by indirect immunofluorescence with an Alexa 633–conjugated secondary Ab (blue). Samples were inspected with a confocal laser-scanning microscope. (C-D) Histochemical staining of PCMCs with toluidine blue (C) or Alcian blue followed by safranin (D). Bars = 10 μm. (E) Secretagogue-induced β-hexosaminidase release. PCMCs were stimulated with increasing concentrations of Substance P or compound 48/80 for 30 minutes at 37°C. (F) FcεRI-dependent β-hexosaminidase release. PCMCs were sensitized with mouse IgE anti-DNP and challenged with DNP-HSA. The percentage of β-hexosaminidase released was plotted against the concentration of the stimulus. Tests for phenotypic and functional analysis of PCMCs were routinely performed after 3 to 5 weeks of culture. Data are from 1 representative experiment of 3.

a more mature phenotype, characterized by a higher content of mature secretory granules.

We next investigated PCMC biologic function by using a standard degranulation assay. We used 2 classes of stimuli: polycationic compounds, such as compound 48/80 and substance P, that induce degranulation specifically in CTMCs (and not in mucosal-type mast cells) or antigen (dinitrophenylated human serum albumin [DNP-HSA]) in IgE-sensitized mast cells. When PCMCs were incubated with increasing concentrations of 48/80 or substance P, they exhibited a strong and dose-dependent degranulation as detected by β-hexosaminidase release (Figure 1E). Moreover, PCMCs, previously sensitized with anti-DNP IgE, degranulated in a dose-dependent fashion when incubated with increasing concentrations of DNP-HSA (Figure 1F). Together these results indicated that PCMCs exhibit connective tissue mast cell functional properties.

Treatment with IFN-γ and IL-4 induces MHC class II molecule expression on PCMC surface

Having phenotypically and functionally characterized PCMCs, we used these cells to investigate whether they could be primed to become APCs for class II–restricted T lymphocytes.

PCMCs expressed neither surface MHC class II molecules nor costimulation molecules (Figure 2). We thus investigated whether these cells might be induced to express class II molecules by using treatments known to prime conventional APCs. Treatment of PCMCs with *Toll*-like receptor ligands (different lipopolysaccharide preparations from Gram-negative bacteria, peptidoglycan, poly I:C, lipoteichoic acid) added to PCMCs for 24, 48, 72, or 96 hours did not result in a detectable increase in I-A^b expression (data not shown).

We next tested whether IFN-γ and IL-4 could induce I-A^b on the PCMC surface. After 72 hours of culture with 50 ng/mL IFN-γ, a significant fraction of PCMCs (20%-35%) expressed I-A^b (Figure 2A-B). Interestingly, although the addition of 10 ng/mL IL-4 alone to PCMC cultures did not affect I-A^b expression level, it increased the frequency of IFN-γ–induced MHC class II expression, resulting in 40% to 50% MHC class II^+ PCMCs (Figure 2A-B). Moreover, the level of I-A^b expression per cell was also significantly increased. I-A^b expression was assessed by using 2 different monoclonal antibodies: M5/114 that recognizes mature and immature forms of MHC class II molecules and KH-74 that mainly binds mature forms of MHC class II.[34] Under these conditions, although individual class II^+ PCMCs expressed fairly high levels of I-A^b, approximately 50% of the PCMC population remained I-A^b negative (Figure 2A-B).

We next investigated by fluorescence-activated cell sorting (FACS) analysis whether IFN-γ plus IL-4 could induce the expression of costimulatory molecules. Untreated PCMCs were negative for several costimulation molecules: CD80, CD86, CD40, ICOSL, 4-1BB-L, and OX40L (Figure 2; supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Treatment with IFN-γ plus IL-4 resulted in the up-regulation of the surface expression of CD86 (Figure 2C) but did not enhance the expression of the other costimulation molecules tested (supplemental Figure 1). To test whether the PCMC population used in this study, although expressing phenotypic and histochemical markers of mast cells, could result from the skewing of peritoneal mast cells toward monocytes, we measured the surface expression of CD11b and F4/80 before and after treatment with IFN-γ plus IL-4. This analysis showed that neither untreated PCMCs nor cytokine-treated

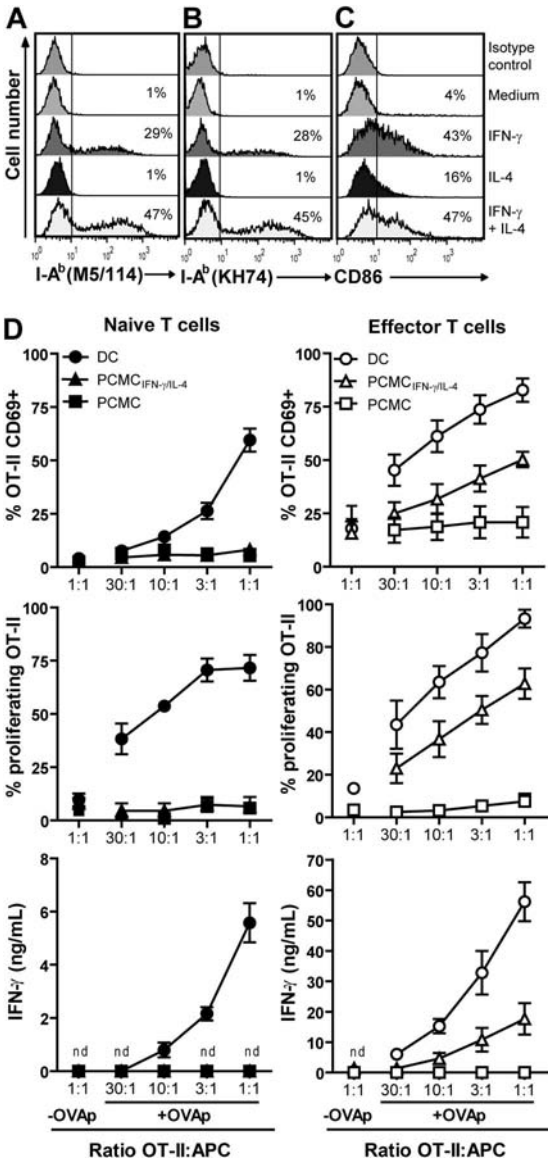

Figure 2. IFN-γ/IL-4–primed PCMCs express MHC class II and CD86 and induce functional responses in effector OT-II T cells but not in their naive counterparts. (A-C) PCMCs were incubated 72 hours with IFN-γ (50 ng/mL) or IL-4 (10 ng/mL) or with IFN-γ + IL-4. CD86 and I-A^b molecule expression (using the KH74 or M5/114 mAb) was analyzed by flow cytometry. The numbers inside the panels indicate the percentage of positive cells. Data are representative of at least 10 experiments. (D) APCs were pulsed or not for 16 hours with 10 μg/mL OVAp and cocultured with naive (left) or effector (right) OT-II T cells. OT-II T-cell activation was monitored by CD69 expression (after 20 hours of coculture), IFN-γ production (after 20 hours of coculture), and proliferation (after 72 hours of coculture). Results are presented as mean ± SEM of 4 independent experiments. nd indicates not detected.

PCMCs expressed detectable levels of CD11b and of F4/80, ruling out the contribution of monocyte-like cells to the T-cell activation we observed (supplemental Figure 2). Together the above-mentioned results indicate that a fraction of the whole PCMC population treated with IFN-γ plus IL-4 (further referred to as PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$) develop into potential APCs.

PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ can serve as APCs for CD4$^+$ T lymphocytes

To examine the antigen-presenting function of PCMCs, we investigated whether they could present the OVAp to CD4$^+$ T cells from OT-II TCR transgenic mice. When freshly purified naive OT-II T cells where cocultured overnight with unprimed PCMCs or with PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ either unpulsed or pulsed with OVAp at different T cell/APC ratios, they did not undergo detectable up-regulation of the T-cell activation marker CD69. Accordingly, neither proliferation nor IFN-γ production (measured after 3 days of coculture) was detected (Figure 2D). Conversely, these cells exhibited CD69 up-regulation, proliferation, and IFN-γ production when cocultured with OVAp-pulsed mature dendritic cells (DCs; Figure 2D).

We next tested whether PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ could serve as APCs for effector T cells. To this end, OT-II T cells were expanded with anti-CD3/anti-CD28 coated beads for 8 to 10 days before coculture with PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ or mature DCs either unpulsed or pulsed with OVAp. These previously activated T cells exhibited effector T-cell phenotype CD62L$^-$CD44^{high} (data not shown). As shown in Figure 2D, effector OT-II T cells up-regulated CD69, proliferated and produced IFN-γ when interacting with OVAp-pulsed DCs, and, although to a lesser extent, also with OVAp-pulsed PCMCs. Similar results were obtained when IL-2 production was measured; conversely, in the same conditions, we did not detect IL-4 production by OT-II T cells (data not shown). Peptide-pulsed PCMCs not primed with IFN-γ and IL-4 were unable to induce detectable responses in the same OT-II T-cell population (Figure 2D).

To further characterize the APC potential of PCMCs we investigated whether they could capture and process ovalbumin. PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ or DCs were incubated 16 hours with ovalbumin alone or with ovalbumin complexed with IgG, and their capacity to process and present ovalbumin was assessed by measuring CD69 up-regulation in effector OT-II T cells. As shown in Figure 3A and B, PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ incubated with a high concentration of ovalbumin (400 μg/mL) efficiently activated OT-II T cells to CD69 up-regulation, indicating that PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ can internalize and process native antigens. When incubated with a lower concentration of ovalbumin (30 μg/mL) in the presence of irrelevant rabbit IgG, PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ failed to induce CD69 up-regulation in OT-II T cells. Conversely, PCMCs were able to present the antigenic determinant to OT-II T cells when pulsed with the same concentration of ovalbumin complexed with anti–ovalbumin IgG, indicating that PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ can uptake antigens via an IgG-assisted mechanism. Similar results were obtained when T-cell proliferation (Figure 3C) and IFN-γ production (Figure 3D) were measured, indicating that the activation of OT-II T cells by PCMCs presenting a native antigen is not limited to CD69 up-regulation.

These results show that PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ can capture and process native antigens and can present antigenic peptides to effector CD4$^+$ T lymphocytes, leading to cytokine production and proliferation.

ISs and T-cell polarization responses at the mast cell/T-cell contact site

Taken together the above-mentioned results show that IFN-γ/IL-4–primed PCMCs can activate CD4$^+$ T cells in an antigen-dependent fashion. These observations do not formally rule out the possibility that bystander professional APCs (possibly present as contaminants of T-cell purification) could uptake antigenic determinants and in turn present them to T cells.[35]

To address this point we aimed to directly visualize T cell/PCMC cognate interactions. In a first round of experiments we measured the formation of conjugates between OT-II T cells and PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ by FACS analysis. As shown in supplemental Figure 3, OT-II T cells formed stable conjugates with PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ (lasting up to 1 hour). Conjugate formation was more pronounced with antigen-pulsed PCMCs than with unpulsed PCMCs$_{IFN\text{-}\gamma/IL\text{-}4}$ (supplemental Figure 3).

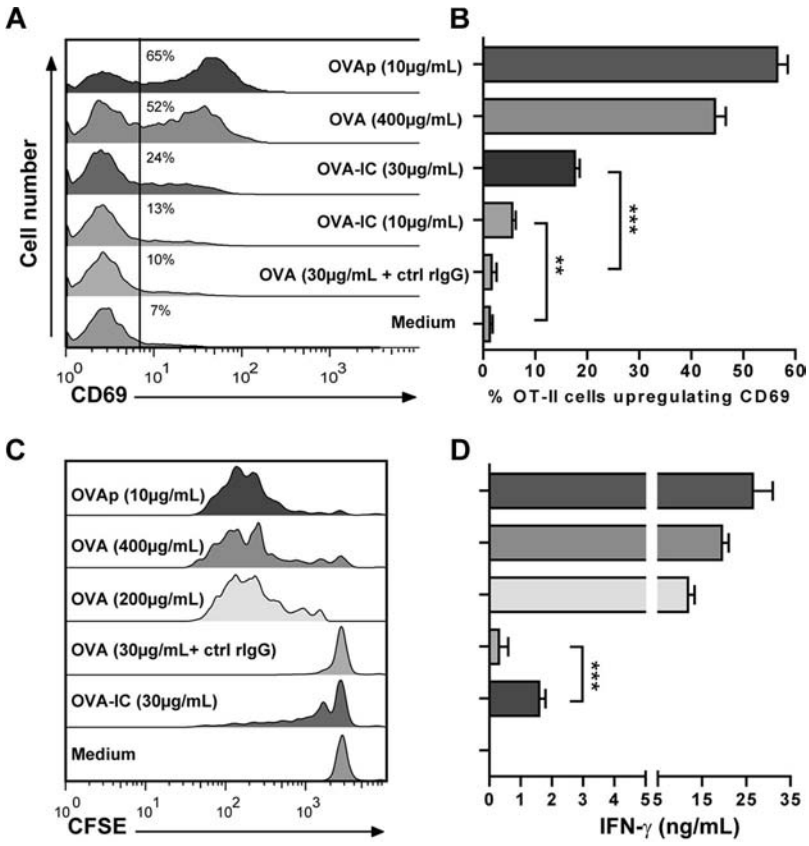

Figure 3. IFN-γ/IL-4–primed PCMCs can capture and process of protein antigen. (A-D) IFN-γ/IL-4–primed PCMCs were incubated for 16 hours with soluble OVA, OVA-IgG immune complexes (ICs), or soluble OVA in the presence of control rabbit IgG (ctrl rIgG) or OVAp. (A-B) Cells were cocultured with effector OT-II T cells for 16 hours, and CD69 up-regulation on OT-II T-cell surface was measured by FACS analysis on Vα2+ gated cells. (A) FACS profiles representative of 3 independent experiments. The numbers inside the panel indicate the percentage of positive cells. (B) Data are presented as percentage of Vα2+ gated cells up-regulating CD69 (mean ± SEM of 3 independent experiments). (C) Proliferation of T cells was monitored by carboxyfluorescein succinimidyl ester dilution after 72 hours of coculture. Panels show FACS profiles from 1 representative experiment of 3. (D) IFN-γ in supernatant was dosed by ELISA after 16 hours of coculture (mean ± SEM of 3 independent experiments). Difference between groups was evaluated by an unpaired Student *t* test with the GraphPad Prism software. It should be noted that DCs were more efficient than PCMCs in inducing functional responses to whole ovalbumin in OT-II T cells. Approximately 95% of T cells proliferated when cultured with DCs previously incubated with OVA (400 μg/mL) and approximately 85% of T cells proliferated when cultured with DCs previously incubated with IgG-OVA (30 μg/mL). IFN-γ production by OT-II T cells when cultured with DCs pulsed with OVA (400 μg/mL) or IgG-OVA (30 μg/mL) was approximately 50 ng/mL and approximately 10 ng/mL, respectively.

We next investigated by confocal microscopy the capacity of OT-II T cells to form ISs with PCMCs. To this end, OT-II T cells were cocultured with $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$ for 20 minutes and stained with anti–phosphotyrosine (PTyr) antibodies to detected PTyr staining (an early marker of signaling at the IS[27]) and with Alexa 488–labeled phalloidin (to detect F-actin). Confocal microscopy analysis showed that OT-II T cells interacting with ovalbumin-pulsed $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$ exhibited F-actin (~ 37% of scored conjugates) and PTyr enrichment (~ 22% of scored conjugates) at the synaptic area (supplemental Figure 4).

To further characterize T-cell activation during interaction with PCMCs, compared with the activation induced by professional APCs, we focused on later steps of the TCR-mediated signaling cascade. OT-II T cells were cocultured with $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$ or mature DCs (either unpulsed or OVAp-pulsed) for 2.5 hours; cells were fixed, permeabilized, and stained with anti-PKCθ, anti-tubulin, and anti–IFN-γ antibodies to detect sustained signaling at the ISs and polarization of the T-cell secretory machinery toward the APCs. As shown in supplemental Figure 5, T cells interacting with OVAp-pulsed DCs exhibited enrichment of PKCθ (~ 60% of scored conjugates) and polarization of IFN-γ secretion (~ 40% of scored conjugates) toward the APCs. Interestingly, also at the T cell/PCMC contact site a significant number of conjugated T cells enriched PKCθ and polarized IFN-γ toward PCMCs (~ 33% enriched PKCθ and ~ 16% enriched PKCθ and polarized IFN-γ of 300 total scored T cells from 3 independent experiments, Figure 4). The above-mentioned results show that, among OT-II T cells exhibiting an enrichment of PKCθ at the IS, a limited fraction only polarized IFN-γ toward PCMCs. This observation can be explained by the fact that the T-cell lines were expanded in nonpolarizing conditions; thus, only a fraction of T cells was of the Th1 phenotype. In some experiments, to better show the establishment of functional ISs between mast cells and T cells, cell conjugates were simultaneously stained with anti-PKCθ, anti–IFN-γ, and avidin-sulforhodamine 101 (which stains mast cell granules[31,32]). This triple staining unambiguously showed T cell/mast cell cognate interactions (supplemental Figure 6). Similar results were obtained when mast cell tryptase staining was used to identify PCMCs (data not show).

To define whether the observed directionality of the T cell/PCMC interactions was due to specific TCR engagement at the IS, we stained cell conjugates with antibodies directed against the TCR/CD3 complex (anti-CD3ε antibodies) and with antibodies against $p56^{lck}$ (a key signaling component of the TCR-mediated signaling cascade). As shown in Figure 5, in a significant fraction of conjugated T cells TCR/CD3 and $p56^{lck}$ were enriched at the contact site with OVAp-pulsed PCMCs (~ 40% enriched both CD3ε and $p56^{lck}$ of ~ 300 total scored T cells from 3 independent experiments).

To further document the formation of cognate interactions between OT-II T cells and PCMCs, we investigated the enrichment of PKCθ at the IS and the polarization of the T-cell secretory machinery in conditions in which $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$ had captured and processed ovalbumin. As shown in Figure 6, OT-II T cells interacting with PCMCs previously incubated for 16 hours with 400 μg/mL ovalbumin (and identified by avidin-sulforhodamine 101 staining) exhibited an enrichment of PKCθ (~ 35%) and IFN-γ–polarized secretion (~ 25%) toward the antigen-presenting PCMCs.

Finally, we used an additional ex vivo model to investigate T cell/mast cell cognate interactions. In a first set of experiments, freshly isolated peritoneal mast cells were tested for their capacity to activate OT-II T cells. As shown in supplemental Figure 7,

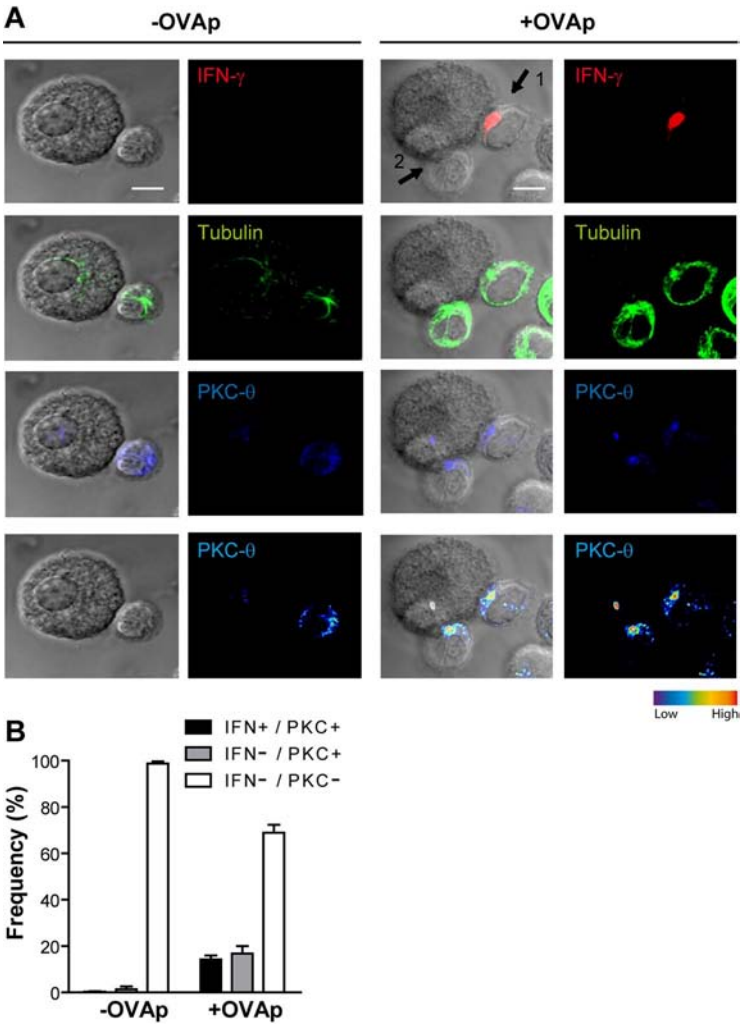

Figure 4. CD4⁺ T cells polarize their secretory machinery toward IFN-γ/IL-4–primed PCMCs. IFN-γ/IL-4–primed PCMCs were pulsed or not with 10 μg/mL OVAp. After washing, APCs were cocultured with effector OT-II T cells for 10 minutes. Cells were settled onto polylysine-coated slides; fixed; and stained for tubulin, PKCθ, and IFN-γ; and analyzed by confocal laser scanning microscopy. (A) Representative staining for IFN-γ (red), tubulin (green), and PKCθ (blue) of a $PCMC_{IFN\text{-}\gamma/IL\text{-}4}$/OT-II T-cell conjugate, each staining is represented alone or merged with DIC images; PKCθ staining is also shown as pseudocolor intensity. Arrow 1 points to a synapse where IFN-γ is polarized toward the PCMCs and PKCθ is enriched at the OT-II T cell/PCMC contact site; arrow 2 points to a synapse where PKCθ only is enriched. (B) OT-II T-cell/PCMC conjugates were randomly selected and scored for the recruitment of PKCθ to the cell–cell contact site either alone or in combination with IFN-γ polarization. ▒ indicates conjugates exhibiting recruitment of PKCθ alone; ■, conjugates exhibiting polarization of both PKCθ and IFN-γ; □, conjugates exhibiting neither PKCθ nor IFN-γ polarization. Approximately 100 conjugates were analyzed per experiment. Histograms represent means ± SEM of 3 independent experiments. Bar = 5μm.

interaction of OT-II T cells with freshly isolated mast cells, primed with IFN-γ and IL-4 (under the same conditions as PCMCs) and pulsed with OVAp, resulted in T-cell activation to IFN-γ production and proliferation. We next investigated whether freshly isolated peritoneal mast cells could form ISs with OT-II T cells. Freshly isolated mast cells were IFN-γ/IL-4 primed, cocultured 16 hours with 400 μg/mL ovalbumin, and conjugated for 2.5 hours with OT-II T cells. In these conditions we detected an enrichment of PKCθ (~ 36%) and polarization of IFN-γ secretion (~ 20%) toward mast cells (Figure 6), comparable with that observed in OT-II T cells interacting with PCMCs, thus validating the use of PCMCs as a model of antigen-presenting mast cells.

Together these results show that CD4⁺ T cells form ISs and polarize their secretory machinery toward both peptide-loaded and native antigen-loaded mast cells, providing the first morphologic evidence in support of a role of mast cells as APCs for T lymphocytes.

PCMCs are functionally activated during cognate interaction with OT-II T cells

To investigate whether PCMCs are functionally activated during cognate interaction with OT-II T cells, we evaluated the effect of antigen-specific T cell/PCMC interactions on DNP-HSA–induced degranulation of IgE-sensitized mast cells. As shown in Figure 7A and B, $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$ pulsed with OVAp and cocultured with effector OT-II exhibited lower threshold responses to DNP-HSA stimulation than did unpulsed $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$. Moreover, $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$ increased their surface expression of the activation marker CD69 after cognate interaction with OT-II T cells (Figure 7C). CD69 up-regulation could not be mimicked by supernatant from activated OT-II cells, indicating that $PCMC_{IFN\text{-}\gamma/IL\text{-}4}$ activation is not mediated by soluble factors but results from cell–cell contact.

Taken together the above-mentioned results indicate that, on cognate interaction with helper T cells, PCMCs receive stimulatory signals that enhance their biologic functions.

Discussion

In the present study we used PCMCs and freshly isolated peritoneal mast cells, primed with IFN-γ and IL-4, as potential APCs for CD4⁺ T cells. We report that cognate interactions are formed between mast cells and effector CD4⁺ T cells, resulting in their activation.

It was previously reported that mouse bone marrow–derived mast cells (BMMCs) could synthesize functional class II molecules[36] and could present antigenic peptides to CD4⁺ T-cell hybridoma.[37] However, a recent study by Kambayashi et al[35]

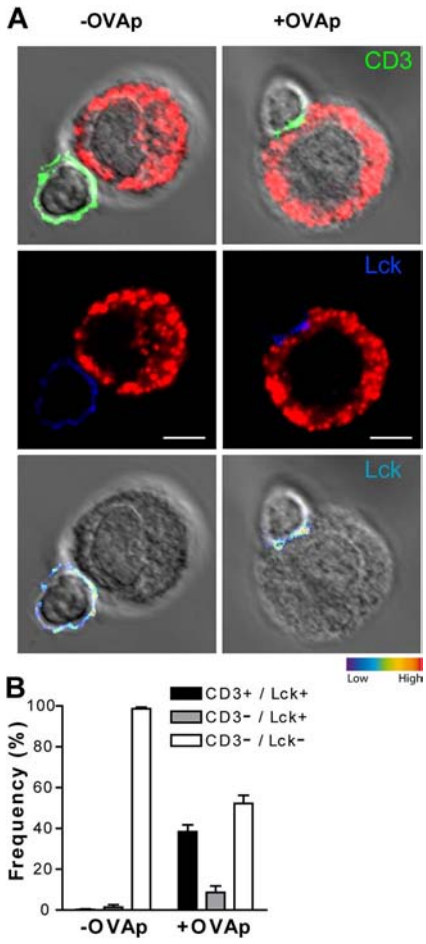

Figure 5. Enrichment of TCR/CD3 complex and of p56^{lck} at the T cell/PCMC$_{IFN\text{-}\gamma/IL\text{-}4}$ contact site. IFN-γ/IL-4–primed PCMCs were pulsed or not with 10 μg/mL OVAp. After washing, APCs were cocultured with effector OT-II T cells for 10 minutes. Cells were settled onto polylysine-coated slides, fixed, and stained for mast cell granules with avidin-sulforhodamine 101 (red), CD3ε (green), and p56^{lck} (blue). (A) Representative staining for CD3ε (green), and p56^{lck} (blue) of a PCMC$_{IFN\text{-}\gamma/IL\text{-}4}$/OT-II T-cell conjugate. Staining is represented merged with DIC images; p56^{lck} staining is also shown as pseudocolor intensity. (B) OT-II T cell/PCMC conjugates were randomly selected and scored for the recruitment of p56^{lck} to the cell-cell contact site either alone or in combination with CD3ε. ▩ indicates conjugates exhibiting recruitment of p56^{lck} alone; ■, conjugates exhibiting polarization of both p56^{lck} and CD3ε; □, conjugates exhibiting neither p56^{lck} nor CD3 polarization. Approximately 100 conjugates were analyzed per experiment. Histograms represent means ± SEM of 3 independent experiments. Bar = 5μm.

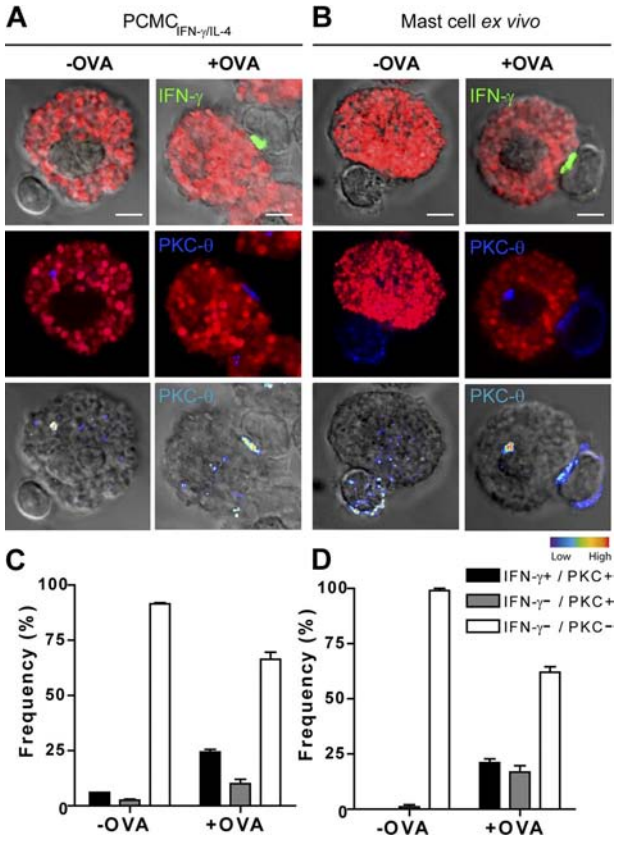

Figure 6. IFN-γ/IL-4–primed PCMCs and freshly isolated mast cells process whole ovalbumin and form functional ISs. IFN-γ/IL-4–primed PCMCs (A) or IFN-γ/IL-4–primed peritoneal mast cells (B) were pulsed or not for 16 hours with 400 μg/mL ovalbumin. After washing, APCs were cocultured with effector OT-II T cells for 2.5 hours. Cells were fixed, permeabilized, and stained for mast cell granules with avidin-sulforhodamine 101 (red), PKCθ (blue), and IFN-γ (green) and analyzed by confocal laser scanning microscopy. (Top) IFN-γ (green) and avidin-sulforhodamine 101 (red) are merged with DIC images; (middle) avidin-sulforhodamine 101 (red) and PKCθ staining (blue) are shown; (bottom) PKCθ staining is shown as pseudocolor intensity merged with DIC images. (C-D) For each condition, OT-II T-cell/APC conjugates were randomly selected and scored for the recruitment of PKCθ to the cell–cell contact site either alone or in combination with IFN-γ polarization. ▩ indicates conjugates exhibiting recruitment of PKCθ alone; ■, conjugates exhibiting polarization of both PKCθ and IFN-γ; □, conjugates exhibiting neither PKCθ nor IFN-γ polarization. Approximately 50 conjugates were analyzed per experiment. Histograms represent means ± SEM of 3 independent experiments. Bar = 5μm.

casted doubts on the possibility that BMMCs might present antigens to CD4+ T cells, by showing that, although BMMCs could uptake antigens, they did not express functional class II molecules on their surface. That study also showed that activation of CD4+ T cells by antigen-loaded BMMCs was indirect and depended on cross-presentation of Ag determinants by adjacent DCs after phagocytosis.[35] More recently, the same investigators reported that BMMCs can, under certain circumstances, activate CD4+ T cells via direct antigen presentation, thus reproposing the issue of direct antigen presentation by mast cells.[38] In addition, recent studies, by showing that basophils, which are closely related to mast cells, can serve as APCs are able to initiate T helper type 2 responses, support the idea that "nonconventional" APCs can play a central role in the development of adaptive immune-responses.[39-41]

Our results extend these previous studies and provide experimental evidence of direct antigen presentation by mast cells at the single-cell level by (1) showing antigen presentation by PCMCs, a cellular system that more closely mimics tissue mast cells[28]; (2) presenting the first morphologic evidence of IS formation and T-cell polarization at the T-cell/PCMC contact site; and (3) showing cognate interactions between T cells and freshly isolated peritoneal mast cells.

We present strong morphologic evidence of the antigen-presenting role of individual mast cells. Our results show that T cells exhibit the enrichment of PTyr and F-actin at the contact site with cognate PCMCs, indicating that these early events of IS formation are triggered at the cell-cell interface. We also show the sustained enrichment of PKCθ (a key signaling component of TCR-mediated signal transduction, that is known to translocate for prolonged periods of time at the IS,[42-44] at the T cell/mast cell contact site. Finally, we provide evidence for the polarization of tubulin cytoskeleton and of IFN-γ–filled Golgi apparatus toward mast cells and of CD3 and p56^{lck} enrichment at the T cell/mast cell interface, showing that individual T cells in conjugation with mast cells are specifically activated via their TCR.

In the present study only effector (that have low activation threshold[45,46]) and not naive OT-II T cells exhibited functional

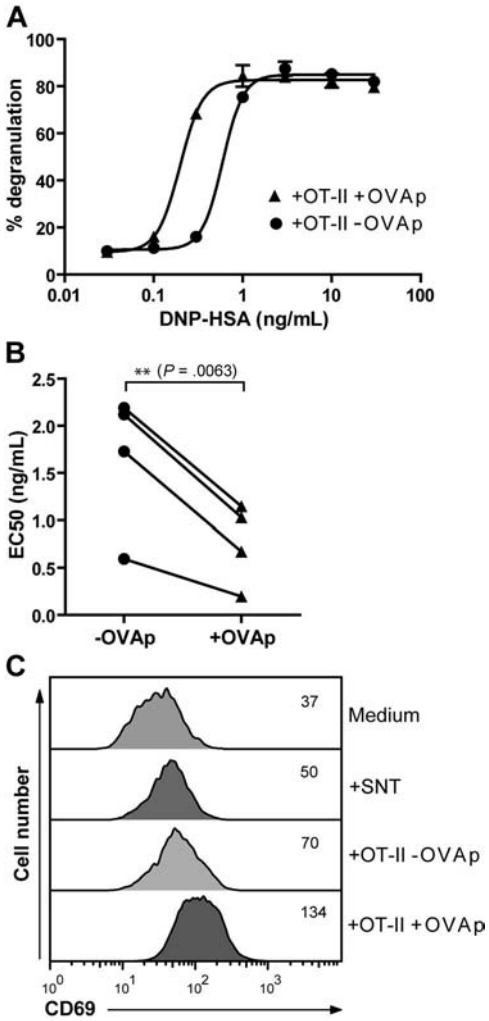

Figure 7. $PCMC_{IFN\text{-}\gamma/IL\text{-}4}$ activation after antigen-specific interaction with OT-II T cells. (A-B) $PCMC_{IFN\text{-}\gamma/IL\text{-}4}$s either unpulsed or pulsed with 10 μg/mL OVAp were cocultured with OT-II T cells in the presence of anti-DNP IgE (1 μg/mL) for 16 hours. (A) Ability to release β-hexosaminidase in response to increasing concentrations of DNP-HSA. Data are from 1 representative experiment of 4 performed in triplicates. (B) EC_{50} (half maximal effective concentration) of DNP-HSA from 4 independent experiments. Difference between groups was evaluated by a paired Student *t* test by using GraphPad Prism software. (C) $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$ either unpulsed or pulsed with 10 μg/mL OVAp were cocultured with OT-II T cells for 16 hours. In parallel samples PCMCs were treated for 16 hours with supernatant from activated OT-II (SNT; 50%, from OT-II cultured for 6 days with anti-CD3/anti-CD28–coated beads) or medium alone. Expression of CD69 on PCMCs was analyzed by flow cytometry; numbers indicate MFI. Data are from 1 representative experiment of 3.

responses when cocultured with peptide-pulsed $PCMCs_{IFN\text{-}\gamma/IL\text{-}4}$. This selective stimulation of effector cells should be sufficient for an antigen-presenting role of mast cells in vivo, considering their preferential location in tissues and, therefore, their susceptibility to encounter effector lymphocytes.

Our results show that the capacity of mast cells to activate $CD4^+$ T cells is quantitatively relevant, as shown by the amount of IFN-γ released after antigen triggering. However, IFN-γ production remained weaker than that obtained using mature DCs. This can be partially explained by the lower level of expression of MHC class II and costimulation molecules on the PCMC surface and by the fact that only a fraction of PCMCs (30%-45%) expressed MHC class II, probably because of the heterogeneity of the PCMC population (Figure 1).

In our cellular model, the best condition to prime PCMCs for MHC class II expression was 3 days of culture in the presence of IFN-γ and IL-4. The role of IFN-γ in positively regulating MHC class I and II expression on different cell types is well documented.[47] Moreover, it is well known that IL-4 and IFN-γ represent the major inducers of class II in the B-lymphoid and monocytic/macrophage lineages, respectively.[48,49] Here, we show an intriguing and unexpected cooperation between IL-4 and IFN-γ on the same cellular subset: although IL-4 cannot by itself induce class II expression on PCMCs, it enhances the IFN-γ–induced expression of these molecules. This indicates that IFN-γ is necessary and sufficient to induce MHC class II expression on a fraction of PCMCs, yet the presence of IL-4 optimizes the APC phenotype of these cells. These observations suggest that by responding to both IFN-γ and IL-4 (possibly produced by local Th1 T cells or natural killer T cells, undergoing simultaneous activation in an inflamed tissue), mast cells could provide platforms for antigen presentation to both Th1 and Th2 cells.

What could be the functional role of the observed cognate interactions between mast cells and $CD4^+$ T lymphocytes? We propose that antigen presentation by mast cells to effector T lymphocytes is instrumental to optimize bidirectional crosstalks between these cells in the course of immune responses (supplemental Figure 8).

From the "T helper cell point of view," the possibility that tissue mast cells might detect, internalize, and present antigens make them important "refueling" cells able to restimulate in situ effector $CD4^+$ T lymphocytes. In other words, mast cells, although less efficient than professional APCs, might offer strategically located platforms for antigen presentation that would locally boost the activation state of effector T lymphocytes, thus favoring their biologic responses. The fact that mast cells produce IL-16 and several chemokines such as CCL3, CCL4, CCL5, CCL20[3,19] that have specific receptors on effector $CD4^+$ lymphocytes, makes T cell–mast cell encounters in tissues likely.

From the "mast cell point of view," the observation that cognate interactions can be established with T cells suggests that activated T cells might boost mast cell functions. Evidence in support of this hypothesis comes from our observation that on cognate interaction with OT-II T cells, PCMCs increase their sensitivity to anti-DNP IgE/DNP-HSA stimulation. Thus, after cognate interaction with Th cells, mast cells might lower their threshold for IgE-mediated stimulation and might become more efficient in antigen presentation to T cells. These observations extend previous studies showing that mast cells exhibit enhanced responses after close contact with activated T cells.[16,17]

It is tempting to speculate that, on one hand, the APC role of tissue mast cells might have a positive effect on the development of adaptive immune responses, but, on the other hand, it might become detrimental in autoimmune diseases (supplemental Figure 8). By responding to Th-derived signals via class II up-regulation and antigen presentation, mast cells can contribute to create an activation loop favorable to the establishment of inflammation. Activated mast cells would then strongly contribute to the inflammatory milieu and would also participate to tissue damage via release of their granule content, thus favoring epitope spreading in the course of the autoimmune process. A recent report showing that regulatory T cells suppress mast cells during inflammatory processes is in agreement with the idea that mast cells might play a role in the establishment of autoimmunity.[50]

In conclusion, our results shed new light on mast cell function. We show that among the heterogeneous mast cell population only a

fraction is able to successfully present antigens to effector CD4+ T lymphocytes, yet this cognate interaction results in cell-cell cooperation at the IS. Further research, looking at mast cell biology from this new angle, will be instrumental to broaden our understanding of their contribution to immunity.

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Authorship

Contribution: N.G., N.E., L.T.M., and E.E. performed experiments; N.G. and E.E. analyzed results and made the figures; R.L. provided reagents and discussed results; and S.V. and E.E. designed the research and wrote the paper.

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Cell-cell cooperation at the T helper cell/mast cell immunological synapse

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Chapter 2
Role of Cytokines

Introduction

Cytokines are small glycoproteins produced by a number of cell types, predominantly leukocytes, that regulate immunity, inflammation and hematopoiesis. They regulate a number of physiological and pathological functions including innate immunity, acquired immunity and a plethora of inflammatory responses. The discovery of cytokines was initiated in the 1950s, but the precise identification of their structure and function took many years. The original discoveries were those of IL-I, IFN and nerve growth factors (NGFs); however, these cytokines were purified and given their names years later. Elucidation of the precise physiological, pathological and pharmacological effects of some of the cytokines is still in progress. The modern techniques of molecular biology were principally responsible for their complete identification and as a consequence, several hundred cytokine proteins and genes have been identified, and the process still continues.

Cytokines are produced from various sources during the effector phases of natural and acquired immune responses and regulate immune and inflammatory responses. They are also secreted during nonimmune events and play a role unrelated to the immune response in many tissues. Generally, their secretion is a brief, self-limited event. They not only are produced by multiple diverse cell types, but also act upon many different cell types and tissues. Cytokines often have multiple effects on the same target cell and may induce or inhibit the synthesis and effects of other cytokines. After binding to specific receptors on the cell surface of the target cells, cytokines produce their specific effects. Multiple signals regulate the expression of cytokine receptors. The target cells respond to cytokines by new mRNA and protein synthesis, which results in a specific biological response.

Interleukin-1

Interleukin-1 was originally discovered as a factor that induced fever, caused damage to joints and regulated bone marrow cells and lymphocytes, it was given several different names by various investigators. Later, the presence of two distinct proteins, IL-1α and IL-1β, was confirmed, which belong to a family of cytokines, the

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IL-1 superfamily. Ten ligands of IL-1 have been identified, termed IL-1F1 to IL-1F10. With the exception of IL-1F4, all of their genes map to the region of chromosome 2. IL-1 plays an important role in both innate and adaptive immunity and is a crucial mediator of the host inflammatory response in natural immunity. The major cell source of IL-1 is the activated mononuclear phagocyte. Other sources include dendritic cells, epithelial cells, endothelial cells, B cells, astrocytes, fibroblasts and Large granular lymphocytes (LGL). Endotoxins, macrophage-derived cytokines such as TNF or IL-1 itself, and contact with $CD4^+$ cells trigger IL-1 production. IL-1 can be found in circulation following Gram-negative bacterial sepsis. It produces the acute-phase response in response to infection. IL-1 induces fever as a result of bacterial and viral infections. It suppresses the appetite and induces muscle proteolysis, which may cause severe muscle "wasting" in patients with chronic infection. IL-1β causes the destruction of β cells leading to type 1 diabetes mellitus. It inhibits the function and promotes the apoptosis of pancreatic β cells. Activation of T-helper cells, resulting in IL-2 secretion, and B-cell activation are mediated by IL-1. It is a stimulator of fibroblast proliferation, which causes wound healing. Autoimmune diseases exhibit increased IL-1 concentrations. It suppresses further IL-1 production via an increase in the synthesis of PGE_2.

IL-1s exert their effects via specific cell surface receptors that include a family of about nine members characterized as IL-1R1 to IL-1R9. All family members with the exception of IL-1R2 have an intracellular TLR domain. Each type of receptor in the family has some common and some unique features. The ligands (Table 2.1) for all of these receptors have not yet been identified.

Kineret (Anakinra)

Kineret is a human IL-1 receptor antagonist and is produced by recombinant DNA technology. It is nonglycosylated and is made up of 153 amino acids. With the exception of an additional methionine residue, it is similar to native human IL-1Ra. Human IL-1Ra is a naturally occurring IL-1 receptor antagonist, a 17-kDa protein, which competes with IL-1 for receptor binding and blocks the activity of IL-1.

Table 2.1 IL-1 Ligands and Their Receptors

Ligand Name	Receptor Name
IL-1F1	IL-1R1, IL-1R2
IL-1F2	IL-1R1, IL-1R2
IL-1F3	IL-1R1, IL-1R2
IL-1F4	IL-1R5
IL-1F5	IL-1R6
IL-1F6	Unknown
IL-1F7	IL-1R5
IL-1F8	Unknown
IL-1F9	IL-1R6
IL-1F10	IL-1R1

Kineret is recommended for the treatment of severely active rheumatoid arthritis for patients 18 years of age or older. It is recommended for patients who have not responded well previously to the disease-modifying antirheumatic drugs. It reduces inflammation, decreases bone and cartilage damage and attacks active rheumatoid arthritis. The drug can be used alone or in combination with other antirheumatic drugs. However, it is not administered in combination with TNF-α antagonists. Kineret also improves glycemia and β-cell secretory function in type 2 diabetes mellitus. It is administered daily at a dose of 100 mg/day by subcutaneous injection.

The most serious side effects of Kineret are infections and neutropenia. Injection site reactions are also common. Other side effects may include headache, nausea, diarrhea, flu-like symptoms and abdominal pain. The increased risk of malignancies has also been observed.

Interleukin-2

IL-2, a single polypeptide chain of 133 amino acid residues, is produced by immune regulatory cells that are principally T cells. When a helper T cell binds to an APC using CD28 and B7, $CD4^+$ cells produce IL-2. IL-2 supports the proliferation and differentiation of any cell that has high-affinity IL-2 receptors. It is necessary for the activation of T cells. Resting T lymphocytes (unstimulated) belonging to either the $CD4^+$ or the $CD8^+$ subsets possess few high-affinity IL-2 receptors, but following stimulation with specific antigen, there is a substantial increase in their numbers. The binding of IL-2 with its receptors on T cells induces their proliferation and differentiation.

IL-2 is the major growth factor for T lymphocytes, and the binding of IL-2 to its specific receptors on TH cells stimulates the proliferation of these cells and the release of a number of cytokines from these cells. IL-2 is required for the generation of $CD8^+$ cytolytic T cells, which are important in antiviral responses. It increases the effector function of NK cells. When peripheral blood lymphocytes are treated with IL-2 for 48–72 h, lymphokine-activated killer (LAK) cells are generated, which can kill a much wider range of targets including the tumor cells. IL-2 enhances the ability of the immune system to kill tumor cells and may also interfere with the blood flow to the tumors. It not only induces lymphoid growth but also maintains peripheral tolerance by generation of regulatory T cells. IL-2 knockout mice produce a wide range of autoantibodies and many die of autoimmune hemolytic anemia, which suggests that it plays a role in immune tolerance.

Interleukin-2 Receptors

The IL-2 receptor occurs in three forms with different affinities for IL-2; the three distinct subunits are the α, β and γ chains. The monomeric IL-2Rα possesses low affinity, the dimeric IL-2Rβγ has intermediate affinity and the trimeric IL-2Rαβγ has high affinity (Table 2.2). The α chain is not expressed on resting T cells but

Table 2.2 IL-2 Receptors

	Low Affinity	Intermediate Affinity	High Affinity
Affinity constant (M)	10^{-8}	10^{-7}	10^{-11}
Dissociation constant (M)	10^{-8}	10^{-9}	10^{-11}
Subunits	IL-2Rα	IL-2Rβγ	IL-2Rαβγ

only on activated T cells and is also called TAC (T cell activation) receptor. Both β and γ chains are required for the signal transduction mediated via IL-2 receptors. The low-affinity and high-affinity IL-2 receptors are expressed by activated $CD4^+$ and $CD8^+$ T cells and in low numbers on activated B cells. The intermediate-affinity IL-2 receptors are expressed on NK cells and in low numbers on resting T cells.

When IL-2 binds to high-affinity receptors, it becomes internalized following receptor-mediated endocytosis. After high-affinity binding, there is an increase in the stimulation of phosphoinositol turnover, redistribution of protein kinase C from the cytoplasm to the cell membrane, and an increased expression of IL-2 receptors, with low-affinity receptors being preferentially increased.

Clinical Uses of Interleukin-2

Immunotherapy for Cancer

Proleukin (Aldesleukin)

Proleukin is a recombinant human IL-2 that received approval for the treatment of renal cell carcinoma in 1992 and for the treatment of metastatic melanoma in 1998. It is also being evaluated for the treatment of non-Hodgkin's lymphoma (NHL). The therapy is restricted to patients with normal cardiac and pulmonary functions.

The treatment generally consists of two treatment cycles, each lasting for 5 days and separated by a rest period. Every 8 h a dose of 600,000 IU/kg (0.037 mg/kg) is administered. The IV infusion period is 15 min and a maximum of 14 doses are administered. After a rest period of 9 days, another 14 doses are administered. Additional treatment can be given following an evaluation after 4 weeks.

The most frequent adverse reactions associated with the administration of proleukin include fever, chills, fatigue, malaise, nausea and vomiting. It has also been associated with capillary leak syndrome (CLS). CLS is defined as a loss of vascular tone and effusion of plasma proteins and fluids into the extravascular space. This leads to hypotension and decreased organ perfusion, which may cause sudden death. Other side effects include anaphylaxis, injection site necrosis and possible autoimmune and inflammatory disorders.

Lymphokine-Activated Killer Cell Therapy

IL-2 has been tested for antitumor effects in cancer patients as part of LAK therapy. LAK cell therapy involves infusion into cancer patients of their own (autologous)

lymphocytes after they have been treated in vitro with IL-2 for a minimum of 48 h to generate LAK cells. IL-2 needs to be administered with LAK cells in doses ranging from 10^3 to 10^6 U/m^2 body area or from 10^4 to 10^5 U/kg body weight.

Interleukin-2 and AIDS

HIV is a retrovirus that infects $CD4^+$ cells. After HIV becomes integrated into the genome of the $CD4^+$ cells, activation of these cells results in the replication of virus, which causes lysis of the host cells. Patients infected with HIV, and with AIDS, generally have reduced numbers of helper T cells and the CD4:CD8 ratio may be as low as 0.5:1 instead of the normal 2:1. As a consequence, very little IL-2 is available to support the growth and proliferation of $CD4^+$ cells despite the presence of effector cells, B cells and cytolytic T cells.

Proleukin has not been approved for the treatment of HIV; however, studies show that proleukin in combination with antiretroviral therapy significantly increases the number of $CD4^+$ cells. Low-frequency doses of subcutaneous proleukin at maintained intervals increased $CD4^+$ cell levels. The CD4 count increased from 520 cells/μl to 1005 cells/μl, and the mean of $CD4^+$ cells present from 27 to 38%. The overall effects of proleukin administration in combination with other anti-HIV drugs are being studied to determine the regulation of immune response as well as a delay in the progression of HIV disease.

Interleukin-4

IL-4 is a pleiotropic cytokine produced by TH_2 cells, mast cells and NK cells. Other specialized subsets of T cells, basophils and eosinophils also produce IL-4. It regulates the differentiation of antigen-activated naïve T cells. These cells then develop to produce IL-4 and a number of other TH_2-type cytokines including IL-5, IL-10 and IL-13. IL-4 suppresses the production of TH_1 cells. It is required for the production of IgE and is the principal cytokine that causes isotype switching of B cells from IgG expression to IgE and IgG4. As a consequence, it regulates allergic disease. IL-4 leads to a protective immunity against helminths and other extracellular parasites. The expression of MHC class II molecules on B cells and the expression of IL-4 receptors are upregulated by IL-4. In combination with TNF, IL-4 increases the expression of VCAM-1 and decreases the expression of E-selectin, which results in eosinophil recruitment in lung inflammation.

IL-4 mediates its effects via specific IL-4 receptors that are expressed on a number of tissues including hematopoietic cells, endothelium, hepatocytes, epithelial cells, fibroblasts, neurons and muscles. The receptor is composed of an α chain, which is the high-affinity receptor, but its signaling requires a second chain, a γ chain (γC), which is also a component of IL-2 receptors. However, the presence of a γ chain does not significantly increase the affinity of the receptor complex for IL-4. IL-4 causes the heterodimerization of the α chain with the γ chain, resulting in IL-4 receptor-dependent signaling pathway. As is the case with other cytokines,

the signaling pathways activated after the binding of IL-4 to its receptors are insulin receptor substrate (IRS-1/2) and Janus family tyrosine kinases–signal transducers and activators of transcription (JAK–STAT) pathways. However, for IL-4, the specificity results from the activation of STAT-6.

The antibodies to IL-4 inhibit allergen-induced airway hyperresponsiveness (AHR), globlet cell metaplasia and pulmonary eosinophilia in animal models. Inhibition of IL-4 by soluble IL-4 receptor (SIL-4R, Nuvance) has proven to be very promising in treating asthma. Clinical trials with recombinant SIL-4R administered by a single weekly dose of 3 mg via nebulization have been effective in controlling the symptoms of moderate persistent asthma.

Interleukin-5

IL-5 is secreted predominantly by TH_2 lymphocytes. However, it can also be found in mast cells and eosinophils. It regulates the growth, differentiation, activation and survival of eosinophils. IL-5 contributes to eosinophil migration, tissue localization and function, and blocks their apoptosis. Eosinophils play a seminal role in the pathogenesis of allergic disease and asthma and in the defense against helminths and arthropods. The proliferation and differentiation of antigen-induced B lymphocytes and the production of IgA are also stimulated by IL-5. TH_2 cytokines IL-4 and IL-5 play a central role in the induction of airway eosinophilia and AHR. It is a main player in inducing and sustaining the eosinophilic airway inflammation.

IL-5 mediates its biological effects after binding to IL-5R, which is a membrane-bound receptor. The receptor is composed of two chains, a ligand-specific α receptor (IL-5Rα) and a shared β receptor (IL-5Rβ). The β chain is also shared by IL-3 and GM-CSF, resulting in overlapping biological activity for these cytokines. The signaling through IL-5R requires receptor-associated kinases. Two different signaling cascades associated with IL-5R include JAK/STAT and Ras/mitogen-activated protein kinase (MAPK) pathways.

IL-5 is usually not present in high levels in humans. However, in a number of disease states where the number of eosinophils is elevated, high levels of IL-5 and its mRNA can be found in the circulation, tissue and bone marrow. These conditions include the diseases of the respiratory tract, hematopoietic system, gut and skin. Some other examples include food and drug allergies, atopic dermatitis, aspirin sensitivity and allergic or nonallergic respiratory diseases.

Another way of interfering with IL-5 or IL-5R synthesis is by the use of antisense oligonucleotides. Antisense oligonucleotides are short synthetic DNA sequences that can hybridize specifically to the mRNA of the cytokine or its receptors. This will result in the inhibition of the transcription and processing of mRNA. The administration of IL-5-specific antisense oligonucleotides results in reduced lung eosinophilia in animal models. However, there is no complete inhibition of antigen-specific late-phase AHR, suggesting that in addition to IL-5, other pathways may also be involved in airway hyperreactivity.

Interleukin-6

IL-6 is a proinflammatory cytokine, which is a member of the family of cytokines termed "the IL-6 type cytokines." The cytokine affects various processes including the immune response, reproduction, bone metabolism and aging. IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts and other cells in response to trauma, burns, tissue damage, inflammation, IL-1 and, to a lesser extent, TNF-α. Pathogen-associated molecular patterns (PAMPs) binding to the TLRs present on macrophage result in the release of IL-6. This cytokine is synthesized by some activated T cells as well. It is also secreted by osteoblasts to stimulate osteoclast formation. Acute-phase response and fever are caused by IL-6, which is also the case for IL-1 and TNF-α. It affects differentiation of B cells and causes neutrophil mobilization. IL-6 is elevated in patients with retroviral infection, autoimmune diseases and certain types of benign or malignant tumors. It stimulates energy mobilization in the muscle and fatty tissue, resulting in an increase in body temperature. IL-6 acts as a myokine — a cytokine produced by muscles — and muscle contraction occurs as a result of elevated IL-6 concentrations. The expression of IL-6 is regulated by various factors, including steroidal hormones, which could be at both transcriptional and posttranscriptional levels. IL-6 mediates its effects via binding to cell surface receptors, IL-6R, which are active in both membrane-bound and soluble forms.

Interleukin-9

Originally described as a mast cell growth factor due to its ability to promote the survival of primary mast cells and as an inducer of IL-6 production, IL-9, which is secreted by TH_2 cells, stimulates the release of a number of mediators of mast cells and promotes the expression of the high-affinity IgE receptors (FcεR1α). IL-9 augments TH_2-induced inflammation and enhances mucus hypersecretion and the expression of its receptors is increased in asthmatic airways. It also promotes eosinophil maturation in synergy with IL-5. IL-9 activates airway epithelial cells by stimulating the production of several chemokines, proteases, mucin genes and ion channels. It is important to point out that, as opposed to the IL-4-induced isotype switching and production of IgE or the IL-5-mediated stimulation of eosinophil maturation, IL-9 induces actions of other cytokines. It is an essential cytokine for asthmatic disease as biopsies from asthmatic patients show an increase in the expression of IL-9 compared to healthy individuals, and therefore it is an important therapeutic target for clinical intervention.

Interleukin-10

First identified as an inhibitor of IFN-γ synthesis in TH_1 cells, IL-10 is an important immunoregulatory cytokine. It is an anti-inflammatory cytokine that was first called

human cytokine synthesis inhibitory factor. IL-10 is secreted by macrophages, TH_2 cells and mast cells. Cytotoxic T cells also release IL-10 to inhibit viral infection-stimulated NK cell activity. IL-10 is a 36-kDa dimer composed of two 160-amino-acid-residue-long chains. Its gene is located on chromosome 1 in humans and consists of five exons. IL-10 inhibits the synthesis of a number of cytokines involved in the inflammatory process including IL-2, IL-3, GM-CSF, TNF-α and IFN-γ. Based on its cytokine-suppressing profile, it also functions as an inhibitor of TH_1 cells and by virtue of inhibiting macrophages, it functions as an inhibitor of antigen presentation. Interestingly, IL-10 can promote the activity of mast cells, B cells and certain T cells.

There are several viral IL-10 homologs: Epstein–Barr virus (BCRF.1), cytomegalovirus, herpesvirus type 2, orf virus and Yaba-like disease virus. Now the IL-10 family of cytokines includes not only IL-10 but also its viral gene homologs and several other cytokines including IL-19, IL-20, IL-22, IL-24, IL-26, IFN-λ1, IFN-λ2 and IFN-λ3. IL-10 mediates its effects after binding to two receptor chains, IL-10R1 (α) and IL-10R2 (β). These receptors are members of the class II or IFN receptor family. The interaction of IL-10 with its receptors is highly complex and the IL-10R2 (β) chain is essential for the production of its effects. Several hundred genes are activated after interaction of IL-10 with its receptors. The tyrosine kinases JAK1 and Tyk2 are activated by the interaction of IL-10 with its receptors, which results in the induction of transcription factors STAT1, STAT3 and STAT5, and eventual gene activation.

The major immunobiological effect of IL-10 is the regulation of the TH_1/TH_2 balance. TH_1 cells are involved in cytotoxic T-cell responses whereas TH_2 cells regulate B-cell activity and function. IL-10 is a promoter of TH_2 response by inhibiting IFN-γ production from TH_1 cells. This effect is mediated via the suppression of IL-12 synthesis in accessory cells. IL-10 is involved in assisting against intestinal parasitic infection, local mucosal infection by costimulating the proliferation and differentiation of B cells. Its indirect effects also include the neutralization of bacterial toxins.

IL-10 is a potent inhibitor of IL-1, IL-6, IL-10 itself, IL-12, IL-18, CSF and TNF. It not only inhibits the production of proinflammatory mediators but also augments the production of anti-inflammatory factors including soluble TNF-α receptors and IL-1RA. IL-10 downregulates the expression of MHC class II molecules (both constitutive and IFN-γ-induced), as well as that of costimulatory molecule, CD86, and adhesion molecule, CD58. It is an inhibitor of IL-12 production from monocytes, which is required for the production of specific cellular defense response. IL-10 enhances the expression of CD16, CD32 and CD64 and augments the phagocytic activity of macrophages. The scavenger receptors, CD14 and CD163, are also upregulated on macrophages by IL-10. It is a stimulator of NK cells, enhances their cytotoxic activity, and also augments the ability of IL-18 to stimulate NK cells. Based on its immunoregulatory function, IL-10 and ligands for its receptors are tempting candidates for therapeutic intervention in a wide variety of disease states, including autoimmune disorders, acute and chronic inflammatory diseases, cancer, infectious disease, psoriasis and allergic disease.

Modest but significant improvement has been observed in patients with chronic hepatitis C, Crohn's disease, psoriasis and rheumatoid arthritis after subcutaneous administration of IL-10 in human clinical trials. The systemic administration of IL-10 produces general immune suppression, inhibition of macrophage and T-cell infiltration, less secretion of IL-12 and TNF-α by monocytes and suppression of nuclear factor (NF)-κB induction. In patients with acute myelogenous leukemia, IL-10 increases the serum levels of TNF-α and IL-1β. The use of IL-10 for human cancer therapy is under investigation and despite its immunosuppressive effects it may serve a role as a facilitator in preconditioning tumors to be recognized by immune effector cells.

Interleukin-11

IL-11, a member of the IL-6 superfamily, is produced by bone marrow stroma and activates B cells, plasmacytomas, hepatocytes and megakaryocytes. The gene for IL-11 is located on chromosome 19. IL-11 induces acute-phase proteins, plays a role in bone cell proliferation and differentiation, increases platelet levels after chemotherapy and modulates antigen–antibody response. It promotes differentiation of progenitor B cells and megakaryocytes. The recovery of neutrophils is accelerated by IL-11 after myelosuppressive therapy. IL-11 also possesses potent anti-inflammatory effects due to its ability to inhibit nuclear translocation of NF-κB. Additional biological effects of this cytokine include epithelial cell growth, osteoclastogenesis and inhibition of adipogenesis. The effects of IL-11 are mainly mediated via the IL-11 receptor α chain. IL-11 forms a high-affinity complex in association with its receptor and associated proteins and induces gp130-dependent signaling.

Oprelvekin (Neumega)

Recombinant human IL-11 (oprelvekin) is a polypeptide of 177 amino acids. It differs from natural IL-11 due to lack of glycosylation and the amino-terminal proline residue. Oprelvekin is administered by subcutaneous injection, usually 6–24 h after chemotherapy, at a dose of 25–50 μg/kg per day. The drug has a half-life of about 7 h. It is used to stimulate bone marrow to induce platelet production in nonmyeloid malignancies in patients undergoing chemotherapy. The common side effects of oprelvekin include fluid retention, tachycardia, edema, nausea, vomiting, diarrhea, shortness of breath and mouth sores. Other side effects include rash at the injection site, blurred vision, paresthesias, headache, fever, cough and bone pain. Rarely, CLS may occur.

Interleukin-13

IL-13 belongs to the same α-helix superfamily as IL-4, and their genes are located 12 kb apart on chromosome 5q31. It was originally identified for its effects on B

cells and monocytes, which included isotype switching from IgG to IgE, inhibition of inflammatory cytokines and enhancement of MHC class II expression. Initially, IL-13 appeared similar to IL-4 until its unique effector functions were recognized. Nevertheless, IL-13 and IL-4 have a number of overlapping effects. IL-13 also plays an essential role in resistance to most GI nematodes.

It regulates mucus production, inflammation, fibrosis and tissue remodeling. IL-13 is a therapeutic target for a number of disease states including asthma, idiopathic pulmonary fibrosis, ulcerative colitis, cancer and others. Its signaling is mediated via IL-4 type 2 receptor. The receptor consists of IL-4Rα and IL-13Rα1 and IL-13Rα2 chains.

IL-13 induces physiological changes in organs infected with parasites that are essential for eliminating the invading pathogen. In the gut, it induces a number of changes that make the surrounding environment of the parasite less hospitable, such as increasing contractions and hypersecretion of glycoproteins from gut epithelial cells. This results in the detachment of the parasites from the wall of the gut and their subsequent removal. IL-13 response in some instances may not resolve infection and may even be deleterious. For example, IL-13 may induce the formation of granulomas after organs such as the gut wall, lungs, liver and central nervous system are infected with the eggs of *Schistosoma mansoni*, which may lead to organ damage and could even be life threatening.

IL-13 is believed to inhibit TH_1 responses, which will inhibit the ability of the host to eliminate the invading pathogens. The role of IL-13 in the etiology/pathogenesis of allergic disease/asthma has drawn broad attention. It induces AHR and goblet cell metaplasia, which result in airway obstruction and cause allergic lung disease. IL-13/chemokine interactions play a key role in the development of AHR and mucus production. IL-13 induces the expression of eotaxins. These chemokines recruit eosinophils into the site of inflammation in synergy with IL-5. Eosinophils release IL-13 and induce the production of IL-13 from TH_2 cells, which is mediated via IL-18. IL-13 then, through its effects on epithelial and smooth muscle cells, aids in the development of AHR and mucus production. In addition to its potent activation of chemokines, IL-13 is also an inducer of adhesion molecules involved in asthma.

Interleukin-18

IL-18 is a member of the IL-1 family that promotes the production of various proinflammatory mediators and plays a role in cancer and various infectious diseases. It was originally identified as IFN-γ-inducing factor and is produced by cells of both hematopoietic and nonhematopoietic lineages, including macrophages, dendritic cells, intestinal epithelial cells, synovial fibroblasts, keratinocytes, Kupffer cells, microglial cells and osteoblasts. The production of IL-18 is structurally homologous to that of IL-1β; it is produced as an inactive precursor of 24 kDa, which lacks a signal peptide. Endoprotease IL-1β-converting enzyme activates it after cleaving pro-IL-18, resulting in a biologically active cytokine. Caspase-1 plays an important role in the processing of IL-18, but is not exclusive since proteinase 3 can also perform the same function.

IL-18 augments T- and NK-cell maturation, cytotoxicity and cytokine production. It stimulates TH differentiation, promotes secretion of TNF-α, IFN-γ and GM-CSF and enhances NK cell cytotoxicity by increasing FasL expression. IL-8-mediated neutrophil chemotaxis is promoted by IL-18 via its effects on TNF-α and IFN-γ, which are stimulatory in action. It plays an important role in maintaining synovial inflammation and inducing joint destruction in rheumatoid arthritis. In synovium of patients with rheumatoid arthritis, enhanced levels of TNF-α and IL-1 are associated with augmented expression of IL-18.

IL-18 also induces IL-4, IL-10 and IL-13 production, increases IgE expression on B cells and in association with IL-2, it enhances stimulus-induced IL-4 production from TH_2 cells. Bone marrow-derived basophils produce IL-4 and IL-13 in response to a stimulus from IL-18 and IL-3. IL-18 in combination with IL-12 induces IFN-γ from dendritic cells and bone marrow-derived macrophages. Adhesion molecules, ICAM-1 and VCAM-1, are induced by this cytokine on synovial fibroblasts and endothelial cells. It inhibits osteoclast formation via its induction of GM-CSF from T cells. The receptors of IL-18, IL-18Rα and IL-18Rβ, share their signaling mechanisms via the IL-1R family. Toll-like receptors also share the downstream signaling pathway of IL-18 and are known to regulate IL-18 expression.

IL-18 plays a critical role in host defense against bacterial, viral, fungal and protozoan infections. One predominant mechanism is the induction of host IFN-γ production, which activates several effector pathways including nitric oxide production, resulting in the clearance of the invading pathogens. A role of IL-18 in robust TH_1 responses against *Mycobacterium tuberculosis* and *Mycobacterium avium* has been suggested. For viral infections, the effects of IL-18 are mediated not only via IFN-γ but also by activation of $CD8^+$ T cells. IL-12 and IL-15 also play a role in its effects in host defense and as mediator of inflammation where IL-18 works in concert with other cytokines and their signaling pathways. Its modulation of inflammation is at multiple checkpoints. IL-18 binding protein (IL-18 BP) is the naturally occurring antagonist that may serve as a negative feedback mechanism for IL-18 as several isoforms of this antagonist have been identified.

Interferons

Although originally identified as proteins with antiviral activity, these inducible cytokines play an important role in regulating innate and acquired immunity. Initially characterized by the secreting cell type, IFNs are now divided into two groups, type I and type II IFNs. Type I IFNs, which are also called IFN-α/β family, are the product of numerous genes and include IFN-α, IFN-β, IFN-ω, IFN-κ, IFN-ε and IFN-λ. Almost all cell types secrete type I IFN; however, hematopoietic cells are the major source of IFN-α and IFN-ω, and fibroblasts are the major producers of IFN-β. Macrophages under appropriate induction also secrete IFN-β. Their structural genes are located on chromosome 9 in humans. The type II IFN IFN-γ is the product of a single gene on chromosome 12 in humans. The stimuli for the production of type I IFNs are viral and microbial infections and double-stranded RNA.

Type I Interferons

Type I IFNs are two distinct groups of proteins, IFN-α (approx. 18 kDa) and IFN-β (20 kDa). IFN-α is subdivided into two subgroups, IFN-α1 and IFN-α2/IFN-ω. Viral infection is the most potent natural signal for the synthesis of type I IFNs.

The principal biological actions of type I IFNs include inhibition of viral replication, inhibition of cell proliferation, increase in the lytic potential of NK cells and the modulation of MHC molecule expression. They increase the expression of MHC class I molecules and decrease the expression of MHC class II molecules.

Type I IFNs exerts their biological effects after binding to distinct heterodimeric cell surface receptors on the target cells. Binding of the agonist to the cell surface receptors results in activation of the Janus-activated kinase (JAK)–STAT signaling pathway. The JAK–STAT activation results in the induction of specific genes. These genes contain IFN-specific response elements or IFN-γ-stimulated sequence. The IFNs have both overlapping and distinct pharmacological activities because some genes overlap partially, whereas some IFNs are produced at different sites.

IFN-α/β mediate antiviral activity by multiple mechanisms. A series of antiviral proteins are produced after IFN-α/β bind to their specific cell surface receptors. The proteins induced by IFNs include a 2′, 5′-oligoadenylate synthetase and a protein kinase; both in the presence of double-stranded RNA can inhibit protein synthesis. A latent cellular endoribonuclease is activated by adenylate oligomers produced by an oligoadenylate synthetase, which breaks down viral as well as cellular single-stranded RNAs. The protein kinase inactivates eukaryotic initiation factor (EIF)-2 after phosphorylation, which is involved in protein synthesis and is also an effector for apoptosis. Furthermore, peptide elongation is prevented as a result of cleaving of transfer RNA by a phosphoesterase that is induced by IFN-α/β. Depending on the family of the virus, multiple steps may be inhibited by IFN to varying degrees.

Clinical Applications of Interferons

Interferon-α

IFN-α may be used for the treatment of condylomata acuminata (venereal or genital warts), malignant melanoma, hairy cell leukemia and hepatitis B and C, and other types of cancer including skin, kidney and bone cancers.

Interferon-α-2a (Roferon-A)

Produced by recombinant DNA technology, IFN-α-2a is used for the treatment of chronic myeloid leukemia, Kaposi sarcoma, lymphoma, hairy cell leukemia, hepatitis B and C and cancer of the skin and kidney. It can only be administered by injection or into the bloodstream, and the most common method is subcutaneous injection. This cytokine can be injected every day; however, commonly it is

administered three times a week. The antiviral or antitumor activity of IFN-α-2a is mediated via inhibition of viral replication and modulation of host immune response as well as its antiproliferative activity. It is filtered through the glomeruli, and its proteolytic degradation takes place during tubular reabsorption. The common side effects include flu-like symptoms of fever, fatigue, chills, dry mouth, GI disorders, changes in mood and temporary effects on the bone marrow. The occasional side effects may include skin rash, hair thinning, loss of appetite and loss of fertility.

Peginterferon-α-2a

Pegylated α-IFN is made by attaching polyethylene glycol (PEG) to the α-IFN. PEG is a large water-soluble molecule that decreases the clearance of α-IFN and also increases the duration of its activity. This modified cytokine is used to treat chronic hepatitis C. However, it is rarely used as a single therapeutic agent for hepatitis C because of its low response rate.

Interferon-α-2b

IFN-α-2b is a water-soluble α-IFN protein produced by recombinant DNA technology. Both IFN-α-2b and -α-2a are pure clones of single IFN subspecies, but they differ by virtue of two amino acids. The potencies of both α-2a and α-2b IFNs are similar. IFN-α-2b is also available in pegylated form. All IFN-α cytokines augment the killing of target cells by lymphocytes and inhibit the replication of virus in infected cells.

Interferon-β

Natural IFN-β is predominantly synthesized by fibroblasts. Its sequence is 30% homologous to that of IFN-α. The receptors for both IFN-α and -β are the same but the fit of the receptor is different for the two agonists. There are also differences between IFN-α and -β in structure (IFN-β is glycosylated on one site, pharmacokinetics and binding to tissues.

IFN-β-1a is used to treat patients with a relapsing form of MS. It is not a cure for MS; however, it may slow some disabling effects of the disease. IFN-β-1a may also decrease the number of relapses of MS. The possible mechanisms of action for the treatment of MS include the antagonism of IL-4 and IFN-γ. It also modifies the mechanics of blood barrier since it inhibits cell adhesion, cell migration and metalloproteinase activity. IFN-β induces IL-10 and TGF-β, which are anti-inflammatory cytokines. It is also used for the treatment of genital warts.

The available preparations for IFN-β-1a include Avonex and Rebif, both synthesized by recombinant DNA technology. They are similar but Rebif is administered more frequently and at a higher dose. A third preparation, Betaseron, is IFN-β-1b.

Type II Interferons

Interferon-γ

IFN-γ modulates a number of components of the immune response. This is the only type II IFN whereas there are more than 20 types of type I IFNs (IFN-α, IFN-β, IFN-ω and IFN-τ). It is not related to type I IFNs, has separate receptors and is encoded by a different chromosomal locus. IFN-γ is produced by activated T lymphocytes (TH_1 and $CD8^+$ cells), NK cells, B cells, NKT cells and professional APCs. It promotes the activity of cytolytic T lymphocytes, macrophages and NK cells. The cell self-activation and activation of nearby cells in part may result from IFN-γ production by professional APCs, which include monocyte/macrophage and dendritic cells. The early host defense against infection is likely to utilize IFN-γ secreted by NK and professional APCs. In acquired immune responses, T lymphocytes are the major source of IFN-γ.

IFN-γ production is regulated by IL-12 and IL-18, both cytokines secreted by APCs. In the innate immune response, a link is established between infection and IFN-γ by these cytokines. IL-12 and chemokines including macrophage inflammatory protein-1α (MIP-1α) are secreted as macrophages recognize pathogens, and NK cells are attracted to the site of inflammation by the chemokines. This is followed by the induction of IFN-γ production and secretion by IL-12. IL-12 and IL-18 further stimulate the production of IFN-γ from macrophages. The production of IFN-γ is inhibited by IL-4, IL-10 and TGF-β.

IFN-γ is a potent activator of mononuclear phagocytes. The expression of both MHC class I and class II molecules is augmented by IFN-γ as IFN-γ-induced upregulation of MHC class I molecules is pivotal for host defense against intracellular pathogens, resulting in an increased susceptibility to cytolytic T cells for recognition and consequent promotion of cell-mediated immune response. The stimulation by IFN-γ results in the addition of "immunoproteosome subunits" and the removal of constitutive proteosome subunits. The unstimulated cells contain β_1, β_2 and β_5 proteosome enzymatic subunits, which are encoded outside the MHC locus. β_1 is replaced by LMP2, β_2 by MECL-1 and β_5 by LMP7, and the expression of the new subunit is stimulated by IFN-γ. This results in the formation of new subunits of proteosomes. This is a potential mechanism utilized by IFN-γ to enhance the characteristics of peptides for MHC class I loading. The ability of immunoproteosomes to cleave peptides enhances the ability of antigen fragments to bind to MHC class I molecules. The diversity of the antigenic fragments is increased, resulting in better immune surveillance. IFN-γ also augments the MHC class II antigen-presenting pathway and results in the activation of $CD4^+$ T cells via peptides. It not only stimulates the expression of MHC class II molecules on cells that constitutively express these antigens but also induces their expression on cells that do not constitutively express their genes. The expression of several other molecules including Ii chain, cathepsins B, H, L, lysosomal proteases and HLA-DM is upregulated by IFN-γ. These molecules are involved in various processes associated with antigen presentation, peptide accessibility and peptide loading.

Table 2.3 Characteristics of Selected Cytokines

Name	Source	Target	Biological Role
IL-1 (IL-1α and -β)	Macrophages, dendritic cells, endothelial cells, other cells	TH and B cells and various other tissues	Activation (other details provided in the text)
IL-2	TH_1 cells	TH, T_C and NK cells	T cell and NK proliferation and induction of activity
IL-3	TH_1 and TH_2 cells, mast cells, NK cells	Hematopoietic and mast cells	Progenitor cell proliferation and differentiation
IL-4	TH_2 cells, mast cells, NK cells	B cells, T cells, mast cells, macrophages	Proliferation, isotype switching, induction of MHC class II expression
IL-5	TH_2 cells, mast cells	Eosinophils	Proliferation and differentiation
IL-6	Macrophages, TH_2 cells	Plasma cells, B cells and others	Differentiation and antibody secretion
IL-8	Bone marrow, thymus (stromal cells)	Neutrophils	Chemoattractant
IL-9	TH_2 cells	TH cells, mast cells, eosinophils	Induces inflammatory responses
IL-10	TH_2 cells	Macrophages, APC	Anti-inflammatory cytokine inhibits cytokine production
IL-11	Bone marrow (stromal cells)	B-cell progenitors and others	Differentiation
IL-12	Macrophages, B cells	T_C, NK and LAK cells	Proliferation and differentiation in synergy with IL-2
IL-13	TH cells	Macrophages, B cells	Inhibition of inflammatory cytokines, regulation of inflammation. Parasitic infections
IL-16	T_C cells	TH cells	Chemotaxis
IL-18	Hematopoietic and nonhematopoietic lineage cells	T cells, NK cells	Proinflammatory cytokine; IFN-γ-inducing factor
IFN-α	Leukocytes		Inhibitor of viral replication
IFN-β	Fibroblasts		Inhibitor of viral replication
IFN-γ	TH_1, T_C, NK	Various cells including macrophages	Inhibitor of viral replication. Inhibitor of cell proliferation. Inhibitor of IL-4-induced isotype switching
TNF-α	Macrophages	Tumor cells, polymorphonuclear leukocytes, macrophages	Cytotoxicity, induction of cytokine secretion
TNF-β	T cells	Tumor cells, neutrophils, macrophages	Cytotoxicity, phagocytosis

IFN-γ is produced by TH_1 cells and shifts the response toward a TH_1 phenotype. This is accomplished by activation of NK cells that promotes innate immunity, augmenting specific cytolytic response and induction of macrophages. The induction of cytotoxic immunity can be direct or indirect via suppression of TH_2 response. Another direct effect of IFN-γ is the differentiation of naïve $CD4^+$ lymphocytes toward a TH_1 phenotype. The cytokines present are very important in this differentiation process. Furthermore, induction of IL-12 and suppression of IL-4 by IFN result in differentiation toward a TH_1 phenotype.

IFN-γ is an inhibitor of cell growth and proliferation. The proliferation is inhibited by augmenting the levels of Cip/Kip, CKIs and Ink4. It increases p21 and p27 CKIs, which inhibit the function of cyclin E:CDK2 and cyclin D:CDK4, respectively. This results in stopping the cell cycle at G1/S interphase. IFN-γ induces apoptosis via activation of STAT-1, which results in the production of large amounts of IRF-1 (IFN regulatory factors). Apoptosis may be needed to kill the invading pathogen-infected macrophages.

IFN-γ also induces the costimulatory molecules on the macrophages, which increases cell-mediated immunity. As a consequence, there is activation and increase in the tumoricidal and antimicrobial activity of mononuclear phagocytes, granulocytes and NK cells. The activation of neutrophils by IFN-γ includes an increase in their respiratory burst. IFN-γ stimulates the cytolytic activity of NK cells. It is an activator of vascular endothelial cells, promoting $CD4^+$ T lymphocyte adhesion and morphological alterations, which facilitates lymphocyte extravasation. IFN-γ promotes opsonization by stimulating the production of IgG subclasses that activate the complement pathway. A summary of the characteristics of selected cytokines is shown in Table 2.3.

Colony-Stimulating Factors

A major cause of morbidity and mortality in patients who receive cytotoxic treatment or radiotherapy for cancer is bacterial and fungal infections. Intensive chemotherapy is associated with fever and infection, and the development of neutropenia further increases this risk of infection. Consequently, maximum doses of some cytotoxic drugs are limited due to bone marrow toxicity. Higher doses of chemotherapy and radiation therapy have become possible due to a reduction in bone marrow damage with the availability of the CSFs for clinical use.

The CSFs are glycoproteins that support hematopoietic colony formation. They influence the survival, proliferation and maturation of hematopoietic progenitor cells and regulate the activities of the mature effector cells. There are three lineage-specific CSFs, granulocyte colony-stimulating factor (G-CSF), monocyte–macrophage colony-stimulating factor (M-CSF) and erythropoietin, and two multipotential CSFs, IL-3 and GM-CSF.

Clinical Uses of Colony-Stimulating Factors

The CSFs prevent chemotherapy-induced neutropenia. They stimulate hematopoiesis in marrow failure. The CSFs promote cell differentiation, assist in marrow transplantation, stimulate monocyte anticancer effects and augment effector cell function.

Granulocyte Colony-Stimulating Factor

G-CSF is a glycoprotein produced by macrophages, endothelium and various leukocytes. It stimulates the bone marrow to produce granulocytes and stem cells and then directs their migration from the bone marrow to the peripheral blood. G-CSF is a growth factor for the proliferation, differentiation, effector function and survival of neutrophils. The gene for G-CSF is located on chromosome 17, locus q11.2-q12.

G-CSF mobilizes bone marrow-derived cells into the bloodstream. These stem cells can migrate to ischemic myocardium and differentiate into cardiomyocytes, smooth muscle cells and endothelial cells. They may also induce metalloproteinases and vascular endothelial growth factor and thus play a role in tissue healing. Furthermore, G-CSF induces proliferation and enhanced survival of cardiomyocytes. This is accomplished via activation of G-CSF receptors in myocardium. G-CSF in association with TGF-β and collagen enhances ventricular expansion in the infarcted area.

G-CSF activates neutrophils, transforming them into cells capable of respiratory burst and release of secretory granules. It also modulates the expression of adhesion molecules on neutrophils as well as CD11b/CD18 and plasma elastase antigen levels. G-CSF induces proliferation of endothelial cells, phagocytic activity of neutrophils, reactive oxygen intermediate production by neutrophils and antibody-dependent cellular toxicity by neutrophils.

Filgrastim (Neupogen)

Recombinant human G-CSF (filgrastim) is a 175-amino-acid glycoprotein. It differs from natural G-CSF due to lack of glycosylation and has an extra N-terminal methionine. Pegylated recombinant human G-CSF (pegfilgrastim) is also available. Filgrastim administered to patients receiving cytotoxic chemotherapy for advanced cancer has resulted in a dose-dependent amelioration of neutropenia associated with cancer chemotherapy. It is well tolerated and may reduce the morbidity and mortality rate associated with chemotherapy, possibly permitting higher doses and a greater antitumor response. Filgrastim is also used after autologous stem cell transplantation to treat neutropenia. It reduces the duration of neutropenia and lessens morbidity secondary to bacterial and fungal infections. Additional use of this drug includes the treatment of severe congenital neutropenias, of neutropenia in patients with AIDS resulting from treatment with zidovudine and of patients donating peripheral blood stem cells for stem cell transplantation.

Filgrastim is administered by intravenous infusion or subcutaneous injection. The doses given are 1–20 μg/kg per day over at least a 30-min period. Generally a dose of 5 μg/kg is used in patients receiving chemotherapy for 14–21 days or longer. The half-life of the drug is 3.5 h. The side effects include bone pain, local skin reactions and rarely cutaneous vasculitis.

Granulocyte–Macrophage Colony-Stimulating Factor

GM-CSF is a glycoprotein produced by macrophages, T cells, mast cells, fibroblasts and endothelial cells. It stimulates stem cells to produce neutrophils, monocytes, eosinophils and basophils. Monocytes migrating into tissue from the circulating blood differentiate into macrophages and undergo maturation.

Sargramostim (Leukine)

Recombinant human GM-CSF (sargramostim) is a 127-amino-acid glycoprotein, which is similar to natural GM-CSF except for variation in glycosylation and presence of a leucine in position 23. It has beneficial effects on bone marrow function in patients receiving high-dose chemotherapy in the setting of autologous bone marrow transplantation as well as for the treatment of advanced cancers. Sargramostim is used in AIDS, myelodysplastic syndrome and aplastic anemia where it stimulates bone marrow function. It has not shown beneficial effects in graft-versus-host disease but may be of value in patients with early graft failure. It has been used in patients donating peripheral blood stem cells because it mobilizes $CD34^+$ progenitor cells. Sargramostim is administered either by slow intravenous infusion or by subcutaneous injection. The doses given are 125–500 $\mu g/m^2$ per day. Intravenous administration requires a period of at least 3–6 h. The half-life with subcutaneous injection is 2–3 h. The side effects with high doses include bone pain, flu-like symptoms, fever, diarrhea, nausea and vomiting. Prolonged administration has produced marked weight gain, generalized edema, capillary leak and hypotension. It also causes a dose-dependent, asymptomatic eosinophilia.

Tumor Necrosis Factor-α

This proinflammatory cytokine was first isolated in 1975, and its name is misleading in that it does not cause the necrosis of all tumors. As a matter of fact, it may stimulate the growth of some tumors. TNF-α is a 185-amino-acid glycoprotein, which is cleaved from a 212-amino-acid peptide, and the cleavage occurs on the cell surface of mononuclear phagocytes. In humans, the genes for TNF-α are present on chromosome 7p21. The major cell source of TNF-α is the macrophage, specifically the endotoxin-activated mononuclear phagocyte. Other sources include endothelium after tissue damage, antigen-stimulated T cells, activated NK cells and activated mast cells. IFN-γ augments TNF-α synthesis.

TNF-α is a mediator of both natural and acquired immunity. Local increasing concentrations of TNF-α cause heat, swelling, redness and pain. TNF-α causes vascular endothelial cells to express new adhesion molecules. It increases the mobilization and effector function of neutrophils and their adhesiveness for endothelial cells. TNF-α induces the production of IL-1, IL-6, TNF-α itself and chemokines via stimulation of macrophages. It exerts an IFN-like protective effect against viruses and augments expression of MHC class I molecules. TNF-α is an endogenous pyrogen that acts on cells in hypothalamic regulatory regions of the brain to induce fever. It suppresses appetite. The hypothalamic–pituitary–adrenal axis is stimulated via the release of corticotrophin-releasing hormone by TNF-α. TNF-α induces acute-phase responses by activating hepatocytes. Acute-phase proteins including C-reactive protein and mannose-binding protein (MBP) are detected in blood in response to an infection. TNF-α suppresses bone marrow stem cell division and reduces tissue perfusion by depressing myocardial contractility.

Tumor Necrosis Factor Receptors

There are two distinct types of TNF receptors, TNF-R1 (CD120a or P55) and TNF-R2 (CD120b or P75). They are implicated in inflammatory processes and both belong to the TNF receptor superfamily. TNF receptors are transmembrane proteins with intracellular domains that lack intrinsic enzymatic activity, and consequently, they require cytoplasmic proteins that help initiate the receptor-induced signaling pathways. TNF-R1 possesses an intracellular death domain and TNF-R2 interacts with molecules of the TNF receptor-associated factor 2 family (TRAF).

The receptors for TNF-α are widely distributed although TNF-R1 is more common in nonhematopoietic cells. Both groups of receptors interact with the ligand TNF-α (soluble form) with similar affinity. TNF-R1 recognizes both the membrane-bound and soluble TNF-α, whereas TNF-R2 binds to membrane-bound TNF-α with greater affinity. The signals initiated by the two receptors are different since there are structural differences between the intracellular domains of the two receptors. The activated TNF-R1 contains a death domain in its cytoplasmic region that recruits the adopter proteins. The downstream signaling involves different pathways that lead to cell death or survival.

After the binding of TNF-α to its receptors, there is induction of two major intracellular signaling pathways. One pathway leads to the transcription of other genes, and the other pathway leads to cell death or apoptosis. The two main transcription factors activated by TNF-α are AP-1 and NF-κB.

Etanercept (Enbrel)

Etanercept is a genetically engineered protein that is soluble TNF-α receptor. Its molecular weight is 75 kDa. It binds to TNF-α. It is used for the treatment of rheumatoid arthritis, juvenile rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis. Structurally, two TNF-α receptors are linked to an Fc portion of an

IgG1 molecule. Consequently, an artificial antibody is constituted with two Fab sites, which are soluble human 75-kDa TNF-α receptors. It competitively inhibits the binding of TNF molecules to the TNF receptor sites. The binding of etanercept to TNF renders the bound TNF biologically inactive, resulting in the reduction of the inflammatory activity. The most frequent adverse side effects are injection site reactions, infections and headache and malignancies are rare. Etanercept is not recommended for patients with serious infections or sepsis and does not appear to result in the reactivation of tuberculosis.

Chemokines

Chemokines are a large family of small heparin-binding chemotactic cytokines released by many cell types. They are composed of four groups called CXC, CC, C and CX3C. The designation and classification is based on the spacing of conserved cysteines and X is an amino acid. Many members constitute the CXC and CC groups, which is not the case for C and CX3C chemokines. Neutrophils and lymphocytes are the targets of CXC chemokines. The targets of CC chemokines are diverse, including basophils, dendritic cells, macrophages and eosinophils. The CXC family includes chemokines CXCL1–CXCL17, the CC family includes CCL1–CCL28, the C family includes XCL1–XCL2 and the CX3C family includes only CX3CL1.

The early signals produced during innate immune responses are the main stimuli for the secretion of chemokines. Various chemokines are secreted by a stimulus resulting from viral infection, bacterial products (e.g., LPS) and proinflammatory cytokines including IL-1 and TNF-α. Consequently, some chemokines are proinflammatory in nature and are produced during an immune response to direct leukocytes to the site of injury/infection, whereas others are homeostatic in nature and control the migration of cells during routine tissue maintenance or development.

CXC Chemokines

Also termed α-chemokines, CXC chemokines are composed of two N-terminal cysteines, separated by one amino acid designated with an “X” in the name. There are 17 different CXC chemokines and they are divided into two groups. One group has a specific motif of glutamic acid–leucine–arginine (ELR) right before the first cysteine of the CXC motif and is called ELR-positive. The other group does not have this motif and is called ELR-negative. Glutamic acid–leucine–arginine-positive CXC chemokines are specific for neutrophils and mediate their effects via CXCR1 and CXCR2 (CXC receptors 1 and 2). IL-8 is an example of ELR-positive chemokines that direct the migration of neutrophils to the infected tissue. Glutamic acid–leucine–arginine-negative CXC chemokines, for example, CXCL13, are chemoattractants for lymphocytes.

CC Chemokines

Also termed β-chemokines, CC Chemokines are composed of two adjacent cysteines near their amino-terminus. There are at least 27 different CC chemokines of which CCL9 and CCL10 are the same. Most of the members of this group possess four cysteines (C4-CC) but a small number have six cysteines (C6-CC). CC chemokines regulate the migration of monocytes, dendritic cells and NK cells. An important chemokine in this group is monocyte chemoattractant protein-1 (MCP-1, also called CCL2), which promotes the migration of monocytes from the bloodstream to the tissue where they differentiate to become macrophages. Other CC chemokines include MIPs, MIP-1α (CCL3) and MIP-1β (CCL4) and RANTES (CCL5). The effects of CC chemokines are mediated via specific cell surface receptors: 10 different types of these receptors (CCR1–CCR10) have been identified.

C Chemokines

Also termed γ-chemokines, C Chemokines are composed of only two cysteines: one on the N-terminus and the other a downstream cysteine. There are two chemokines in this group, lymphotactin-α (XCL1) and lymphotactin-β (XCL2). Their function is the attraction of T-cell precursors to the thymus.

CX3C Chemokines

Also termed δ-chemokines, CX3C Chemokines are composed of three amino acids between the two cysteines. This subgroup has only one member, fractalkine (CX3CL1). CX3C chemokines are secreted as well as present on the cell surface and serve both as a chemoattractant and as an adhesion molecule.

Biological Role of Chemokines

The primary function of chemokines is to induce the migration of leukocytes. A signal directs these cells toward the chemokines. During immunological surveillance, chemokines direct lymphocytes to the lymph nodes, which allows them to interact with the APCs and detect any invading pathogens. Such chemokines are called homeostatic chemokines and do not require a stimulus for their secretion. Some chemokines are proinflammatory in nature and require specific stimulus for their release. These stimuli include viral infection, bacterial products as well as other chemical agents. Proinflammatory cytokines including IL-1 and TNF-α promote their release. These chemokines are chemoattractants for neutrophils, leukocytes, monocytes and some effector cells, and they direct the migration of these leukocytes to the site of injury/infection. Some proinflammatory chemokines are also involved in wound healing similar to the proinflammatory cytokines. Chemokines are also

capable of activating leukocytes to initiate an immune response and are involved in both innate and acquired immunity. Other chemokines play a role in development and are involved in angiogenesis and cell maturation.

Chemokine Receptors

Chemokine receptors are a family of G protein-coupled receptors that contain seven transmembrane domains. Chemokine receptors are present on the cell surface membrane of leukocytes. As was the case for chemokines, these receptors are also divided into four subgroups: CCR is specific for CC chemokines, CXCR for CXC chemokines, XCR1 for C chemokines and CX3CR1 for CX3C chemokines. The CC chemokine receptor family has eleven members, the CXC chemokine receptor family has seven members, and both the C chemokine receptor family and the CX3C chemokine receptor family have one member each. The signal transduction is mediated via the standard G protein-dependent pathway.

Chemokines and Disease States

Human Immunodeficiency Virus Infection

Human immunodeficiency virus requires CD4 and either CXCR4 or CCR5 to enter target cells. This allows the entry of HIV into $CD4^+$ T cells or macrophages, which eventually leads to the destruction of $CD4^+$ T cells and almost total inhibition of antiviral activity. Individuals who possess a nonfunctional variant of CCR5 and are homozygous for this gene remain uninfected despite multiple exposures to HIV. Clinical trials are under way to develop antagonists of these chemokine receptors as potential therapeutic agents for HIV infection and AIDS.

Diabetes with Insulin Resistance

Cytokines and chemokines have been implicated in insulin resistance. The cytokines which may play a role include IL-6 and TNF-α. CCR2 are present on adipocytes, and activation of inflammatory genes by the interaction of CCR2 with the ligand CCL2 results in impaired uptake of insulin-dependent glucose. Adipocytes also synthesize CCL2, resulting in the recruitment of macrophages. CCL3 may also be involved in insulin resistance.

Atherosclerosis

CCL2 is present in lipid-laden macrophages and atherosclerotic plaques that are rich in these macrophages. The production of CCL2 in endothelial and smooth muscle cells is stimulated by minimally oxidized low-density lipoproteins (LDLs). As a consequence, CCL2 is involved in the recruitment of foam cells to the vessel

wall. Patients who are homozygous for the polymorphism in the promoter of CCL2 appear to have a high risk for developing coronary artery disease as opposed to patients who are heterozygous. CXCR2 and CX3CR1 are also implicated in cardiovascular disease.

Inflammatory Diseases

Chemokines are involved in various inflammatory diseases including asthma, arthritis, psoriasis and MS. Chemokine CC11 (eotaxin) and its receptors CCR3 are involved in the recruitment of eosinophils to the lungs, contributing to the etiology/pathogenesis of allergic disease/asthma. Elevated levels of CCL2, CCL3 and CCL5 are found in the joints of patients with rheumatoid arthritis, and are involved in the migration of monocytes and T cells to the inflamed joint. In psoriasis, CCR4 is expressed on infiltrating effector T cells, and cutaneous cells produce CCL17 and CCL22, which are ligands for CCR4. CXCR3 also plays a role in psoriasis. In MS, the levels of CXCL10 are elevated but there are lower levels of CCL2, and CXCR3 may also play a role. MS lesions contain many chemokines including CXCL10, CCL3, CCL4 and CCL8, where they may be predominantly involved in the migration of monocytes and macrophages from the peripheral blood into the tissue and lesions. The infiltrating monocytes express both CCR1 and CCR5.

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Cytokines orchestrating the immune response

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Summary

The author summarises the current knowledge of the major immune cytokines, their receptors and functions, and illustrates the pivotal role of cytokines in regulating immune responses. As researchers explore the factors which influence the genetics of disease resistance in livestock and poultry, alleles associated with differences in the expression of, and responsiveness to, cytokines will inevitably be defined. Variations in cytokine receptors, as well as in sensitivity to the rapidly expanding array of cytokine agonists and antagonists, will also be identified. These differences influence not only disease resistance but also potential disease pathology and speed of recovery from infection.

The author concludes with a discussion of some uses of cytokines in clinical practice. This area is the subject of active exploration with clinical trials in many species, addressing issues such as immune system stimulation and disease treatment with cytokine proteins. As veterinarians use such new biotherapeutics, the issue of genetic control of responses to deliberate cytokine stimulation will become important to producers.

Keywords

Biotherapeutics – Colony-stimulating factors – Disease resistance – Genetics – Haematopoiesis – Immune regulation – Innate immunity – Interferons – Interleukins – Receptors.

Introduction

This decade has seen an explosion of information on cytokines, the molecules which control the complex interactions that moderate the development and maturation of a healthy immune system and lead to effective immune responses. This group includes the interferons, the interleukins, chemokines and many haematopoietic factors, such as the colony-stimulating factors (CSFs). These molecules are intimately involved in orchestrating immune responses. Extensive literature on cytokines is available (1, 47, 68), including books on the role of cytokines in regulating disease responses and in potentiating vaccine and immune responses (4, 44). In addition to a recent monograph (44), there have been several general reviews of cytokines in livestock and poultry (31, 41, 42, 43, 76). A database on cytokines of livestock and poultry has been developed through the Veterinary Immunology Committee of the International Union of Immunological Scientists (40).

General properties of cytokines

Cytokines are proteins which are active in picomolar to nanomolar concentrations and affect the growth and metabolism of a wide array of cells through both autocrine and paracrine activities. Common properties of cytokines are outlined in Table I. Not every cytokine exhibits all these properties; indeed for each property listed in Table I there is usually one, if not more, exception(s). Most cytokines are < 30 kiloDalton (kDa) polypeptides; only one interleukin (IL), IL-12, is known to be a heterodimer. Unlike hormones, which are secreted by only one type of specialised cell, most cytokines are produced by a broad range of cells. Many cytokines exhibit apparently redundant activities: in fact, the activities of structurally quite different cytokines may overlap yet may not be synergistic. Most cytokines are effective on multiple target cells (73).

Redundancy, ambiguity and pleiotropism are the hallmarks of cytokine action and account for the effectiveness of these

Table I
General characteristics of cytokines

General characteristics
Low molecular weight proteins/glycoproteins
Low/no constitutive expression
Production regulated at transcriptional and translational level
Active in picomolar to nanomolar concentrations in autocrine or paracrine manner
Expression transient and locally limited
Bind to high affinity ($K_d = 10^{-9} - 10^{-12}$ M) cell surface receptors
Common subunits and conserved protein structure of cell surface receptors
Receptor expression tightly regulated
Pleiotropic activity due to overlapping effects on disparate tissues and cell types
Stimulate altered pattern of gene expression in target cell, including expression of other cytokines
Cause proliferation, altered function and/or differentiation of target cell
Exert multiple activities on same target cell over time of action
Redundancy in some intracellular signalling pathways
Interact with other cytokines or growth factors to produce synergistic, or inhibitory, effects

proteins in regulating immune responses. The essential role of cytokines in orchestrating normal immune system maturation and in regulating defence against infectious disease is well established (24). However, the intricacies of the complex interactions of cytokines, their receptors and the stimulatory and/or counter-regulatory effects of other cytokines are only now being fully dissected. As an example, Mantovani *et al.* discussed the role of more than a dozen cytokines in activating distinct, but overlapping, sets of functions in endothelial cells (36). These cells in turn produce a diverse array of cytokines and chemokines which help moderate subsequent immune responses.

The availability of cytokine gene-knockout mice, and of a wide array of molecular reagents for individual cytokine gene evaluation, has helped to delineate the exact effects of each cytokine and to expand knowledge of the complexity and redundancy of regulators of normal immune responses (73). Gene targeting studies produced gene-knockout mice in which the cytokine, or cytokine receptor, gene had been disrupted. Most of these mice exhibited normal development, a testimony to the redundancy of the cytokine network; only IL-10 and transforming growth factor-β1 (TGF-β1) knockout mice experienced growth retardation and early death due to inflammatory reactions, respectively. With infectious disease stress, however, many of these knockout mice exhibited impaired immune responses, such as increased sensitivity to mycobacterial infections with interferon-gamma (IFN-γ) knockout mice, to viral infections for IFN-α/β-receptor (IFN-α/β R) knockout mice and to many infections in IFN-γR knockouts (73). Recent use of 'conditional knockout' strategies, which allow genes to be inactivated in specific cells or at specific timepoints, should help to elucidate further the exact cytokine control mechanisms (24).

Cytokines were originally named after the cell which was determined as their source, the lymphokines from lymphocytes, monokines from monocytes, etc. However, in many cases more detailed studies proved that a wider array of cells actually produced each protein and thus the broader terms, interleukins and cytokines, became common usage. Based on internationally accepted standards, interleukins must mediate potentially important immune responses and must be natural products of immune system cells (48). Interleukins numbering up to interleukin-18 have been described and many more are expected as immune studies probe the details of local immune responses. Table II provides a list of the major cytokines and some of their known functions.

Cytokines and natural immunity

The innate immune system serves as the first line of defence against an unknown antigen. A series of proinflammatory cytokines, including IL-1α/β, IL-6, tumour necrosis factor-α (TNF-α), the chemokines (such as IL-8) and the interferons, are synthesised *de novo* following bacterial or viral infections. These cytokines are active in stimulating phagocytic cells, monocytes, macrophages, neutrophils and endothelial cells to react against, or bind to, micro-organisms, and to summon other immune cells to the site of infection. IL-1 is among the most broadly active of cytokines, inducing subsequent cytokine secretion and activation of everything from immune B, T and natural killer (NK) cells to synovial cells, hepatocytes and osteoclasts. Thus, as scientists consider the genetics of cytokine action, the question should be raised of whether or not expression of IL-1R on each of these cell types will become a factor influencing disease responses as well as normal growth and differentiation in the tissue.

Chemokines are small molecular weight (8 to 11 kDa) proteins which are initiators and promulgators of inflammatory reactions. Chemokine synthesis is usually induced by proinflammatory cytokines (46). Chemokines serve as important chemoattractants, promoting the localisation and subsequent activation of immune cells at local tissue sites.

The interferons are major factors in successful clearing of viral and intracellular parasitic infections. IFN-γ regulates such a broad array of genes that a world-wide web document has recently been generated to keep track of them (http://www.annrevu.org/sup/material.htm) (10). Genetic regulation of the expression of IFN-ω in the trophoblast may affect trophoblast implantation in pigs (33), whereas the local expression of an entire array of cytokines, known as the T helper type 2 (Th2 or type 2) cytokines, clearly affects the overall success of human and murine pregnancies (54) and thus the cytokines are likely candidates for influencing pregnancy success in livestock.

Table II
Selected activities of major cytokines [a]

Cytokine	Abbreviation	Target cells	Cytokine activity	Reference
Interferon-α/β	IFN-α/β	All	Antiviral, antiproliferative	8
		NK cells	Activation	
Interferon-γ	IFN-γ	Mononuclear phagocytes, endothelial cells, NK cells	Activation, up-regulation of MHC I and II expression	10
Interferon-ω	IFN-ω	Trophoblast	Activation, conceptus maintenance	33
Tumour necrosis factor-α/β	TNF-α/β	Hypothalamus	Fever	72
		Liver cells	Acute-phase protein release	
		Neutrophils, endothelial cells, thymocytes	Activation, proliferation, co-factor immune stimulation	
		Muscle, fat	Catabolism (cachexia)	
Interleukin-1-α/β	IL-1α/β	Liver cells	Acute-phase protein release	18
		Endothelial cells, lymphocytes	Activation, proliferation, co-factor immune stimulation	
		Muscle, fat	Catabolism (cachexia)	
Interleukin-2	IL-2	T cells	Growth factor, cytokine production	67
		B cells, NK cells	Growth, activation	
Interleukin-3	IL-3	Immature progenitor cells	Multicolony-stimulating factor, haematopoietic growth factor	
Interleukin-4	IL-4	B cells	Th2 cytokine stimulant, promotion of IgG and IgE secretion	23, 29
		Mononuclear phagocytes	Activation inhibition	
Interleukin-5	IL-5	Eosinophils	Activation	58
		B cells	Growth, differentiation, IgM secretion, activation for IgG secretion	
Interleukin-6	IL-6	Thymocyte	Co-factor immune stimulation	66, 69
		Liver cells	Acute-phase protein synthesis	
		Mature B cells	Growth factor	
		Haematopoietic progenitors	Proliferation and differentiation	
Interleukin-7	IL-7	Immature progenitor cells	Growth and differentiation to mature B cells or T cells	
Interleukin-8	IL-8	Monocytes, lymphocytes	Chemotactic factor	46
		Progenitor cells	Growth modulation	
Interleukin-9	IL-9	Th2 T cells	Growth support	
		Erythroid cells, mast cells	Co-stimulatory signal with other cytokines	
Interleukin-10	IL-10	Th1 cells	Th1 cytokine synthesis inhibition	38, 53
		Monocytes, neutrophils	Cytokine synthesis inhibition	
		Mast cells, eosinophils	Inhibition of differentiation	
Interleukin-11	IL-11	Progenitor cells	Stimulation of megakaryopoiesis, enhancement of macrophage development	
Interleukin-12	IL-12	Macrophages	Induction of IFN-γ production	69
		NK cells	Induction of IFN-γ production, activation	
		T cells	Stimulation of type 1 commitment	
Interleukin-13	IL-13	Premyeloid cells	Induction of proliferation	
		Monocytes, B cells	Induction of activation and proliferation	
		Activated monocytes	Inhibition of inflammatory cytokine production	
Interleukin-14	IL-14	B cells	Growth and expansion, inhibition of Ig secretion	
Interleukin-15	IL-15	T cells	Stimulation of proliferation	
		Multiple cell targets	Proliferation	
Interleukin-16	IL-16	$CD4^+$ T cells	Chemotaxsis, activation	16
		$CD4^+$ monocytes	Chemotaxsis, activation	
		$CD4^+$ eosinophils	Potent chemotaxsis, activation	
Interleukin-17	IL-17	Stromal cells	Induction of IL-6, IL-8 secretion	61
		Cartilage	Augmentation of nitric oxide production	
		Fibroblast	Enhancement of ICAM-1 expression	
Interleukin-18	IL-18	T cells	Induction of IFN-γ production	71
		NK cells	Induction of IFN-γ production and NK activity	
Transforming growth factors	TGF-β1 - TGF-β5	Macrophages, endothelial cells	Activation	55
		B cells, T cells, NK cells	Inhibition of activity	
		Angiogenesis	Stimulation of extracellular matrix	
Granulocyte-macrophage CSF	GM-CSF	Bone marrow progenitor cells	Growth and differentiation to granulocytes and macrophages	63
Monocyte CSF	M-CSF	Bone marrow progenitor cells	Growth and differentiation to macrophages	51
Granulocyte CSF	G-CSF	Bone marrow progenitor cells	Growth and differentiation to granulocytes	51
c-kit ligand		Bone marrow stem cells	Activation, increase in responsiveness of stem cells to CSFs	

a) Summarised from Abbas *et al.* (1), Aggrawal and Puri (4), Paul (47), Sigal and Ron (60), and sources noted in the Table

NK : natural killer
MHC : major histocompatibility complex
Th1 : T helper 1
Th2 : T helper 2
Ig : immunoglobulin
$CD4^+$: cluster of differentiation antigen 4^+
ICAM : intercellular adhesion molecule
CSF : colony-stimulating factor

Cytokines and lymphocyte responses

The adaptive immune system involves both B-cell mediated and T-cell mediated immune mechanisms which contribute to defence against infectious agents. The development of these cellular responses is enhanced each time the infectious agent reappears because of the memory functions of selected T and B lymphocytes. B lymphocytes require activation for secretion of immunoglobulins (Igs). A wide range of cytokines, including IL-6, IL-5 and IL-7, are involved in early B-cell growth, stimulation of mature B-cell growth and differentiation and regulation of B-cell apoptosis and chemotaxsis (50). The type 1 and type 2 cytokines regulate events which control immunoglobulin secretion and isotype specific responses, and B-cell co-stimulation of T-cell responses.

$CD4^+$ and $CD8^+$ T cells respond to infections by producing a range of cytokines, including the interleukins and interferons. Antigen-specific $CD8^+$ cytolytic T cells kill cells infected with invading pathogens. In addition, these cells secrete specific sets of cytokines in response to infections. Mosmann and Coffman showed in murine systems that $CD4^+$ T-cell cytokine responses are biased (39); T helper type 1 (Th1), or type 1, cytokines (such as IL-2 and IFN-γ) stimulate responses against intracellular pathogens, whereas Th2, or type 2, cytokines, such as IL-4, IL-5 and IL-13, stimulate differential B-cell and anti-nematode parasite responses (23). Later studies identified IL-12 as a Th1, or type 1, associated cytokine but clearly showed that it was not secreted by T cells but rather by macrophages and NK cells (70). As the cytokine secretion patterns of $CD8^+$ cytotoxic T cells (Tc) were analysed, it became clear that there were similarly Tc1 and Tc2 biased cytokine responses.

Kelso analysed T-cell clones in detail and proved that a simple division of T-cell cytokine secretion patterns into Th1 and Th2 subsets may not be representative of actual cytokine responses of lymph node T cells (30). The T-cell clones studied exhibited a greater variety of cytokine secretion patterns. Allen and Maizels further cautioned against categorisation into Th1-type or Th2-type responses, and suggested that a more rigorous inspection of actual cytokine profiles would reveal the exact pattern of cytokines involved in each disease response (5). Indeed, Allen and Maizels point out that the combined pattern of Th1- and Th2-type responses would result in a proper balance between the extremes of Th1- and Th2-type responses and thus help avoid infection-induced immunopathology. Romagnani reviewed the role of cytokines in normal and autoimmune disease responses in humans, and indicated that the T1 and T2 biased responses of both $CD4^+$ and $CD8^+$ cells result in significant production of cytokines, some of which are quite different from the standard murine groupings (56). As an example, IL-2 appears to be secreted by all human T cells. Strober *et al.* suggest that control of mucosal immune responses may be balanced by different cytokines, by the proinflammatory IFN-γ and anti-inflammatory TGF-β (65). During pregnancy, the foetoplacental tissues clearly exhibit a Th2 cytokine profile (54); evidence suggests that such an immune bias in these local tissues is essential for successful pregnancies.

As noted by Trinchieri, IL-12 actually serves as a bridge between the innate and adaptive immune responses and stimulates cells involved in innate immunity so that adaptive immune responses are subsequently activated (70). Thus, even though IL-12 is usually thought of as a type 1 cytokine, in response to bacterial lipopolysaccharide (LPS) stimulation of macrophages it is one of several cytokines which is secreted. IL-12 binds to NK cells and activates them, resulting in the secretion of high levels of IFN-γ. One of the most recently described cytokines, IL-18, is an excellent example of the redundancy of cytokine action. IL-18 mimics the activity of IL-12 in stimulating IFN-γ production, yet bears no sequence homology to IL-12 and reacts with different cells, such as T cells, in addition to NK cells. IL-18 is secreted as an inactive precursor polypeptide and is activated by the IL-1β converting enzyme, whereas IL-12 is secreted as an active heterodimer (71). Thus, even individuals with low IL-12 reactivity may have appropriate LPS responses because they compensate with heightened IL-18 responses.

Haematopoietic cytokines

Cytokines which stimulate growth and differentiation of bone marrow progenitor cells are referred to as CSFs. These cytokines regulate the immune system, and tissue and bone development (15, 25, 37). These include c-kit ligand, IL-3 (multi-CSF), granulocyte-macrophage CSF (GM-CSF), M-CSF, G-CSF and IL-7. These cytokines act to stimulate bone marrow cells at different stages of maturation. Only c-kit ligand has been proposed to serve as a pluripotent stem cell growth factor. Certain cytokines, such as TNF-α, IFN-γ and TGF-β, express as one element of their activity the ability to inhibit growth of progenitor cells, whereas other cytokines, such as IL-1 and IL-6, enhance progenitor cell responses to CSFs. Genetic differences in expression of these CSFs, and in speed and intensity of response to their activities, could have major implications for normal immune system development as well as for disease responses.

Cytokine receptors

Cytokines bind to the cell surface by means of specific receptors which then transmit the extracellular binding event into an intracellular signal (3). Many cytokines, such as IFN-γ, bind to one chain of a multichain receptor complex (6). Aggregation of multichain receptor complexes serves to enhance signal transduction (66). Signal transduction of cytokine receptor binding occurs through a series of

intracellular enzymes and usually results in altered patterns of gene expression in the target cell, often including modulation of expression of other cytokines. These intracellular signals cause messenger ribonucleic acid (mRNA) and protein synthesis, and lead to cell proliferation, altered cell function and/or differentiation of the target cell. These events can occur within minutes or hours, but could take effect days later.

In characterising the receptors for cytokines, scientists have revealed a system of shared receptor subunit polypeptides with common structural features, as outlined in Table III. Many receptors consist of two or more polypeptide chains, a cytokine-specific α chain and a signal-transducing β, and possibly γ, chain. This sharing of receptor polypeptides accounts for some of the overlapping activities of different cytokines, as has recently become clear for the IL-4 receptor (IL-4R) and IL-13R, which share the IL-4R α-chain and display similar activities (13). Most cytokine receptors have a typical structure of an extracellular domain, a membrane-spanning domain and a cytoplasmic domain. Based on conserved polypeptide folding motifs of these domains, cytokine receptors can be grouped into the five families outlined in Table III.

Intracellular signalling by these diverse cytokine receptors can occur through different pathways. This can be direct, as occurs for IL-4R and G-CSFR (13). Signalling can be transmitted through a second polypeptide chain, as occurs with IL-3R, IL-5R and GM-CSFR. These cytokines all bind to the α-subunit of their receptors and use their β-subunit to transduce the binding signal intracellularly. IL-6 and related cytokines, such as IL-11, use homo- or hetero-dimerisation of the cytokine receptor glycoprotein 130 (gp 130) (66). Many cytokine receptors display their own kinase activity while others, such as IL-6R and IL-11R, must rely on proteins which, when bound to the receptor, express this intracellular signalling activity. The use of common intracellular signalling pathways is one reason why certain cytokines never act synergistically.

Divergence of signalling pathways may be explained by the fact that the dominant type 1 and type 2 cytokine inducers, IL-12 and IL-4 respectively, cause rapid tyrosine phosphorylation of different signal transducers and activators of transcription (STATs) after binding to their receptors; IL-4 activates STAT6 whereas IL-12 activates STAT4. STAT gene knockout mice confirm these preferences in that STAT6 knockout mice are deficient in Th2 responses (57).

Besides the cell surface receptor, many cytokines bind to soluble receptors. Some of these are the released ligand binding domain of the actual surface receptor, while other soluble receptors are unrelated to the original receptor, such as the viral receptor-like molecules which bind IL-1 (62). A similar molecule has recently been found in a porcine virus (52). Naturally occurring soluble TNF receptor (TNFR), composed of extracellular domains of both TNFR p55 and p75, clearly blocks TNF-mediated inflammatory processes (69). Clinical studies for effectivity of a wide range of soluble cytokine receptors are being actively pursued.

Cytokines and disease

Advances in knowledge of the role of cytokines in immune system maturation and regulation have helped scientists to determine which cytokines, or groups of cytokines, are synthesised in response to infection, cause disease pathology and/or modulate disease progression. The proinflammatory cytokines, the CSFs and two type 1 cytokines, IL-12 and IFN-γ, have clearly been recognised as influencing resistance to a wide range of bacterial, viral and parasitic infections. By

Table III
Cytokine receptor families

Polypeptide folding motif	Cytokine receptor examples	Number of motifs
Immunoglobulin superfamily (Ig-SF)	IL-1R (CD121), PDGFR (CD140)	Multiple Ig domains
Cytokine/haematopoietic growth factor superfamily (CKR-SF)	IL-6R (CD126), IL-4R (CD124), IL-2R β, IL-15R (CD122), IL-2R γ GM-CSFR (CD116), G-CSFR (CD114), IFN-γR (CD119), IFN-α/βR (CD118)	Combination of cytokine receptor domain with fibronectin type III domains, and sometimes Ig domains, and transmembrane tail; may be bound to 1-2 other receptor chains
Tumour necrosis factor receptor superfamily (TNFR-SF)	TNFR p55, p75 (CD120a,b), Fas (CD95), NGF-R	Cys-rich domains
Transmembrane helix, α-chemokine R family	IL-8R (CDw128)	Multiple transmembrane helices
Transforming growth factor R	TGF-b R (CD105)	Transmembrane glycoprotein

IL : interleukin
CD : cluster of differentiation antigen
PDGFR : platelet-derived growth factor receptor
GM-CSFR : granulocyte macrophage colony-stimulating factor receptor
IFN : interferon

extrapolating data from well-described murine models, it is evident that the generation of IFN-γ producing type 1 lymphocytes is a primary step in the immune response against many of these infections and that this differentiation is dependent on the 'local cytokine milieu' (70). Murine responses to gastrointestinal nematode infections are usually biased to the type 2 cytokines (23). Thus, treatment of individuals with cytokines or drugs which stimulate a type-specific response may be very beneficial for patients fighting certain infections, tumours or autoimmune diseases (20, 64).

A wide variety of studies in domestic animals is now appearing. These studies document the expression of an array of cytokines in response to infections with different organisms (2, 12, 14, 26, 28, 32, 45, 74, 75, 78, 79). The challenge for the future in each of these systems will be to identify which cytokine(s) is required for stimulation of protective immunity. The fact that there will be many cytokines which are synthesised in response to the infection is reasonably clear, but it is essential to delineate which cytokines are involved in establishing protective responses, and which cause pathology or divert the immune response away from protective immunity.

The effects of treating human patients with cytokines to stimulate neonatal defences or help fight active infections are important topics of current clinical research (49, 64). Similarly, the focus of animal cytokine studies has been to learn how to stimulate development of the neonatal immune system and to produce the appropriate cytokine milieu. Blecha and colleagues were the first to test the effects of recombinant cytokines on livestock disease responses (9, 59). This research demonstrated that multiple recombinant bovine IL-1 (rBoIL-1) stimulated porcine immune responses and protected the pigs against a *Streptococcus suis* infection. However, disease protection in this system was only possible at doses which caused significant side effects. Lowenthal *et al.* recently showed that recombinant chicken IFN-γ (rChIFN-γ) was effective in protecting chickens against challenge with *Eimeria acervulina* (34). Further studies will determine the efficacy and cost/benefit ratio of such disease treatments. In addition, controlled trials will determine the actual genetic diversity of how animals from various breeding lines and stocks respond to external cytokine stimulation. Studies in humans are beginning to attest to major genetically based differences in responsiveness.

Peptides of cytokines and of their receptors exhibit agonistic and antagonistic activities. One such agonist, the IL-1β peptide 163-171, is immunomodulatory but is not pyrogenic (11). The use of these novel reagents as biotherapeutics could result in major modifications of livestock health management, without the potential side effects induced by the whole cytokine protein. If immune therapies could be designed to induce specific cytokines, then the question becomes whether they could be targeted so that specific infectious diseases could be prevented. Such developments would help producers to control the spread of infections and thus alleviate disease morbidity and mortality as well as associated production losses. Many cytokines have enzymes which alter their activity; Dinarello and Margolis suggested that a new approach for drug and disease therapy will involve targeting these reagents (19). For livestock, variations in alleles at such enzyme loci could easily influence the levels of active cytokines produced normally or in response to infectious diseases.

Cytokine genetics

Most of the cytokines and their receptors have been mapped to specific chromosomal locations in humans and mice, as well as in many livestock species. Interestingly, many of the cytokine genes are found localised in clusters in the genome. Mapping information is readily available through international mapping databases and will not be summarised here. Duff noted that early mapping studies only determined the genomic location of specific cytokines (20). Studies assessing the allelic variation in cytokine gene expression and receptor expression are only being analysed now. In humans, the targets of such studies are the autoimmune defects, whereas studies in animals will focus on responses to infectious disease challenge (20). Such studies should help breeders to identify animals with germplasm which encodes enhanced disease responses. The disease-resistant genetic stock should be useful to producers in the geographic areas at risk for the specific disease to avoid significant production losses.

As researchers attempt to highlight uses for cytokines, focus is placed on developmental timepoints during which animals face major infectious disease challenges, e.g., weaning and shipping stress. Animals which exhibit an ability to respond quickly to therapeutic doses of external cytokines may have greater resistance to the infectious diseases which so often accompany weaning and shipping. These animals would thus be less likely to exhibit stress-associated production losses, as confirmed by Lowenthal *et al.* in cytokine-treated chickens (34). Alternatively, selecting for animals with enhanced natural disease responsiveness, based on their innate immune responsiveness or ability to produce appropriate cytokine profiles after infection, has been predicted (7). Thus animals selected for higher interferon expression would be expected to be more resistant to bacterial infection (27), although the fact that in humans IFN-α is encoded by 18 separate genes whereas IFN-β is encoded by only one gene may complicate genetic selection efforts. Edfors-Lilja *et al.* analysed the allele variation in specific immune responses associated with disease resistance (21, 22). Mallard *et al.* attempted to select more immune responsive animals which they predicted would be more disease resistant (35). In selecting lines of animals for heightened responses to specific infections, such

as gastrointestinal parasitic infections, altered cytokine responses will possibly also be selected (14, 17, 77).

Conclusion

Cytokines control the type and duration of the immune response. Thus, an understanding of the developing immune system and the regulatory cytokine network will serve as a foundation for planning new strategies to modulate immunity. As more details of the genetic differences in individual cytokine expression and responses, and in receptor signalling capacity, are revealed, scientists will better understand natural resistance to infection and regulation of immune memory responses. Revelations about the mechanisms through which cytokines transmit their regulatory signals will further aid this understanding. Knowledge gained from these studies will enable researchers to identify animals which are healthier and more disease resistant under modern management conditions. This should result in greater numbers of disease- and drug-free livestock and poultry due to genetic selection for enhanced disease resistance based on natural immune parameters, e.g., cytokine profiles and associated immune responsiveness.

■

Orchestration de la réponse immune par les cytokines

J.K. Lunney

Résumé

L'auteur fait le point sur l'état actuel des connaissances concernant les principales cytokines de l'immunité, leurs récepteurs et leurs fonctions, et décrit leur rôle déterminant dans la régulation de la réponse immune. Les travaux conduits par des chercheurs qui concernent actuellement les facteurs exerçant une influence sur la génétique de la résistance aux maladies chez les bovins et les volailles vont entraîner inévitablement la définition des allèles associés aux différences d'expression des cytokines et de réponse à celles-ci. Des variations dans les récepteurs des cytokines, ainsi que dans la sensibilité à l'ensemble en expansion rapide d'agonistes et antagonistes des cytokines, seront également identifiées. Ces différences influencent non seulement la résistance aux maladies mais également les manifestations pathologiques potentielles et la rapidité de guérison.

L'auteur conclut par une discussion sur certains usages des cytokines dans la pratique clinique. Ce domaine fait l'objet de nombreux travaux de recherche avec essais cliniques, portant notamment sur la stimulation du système immunitaire et le traitement des maladies à l'aide des protéines des cytokines, chez de nombreuses espèces. Comme les vétérinaires sont appelés à utiliser ces nouvelles biothérapies, le contrôle génétique des réponses à une stimulation délibérée des cytokines aura des conséquences importantes pour les éleveurs.

Mots-clés

Biothérapie – Facteurs stimulateurs de colonies – Génétique – Hématopoïèse – Immunité innée – Interférons – Interleukines – Récepteurs – Régulation de la réponse immune – Résistance aux maladies.

■

Citoquinas que orquestan la respuesta inmunitaria

J.K. Lunney

Resumen

El autor resume los conocimientos actuales sobre las principales citoquinas inmunitarias, sus receptores y funciones, e ilustra el papel central que desempeñan las citoquinas en la regulación de la respuesta inmune. Inevitablemente, a medida que los investigadores progresen en el estudio de los factores genéticos que determinan la resistencia a la enfermedad en el ganado y las aves de corral, irán hallándose y definiéndose alelos ligados a diferencias en la expresión de citoquinas y en la capacidad de respuesta a ellas. También se identificarán variaciones en los receptores de citoquinas y en la sensibilidad al vasto y creciente repertorio de agonistas y antagonistas de las citoquinas. Estas diferencias inciden no sólo en la resistencia a la enfermedad sino también en los tipos de patología contraída y en la velocidad de recuperación tras una infección.

A modo de conclusión, el autor examina algunas aplicaciones de las citoquinas a la práctica veterinaria. Este campo es objeto de intensas investigaciones, con pruebas clínicas sobre muchas especies, que investigan temas como la estimulación del sistema inmunitario o el tratamiento de ciertas enfermedades mediante proteínas de citoquinas. Con el uso veterinario de estas nuevas terapias biológicas, la cuestión de la regulación genética de la respuesta a un estímulo deliberado de las citoquinas va a cobrar un gran interés, sobre todo a ojos de los ganaderos.

Palabras clave

Factores de estimulación colonial – Genética – Hematopoyesis – Inmunidad innata – Interferones – Interleuquinas – Receptores – Regulación inmunitaria – Resistencia a la enfermedad – Terapia biológica.

■

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Endogenous Modulators of Inflammatory Cell Recruitment

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Abstract

Leukocyte recruitment is a central immune process. Multiple factors have been described to promote leukocyte infiltration into inflamed tissues, but only recently has evidence for endogenous negative modulators of this inflammatory process emerged. The discovery of several locally produced modulators has emerged into a new field of endogenous inhibitors of leukocyte extravasation. Recent findings from several inflammatory disease models show that tissues can self-regulate the recruitment of inflammatory cells, suggesting that local tissues may have a greater "regulatory say" over the immune response than previously appreciated. Here, we propose that locally produced modulators of leukocyte recruitment may represent local homeostatic mechanisms that tissues and organs may have evolved for protection against the destructive potential of the immune system.

Leukocyte recruitment

All inflammatory processes involve or depend upon leukocyte recruitment to the inflamed tissue. Inflammatory or infectious stimuli trigger the extravasation of initially circulating neutrophils–and in some cases of monocytes– that are crawling on the endothelial cell surface and patrolling the vasculature, from the vessel into the parenchymal tissue [1–7]. This initial inflammatory wave is followed by the recruitment of monocytes and inflammatory lymphocytes [1–6]. Leukocyte recruitment is orchestrated by different tissue-derived stimuli, such as chemokines or cytokines, and is perpetuated by further leukocyte-derived inflammatory stimuli [1–6,8].

A tightly regulated cascade of low- and high-affinity binding and adhesive interactions between the inflammatory cells and the endothelium that lines the vascular bed of the inflamed tissue coordinates leukocyte extravasation [4,6]. Specifically, leukocyte adhesion to the endothelium involves the following steps: 1) The initial contact between leukocytes and the endothelium is mediated by the tethering and rolling of leukocytes on the endothelial cell surface. Rolling interactions only take place under flow conditions, are transient and are dependent on the binding of P- or E-selectin on the endothelial cell surface to their leukocyte glycoprotein ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1) [4,9]; 2) Subsequently, the velocity of the rolling leukocytes is reduced, resulting in slow rolling,

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which is considered an independent step, as it is mediated by both selectin- and integrin-dependent adhesive interactions [4]; 3) Leukocytes then firm arrest on the endothelium. This step of firm adhesion to the endothelium and the subsequent step of slow locomotion (or crawling) on the endothelial cell surface, during which leukocytes seek an appropriate site for transmigration, are mediated by leukocyte integrins, such as VLA-4 ($\alpha 4\beta 1$), LFA-1 ($\alpha L\beta 2$; CD11a/CD18) or Mac-1 (CD11b/CD18) that interact with endothelial ligands, including VCAM-1, ICAM-1, ICAM-2, or RAGE [4,5,10–12]. The integrin-dependent adhesive interactions involve a complex activation of the avidity of integrins that can thereby bind stronger to their endothelial ligands; activation of integrins can take place either at the level of the affinity of the single integrin receptor for its ligand involving conformational changes, or via clustering of the integrins, usually induced by ligand binding [1,4,13,14]; and 4) Finally, the step of diapedesis or transmigration of leukocytes through the endothelial monolayer mostly occurs via passing through endothelial junctions and involves the participation of several adhesion receptors, such as integrins, Junctional Adhesion Molecules (JAM), PECAM-1 or CD99, capable of interacting in a heterophilic or homophilic manner [1,4,5,10,15]. These different steps of leukocyte extravasation can be modulated by cytokines or chemokines: Whereas tissue-derived cytokines can upregulate endothelial adhesion molecule expression, tissue-derived chemokines present on the apical endothelial cell surface can trigger conformational changes on leukocyte integrins leading to their activation [4,5,8].

Multiple chemokine and adhesion receptors (>20) have been identified to promote leukocyte recruitment, by acting on the various steps of the leukocyte adhesion cascade. More recently, several endogenous negative regulators of leukocyte extravasation have been identified. Here we consider the idea that as tissue-derived chemokines and cytokines trigger leukocyte extravasation and inflammation, these tissues may also possess mechanisms to homeostatically control this inflammatory process. These mechanisms may involve local tissue production of appropriate inhibitors or local release of such inhibitors by the recruited leukocytes upon their interaction with the endothelium.

Endogenous inhibitors of leukocyte recruitment

Pentraxin-3 (PTX-3)

Recently, PTX-3 was identified as inhibitor of the first step of the leukocyte adhesion cascade, *i.e.*, leukocyte-endothelial rolling [16]. Pentraxins constitute a conserved family of soluble pattern-recognition molecules characterized by a radial pentameric structure [17]. Whereas serum amyloid P or C reactive protein belong to the short pentraxins, PTX-3 is a prototypic member of the long pentraxin subfamily [17]. The expression of PTX-3 can be induced by Toll-like receptor ligation in immune or stromal cells [17]. In neutrophils, PTX3 is constitutively stored in the specific granules and can be released locally at the leukocyte-endothelial cell interface by several stimuli including Toll-like receptor agonists [18]. PTX3 participates in various aspects of the innate immune response, such as pathogen recognition, opsonization, and regulation of complement function [19,20].

The anti-inflammatory activity of PTX-3 was inferred by early studies of PTX-3-deficient mice that displayed elevated cardiac tissue damage associated with higher inflammation in the course of ischemia-reperfusion injury of the heart [21]. More recently, it was demonstrated that PTX-3 binds to P-selectin present on endothelial cells, thereby interfering with the interaction of P-selectin with its leukocyte ligand, PSGL-1 (Figure 1) [16]. PTX-3 thereby potently inhibits neutrophil rolling on the endothelium under shear stress. Glycosylation of PTX-3 was required for mediating binding to P-selectin, which is in keeping with the preference of selectins for carbohydrate ligands, such as PSGL-1 [16,22]. The regulatory function of PTX-3 in leukocyte extravasation was demonstrated in mouse

models of inflammation, where PTX-3-deficient mice displayed elevated neutrophil recruitment as compared to PTX-3-sufficient controls. In particular, in a model of pleural inflammation and acid-induced acute lung injury, neutrophil infiltration was higher in mice deficient in PTX-3 as compared to PTX-3-sufficient mice [16]. In bone marrow chimera experiments, hematopoietic-specific deficiency of PTX-3 led to increased inflammation showing that PTX-3 is leukocyte-derived [16].

In addition to its role in leukocyte rolling, PSGL-1 binding to P-selectin mediates leukocyte-platelet interactions [3,23], which additionally involve the binding of leukocyte Mac-1 to JAM-C or glycoprotein Ib on platelets [24,25]. The leukocyte-platelet interaction is involved in several inflammatory processes, including autoimmunity or acid-induced acute lung injury [26,27]. Interestingly, blocking P-selectin-dependent neutrophil-platelet aggregation reverses lung injury [27]. Therefore, PTX-3 may antagonize inflammatory reactions mediated by leukocyte-platelet aggregates. Taken together, PTX-3 is a specific inhibitor of the PSGL-1/P-selectin interaction and can thereby interfere with leukocyte rolling or other PSGL-1-dependent functions of leukocytes.

Galectins

Galectins comprise a family of β-galactoside-binding lectins with at least one carbohydrate-recognizing domain. They interact with host carbohydrate ligands in embryogenesis and development but can also function as immunomodulatory pattern-recognition molecules that can, for instance, interact with glycans on microbial surfaces [28]. Additionally, certain galectins are implicated in the regulation of leukocyte recruitment and inflammation. For instance, endothelial galectin-1 inhibits neutrophil rolling and adhesion under flow in vitro and neutrophil recruitment in vivo. The underlying mechanism may involve downregulation of expression of adhesion molecules, such as CD11b, on neutrophils [29,30]. Consistent with this, neutrophil recruitment was enhanced in galectin-1-deficient mice in a model of IL-1β-induced inflammation of the vascular bed of the cremaster muscle [29]. The anti-inflammatory role of recombinant galectin-1 has been demonstrated in different mouse models of autoimmunity [31,32]. In contrast, galectin-3, expressed in neutrophils and macrophages among other cell types, seems to have a proinflammatory action [33,34]. Moreover, galectin-3 may promote eosinophil rolling and adhesion [35]. However, galectin-3 has also been reported as a negative regulator of LPS-induced inflammation in mice [36], implying that the regulatory effects of galectin-3 (and possibly other galectins) on inflammation may be context- and/or tissue-dependent.

Developmental endothelial locus-1 (Del-1)

Del-1, also known as EGF-like repeats and discoidin I-like domains 3 (EDIL3) is an endothelial cell-expressed 52-kDa glycoprotein, originally described for its role in vascularization [37]. Although secreted by endothelial cells, Del-1 can become associated with the endothelial cell surface by binding to surface proteoglycans or to integrin αvβ3 by means of an Arg-Gly-Asp (RGD) motif on the second EGF-like repeat [1,38]. More recently, Del-1 was shown to be a novel ligand for the LFA-1 integrin but, unlike ICAM-1, it antagonizes LFA-1-dependent leukocyte adhesion onto the vascular endothelium (Figure 1) [39]. Importantly, at equimolar amounts, Del-1 outcompetes ICAM-1 for binding to LFA-1 on leukocytes and thereby inhibits the adhesion and diapedesis of neutrophils [39]. These findings suggest that Del-1 acts homeostatically to regulate local inflammation. This concept was demonstrated in an animal model of periodontitis [40], a chronic inflammatory disease, which leads to the destruction of the tissues that surround and support the teeth (periodontium) and constitutes a risk factor for systemic diseases (*e.g.*, atherosclerosis, rheumatoid arthritis, adverse pregnancy outcomes, diabetes, and aspiration pneumonia) [41–46].

The role of Del-1 in periodontal homeostasis was established in distinct models. Del-1 expression is diminished in gingival tissue in old age, correlating with excessive neutrophil recruitment and IL-17–dependent inflammatory bone loss, the hallmark of periodontitis [40]. Consistent with this, Del-1-deficient mice develop spontaneous inflammatory bone loss at young age accompanied by heavy neutrophil infiltration in the gingiva, and inflammation is dependent on LFA-1 and the IL-17 receptor. Whereas Del-1 inhibits LFA-1–dependent neutrophil recruitment and IL-17 production, IL-17 downregulates Del-1 expression in endothelial cells and thereby promotes neutrophil recruitment [40]. Therefore, Del-1 and IL-17 are reciprocally cross-regulated and, moreover, the inhibition of Del-1 by IL-17 is a novel mechanism by which IL-17 can facilitate neutrophil recruitment to sites of inflammation. Other, previously established mechanisms include the capacities of IL-17 to orchestrate granulopoiesis and neutrophil mobilization and chemotaxis [47–49]. The inverse expression of Del-1 and IL-17 is also observed in human gingival biopsy samples, with Del-1 dominating in healthy gingiva and IL-17 in inflamed gingiva [40]. Moreover, human Del-1 inhibits LFA-1–dependent transendothelial migration of human neutrophils in vitro [40].

These findings indicate that Del-1 serves as a mechanism whereby a tissue can locally self-regulate persistent inflammation associated with chronic recruitment of neutrophils. This concept may be exploited therapeutically. Proof-of-concept was established by showing that local periodontal treatment with soluble Del-1 in old mice inhibits IL-17 production, LFA-1-dependent neutrophil infiltration, and bone loss [40].

GDF-15

Growth differentiation factor-15 (GDF-15), a member of the TGFβ superfamily, is another local tissue-expressed inhibitor of leukocyte extravasation. GDF-15 is upregulated in cardiomyocytes upon myocardial infarction [50]. This is likely a protective homeostatic mechanism in myocardial infarction, as GDF-15 restrains the recruitment of neutrophils and thereby attenuates the inflammatory response in the heart leading to reduced infarct size [51]. Conversely, GDF-15–deficient mice exhibit increased myocardial rupture and mortality after infarction [51]. The inhibition of neutrophil recruitment by GDF-15 is dependent upon its ability to interfere with leukocyte integrin activation. Specifically, GDF-15 blocks chemokine-induced activation of β2-integrin affinity and clustering, thereby preventing neutrophil adhesion to ICAM-1 and transendothelial migration [51]. Mechanistically, the inhibitory effect of GDF-15 on integrin activation is mediated through regulation of small GTPases, involving activation of Cdc42 and deactivation of Rap1 (Figure 1) [51]. Therefore, GDF-15 upregulation in cardiomyocytes upon myocardial infarction appears to be a local tissue mechanism to attenuate inflammation and myocardial damage.

In contrast to the direct mechanism of GDF-15 action (Figure 1), other cytokines such as IL-4, IL-10, and TGFβ exert indirect and complex effects to regulate inflammatory cell recruitment, including transcription regulation of molecules that promote or inhibit endothelial cell-leukocyte interactions [52,53]. Therefore, at least in principle, the different mechanisms of action of these regulators could be exploited for complementary therapeutic approaches to inflammatory diseases.

Endogenous pro-resolving agents

It is now well established that resolution of inflammation is an active process mediated by specific pro-resolving agonists of endogenous origin. Such agonists include small lipid molecules, such as lipoxins, resolvins, and protectins, which are derived from arachidonic acid and other polyunsaturated fatty acids [54]. In contrast to Del-1, which at the

endothelial-leukocyte interface is produced by endothelial cells [39,40], lipid mediators involved in the resolution of inflammation are generated through transcellular biosynthesis involving both endothelial cells and leukocytes [55]. For instance, endothelial cells can act as donor cells that convert a precursor compound (*e.g.*, eicosapentaenoic acid) into an intermediate product (18*R*-hydroxyeicosapentaenoic acid) that is converted into the final active compound (resolvin E1) by activated 5 lipoxygenase in neutrophils, which act as acceptor cells when they interact with endothelial cells within the vasculature [55]. When generated within the vascular lumen, lipoxins and resolvins (derived from docosahexaenoic acid [D series] or eicosapentaenoic acid (E series]) arrest neutrophil diapedesis and recruitment into the tissues. The underlying mechanisms involve primarily modulation of adhesion receptor expression in both neutrophils and endothelial cells. For example, lipoxin A_4 inhibits leukotriene- or peptidoleukotriene-induced neutrophil adhesion and transmigration by inhibiting the expression of P-selectin on endothelial cells and of β2 integrin (CD11b/CD18) on neutrophils [56,57]. Other activities by lipoxins or resolvins that inhibit the extravasation of neutrophils include inhibition of L-selectin shedding and of ICAM-1 expression, and upregulation of endothelium-expressed nitric oxide, an established anti-adhesive factor [58,59].

In addition to resolvins, resolving exudates from various models of inflammation also contain a biochemically distinct family of docosahexaenoic acid-derived pro-resolving mediators termed protectins [55]. Recently, it was shown that D series resolvins (RVD1 and RVD5) and protectin D1 are temporally and differentially regulated in the course of bacterial infections and collectively enhance phagocytosis, downregulate proinflammatory mediators and, moreover, lower the antibiotic requirements for bacterial clearance [60].

In contrast to their inhibitory effects on neutrophils, pro-resolving lipid mediators actually promote the recruitment and activation of monocytes, albeit for nonphlogistic phagocytosis of apoptotic neutrophils by activated monocyte-derived macrophages [61,62]. These and other anti-inflammatory or pro-resolving actions of lipoxins, resolvins, and protectins explain their protective role in several models of inflammatory diseases, including colitis, peritonitis, periodontitis, ischaemia–reperfusion injury, and asthma [54].

Concluding remarks: Local tissue in control of inflammation

Tissues may have greater control, than traditionally appreciated, over the initiation and type of the immune response by virtue of their capacity to communicate with the immune system via tissue-derived signals (*e.g.*, cytokines, chemokines, antimicrobial peptides, and growth factors) [63,64]. In this context, tissues may not be passive recipients of immune protection since tissue-derived “alarm” signals can educate and appropriately activate antigen-presenting cells to promote a certain effector class of host response [63]. Conversely, when no longer needed, the host response can be downregulated by local “health” signals (*e.g.*, resolvins) to promote resolution of inflammation and tissue regeneration [54,63,64].

These notions for proactive involvement of the tissues in immune regulation is consistent with evidence that immune-privileged sites, such as the brain and the eye, have built-in mechanisms to favor the appropriate class of immune response that may protect the tissue against infection without destructive inflammation [63,65,66].

The hypothesis that tissues may have a “regulatory say” over the immune response is strongly supported by the recent advancements on the locally released endogenous modulators of the leukocyte adhesion cascade. It is now clear that tissues can locally regulate the recruitment of inflammatory cells [39,40,51,59]. In fact, the spontaneous periodontal inflammatory phenotype of Del-1–deficient mice suggests that Del-1–expressing tissues may have greater control over the local host inflammatory response than the immune

system itself. Intriguingly, immune-privileged tissues/organs, such as the eye and brain, display abundant expression of Del-1 at significantly higher levels than peripheral tissues, such as the lung or the gingiva [39,40]. This suggests that these tissues may "prefer to take their chances" with infection rather than inflammation. Although Del-1 is not expressed in the heart [39], as discussed above local inflammation is regulated through increased expression of GDF-15 in cardiomyocytes [51]. Hence, distinct mechanisms at different tissues may be employed to self-regulate local inflammatory responses. Understanding the tissue-specific promoters and inhibitors of leukocyte recruitment and their regulation becomes therefore imperative and warrants further research (Box 1). We propose that the above discussed and other, yet to be identified, inhibitors of the leukocyte adhesion cascade may represent local homeostatic mechanisms that tissues and organs may have evolved for protection against the destructive potential of the immune system.

Box 1

Outstanding questions

- What is the tissue specificity and relative abundance of the various endogenous modulators of inflammatory cell recruitment? Is there a correlation between their expression levels and the immune privilege or susceptibility of a tissue or an organ?
- Is the expression of these endogenous modulators regulated by signals derived from the recruited inflammatory cells in a negative feedback loop?
- Is there a crosstalk or cooperation between different classes of endogenous modulators, *e.g.*, do resolvins or lipoxins regulate Del-1 or GDF-15 expression?
- Does aging adversely affect the expression of endogenous modulators? If so, can local treatment reverse or mitigate age-associated inflammatory conditions? By the same rationale, can endogenous modulators be used locally to treat inflammatory diseases affecting tissues that do not normally express them?

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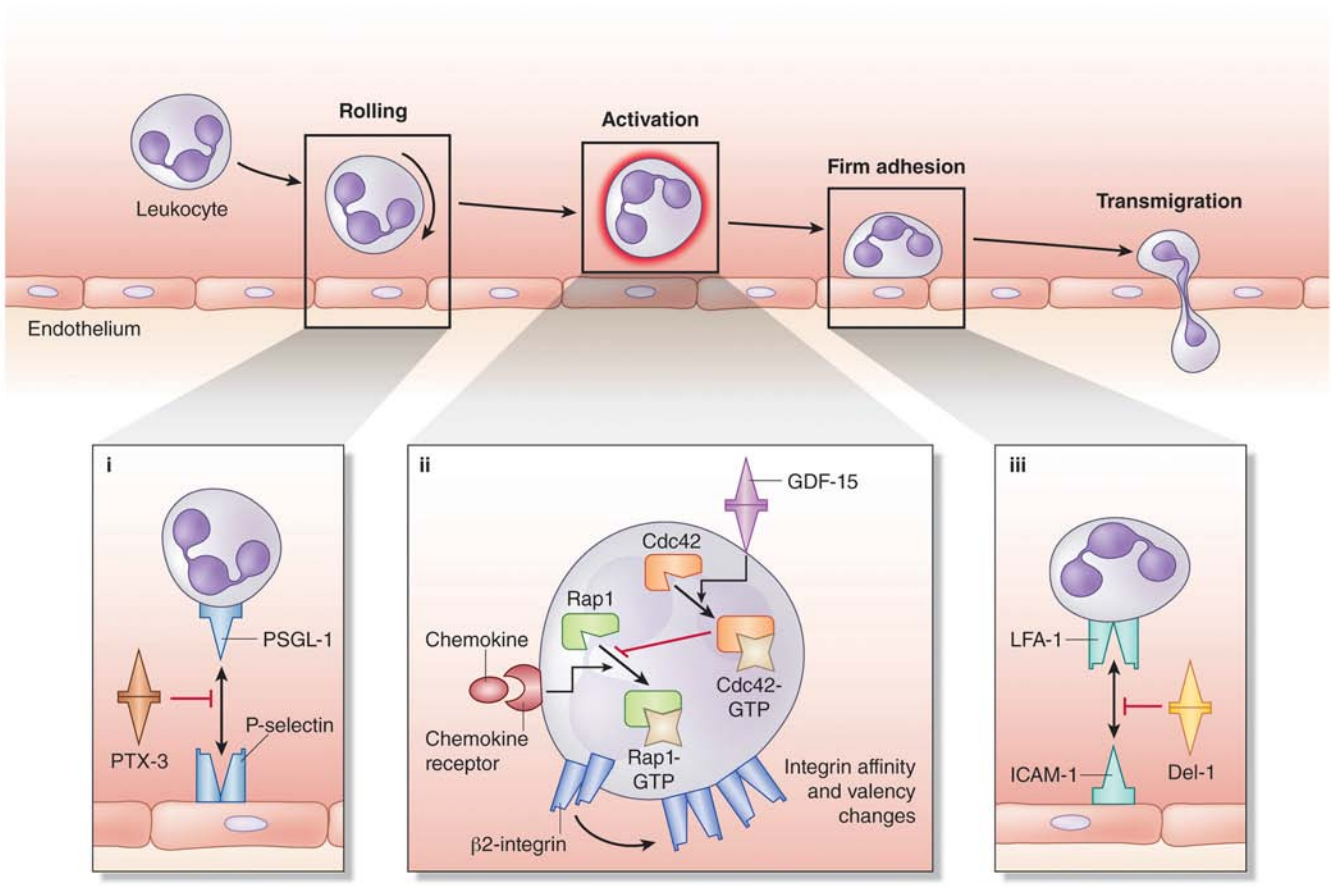

Figure 1. Endogenous modulators of leukocyte recruitment

(a) Leukocyte-derived PTX-3 binds to endothelial P-selectin thereby inhibiting the PSGL-1/P-selectin–dependent rolling. (b) GDF-15 counteracts the chemokine-induced activation of β2-integrins by targeting both affinity (involving conformational changes in a single integrin receptor resulting in higher binding to its ligand) and valency (involving the clustering of more integrin receptors). The inhibitory action of GDF-15 on integrin activation is mediated via upregulation of Cdc42-GTPase activity that antagonizes the activity of Rap1-GTPase. (c) Finally, endothelial cell-associated Del-1 inhibits LFA-1-dependent leukocyte adhesion. The adhesive interactions between LFA-1 on leukocytes and ICAM-1 on endothelial cells constitute a major mechanism mediating firm leukocyte arrest on the endothelium and the subsequent transmigration process.

Dendritic cell–B-cell interaction: dendritic cells provide B cells with CD40-independent proliferation signals and CD40-dependent survival signals

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SUMMARY

Dendritic cells (DC) have recently been shown to play an important role in B-cell function. We have previously shown that DC can capture and retain unprocessed antigen *in vitro* and *in vivo*, and can transfer this antigen to naive B cells to initiate antigen-specific antibody responses. We also demonstrated that DC were providing B cells with isotype-switch signals independent of T cells but that T-cell help was essential for antibody production. In this study, using B cells and DC from wild type (WT) and CD40 knockout (CD40KO) mice we show that DC initiate proliferation of B cells independently of CD40, because WT or CD40KO DC could induce proliferation of WT or CD40KO B cells, but proliferation was greater in the absence of CD40. DC also provide B cells with survival signals as WT DC improved viability of B cells after a 5-day culture but survival was reduced in the absence of CD40 expression.

Studies by our group have shown that dendritic cells (DC) can retain and transport intact antigen (Ag), and that both *in vivo* and *in vitro* DC can transfer retained Ag to naive B cells.[1] This has also been shown for a novel population of DC-related cells.[2] In addition DC can give cell-bound signal(s) to B cells that are required for subsequent class switching.[1] This DC–B-cell interaction occurs in a primary antibody (Ab) response and is independent of any T-cell intervention, although T-cell help is essential for Ab synthesis. Similarly, human DC can influence Ab synthesis and proliferation of tonsillar B cells.[3–7] As we have shown that murine B cells express CD40L,[8] and our (unpublished) and other studies have shown that DC can express CD40L,[9] here we investigate the effects of murine DC on naïve murine B cells.

To determine whether CD40 has a role in DC–B-cell interaction, DC and B cells from wild type (WT) and CD40 knockout (CD40KO) mice[10] were cultured over several days in replicate wells. At several time points, uptake of ^{3}H-thymidine ([^{3}H]TdR) was measured as an indication of B-cell proliferation. Figure 1 illustrates how WT and CD40KO B cells show low proliferation in the absence of any stimulation, although the growth of the latter is always marginally higher. However, in the first 24 hr, WT DC initiate proliferation of WT B cells to the same levels as lipopolysaccharide (LPS) (Fig. 1a). In the absence of CD40 expression on DC or B cells, proliferation is up to 5·5-fold higher. After 3 days, proliferation of WT B cells is reduced to 5% of LPS-induced proliferation while the proliferation of CD40KO B cells is 33–47%.

During these studies we also noticed that the viability of B cells was higher when cultured with DC. To investigate the role of CD40 in B-cell survival, purified DC and B cells from WT or CD40KO mice were cultured together for 5 days and the proportion of viable cells was assessed by the proportion of cells that excluded 7AAD (Fig. 2). Approximately 62% of unstimulated CD40KO and WT B cells are viable after 5 days in culture. While WTDC improved viability of B cells to greater than 92%, only 58% of B cells were viable following culture with CD40KO DC.

Dendritic cells have been shown to have a role in T-cell and B-cell functions, including proliferation and antibody isotype switching.[1,3–7] However, the molecules involved in these interactions are unknown. CD40 expression on DC and B cells is well established and it has been assumed that these cells then interact with CD40 ligand (CD40L) on activated T cells. However, we have shown that murine B cells express CD40L[8] as do murine DC (unpublished), human B cells,[11] and human DC.[9] This suggested the possibility of CD40L on DC, and B cells interacting with CD40 on B cells and DC, respectively. An earlier study found that human DC initiated proliferation of B cells, and it was proposed that CD40L on DC might provide B-cell CD40 with the proliferation signal.[6] Moreover, CD40 has been shown to have a role in B-cell survival[12] but it was assumed to be via T cells.

Here we examined the role of DC and CD40 on B-cell proliferation and survival using DC and B cells from WT and CD40KO mice. We found that the culture of purified DC with

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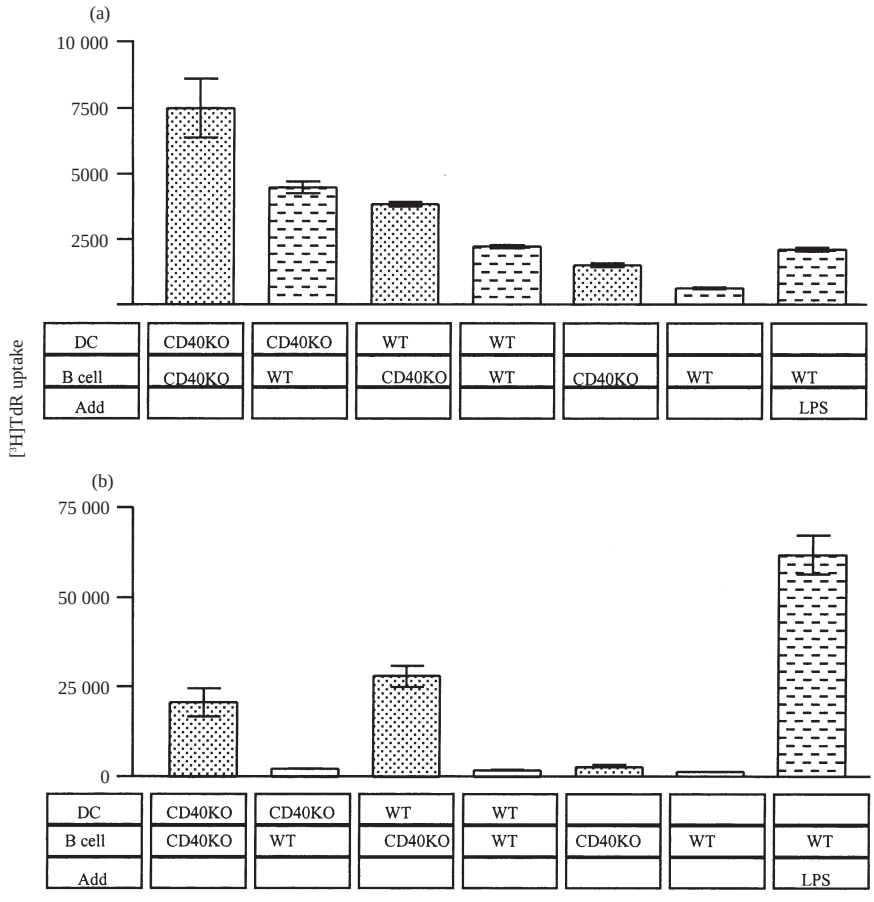

Figure 1. Proliferation of Wild type (WT) and CD40 knockout (CD40KO) B cells cultured with WT and CD40KO DC for (a) 24 hr and (b) 3 days. Purified, irradiated DC[14] and purified B cells[8] were cultured at a ratio of 10 B cells to 1 DC. The cells were cultured in 96-well, round-bottomed plates for the required period, 1 μCi [^{3}H]TdR added and cells harvested after 18 hr.

purified B cells initiated proliferation of B cells as measured by [^{3}H]TdR uptake. The levels of proliferation induced by WT DC were similar to LPS stimulation during the first 24 hr but dropped to 5% by day 3. Proliferation induced by DC is independent of CD40 on DC or B cells, as B cells from WT and CD40KO mice could be induced to proliferate by DC from WT and CD40KO mice. Surprisingly, day 3 proliferation was greatest in the absence of CD40 on DC and B cells, or when WT DC were cultured with CD40KO B cells. This probably reflects the proliferation of B cells that increases the initial ratio of 10 B cells to 1 DC. In this case, WT B cells could use neighbouring B-cell CD40 instead of DC CD40 to inhibit proliferation. This suggested that CD40 can down-regulate proliferation as in its absence proliferation increases. This observation that anti-CD40 signals to B cells inhibit proliferation is perhaps not surprising as it has been shown that anti-CD40 signals can arrest terminal B-cells differentiation and antibody secretion, which could not be overcome by cytokines or mitogens.[13]

We also examined the role of CD40 in DC-mediated survival of B cells. Survival of WT DC/B cells was compared with CD40KO DC/B cells after a 5-day culture. We used CD40KO DC and B cells because CD40 is present on both cell types and in the absence on one cell type, CD40 on neighbouring cells may be used. While approximately 62% of unstimulated WT B cells are viable after 5 days in culture, viability is increased to

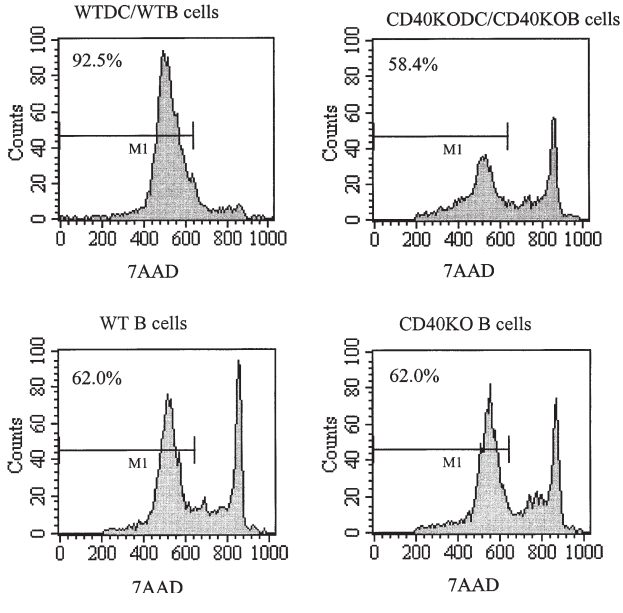

Figure 2. Flow cytometry profiles of WT and CD40KO B cells either unstimulated or cultured with DC. Purified DC and B cells were cultured for 5 days and then treated with fluorescein isothiocyanate (FITC)–B220 and 7AAD to measure viability. The histograms show 7AAD exclusion of B220-expressing cells.

greater than 92% by culture with DC. However, while 62% of unstimulated CD40KO B cells are viable, CD40KO DC do not improve viability. This shows that CD40 signalling is involved in B-cell survival. As we cultured CD40KODC with CD40KO B cells, we have not established whether DC provide CD40L or CD40 signals to B cells. We suggest that because CD40L is present on these DC, but B cells only express CD40L after encountering antigen,[8] survival is provided by CD40L on DC to B-cell CD40.

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How B cells capture, process and present antigens: a crucial role for cell polarity

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Abstract | B cells are key components of the adaptive immune response. Their differentiation into either specific memory B cells or antibody-secreting plasma cells is a consequence of activation steps that involve the processing and presentation of antigens. The engagement of B cell receptors by surface-tethered antigens leads to the formation of an immunological synapse that coordinates cell signalling events and that promotes antigen uptake for presentation on MHC class II molecules. In this Review, we discuss membrane trafficking and the associated molecular mechanisms that are involved in antigen extraction and processing at the B cell synapse, and we highlight how B cells use cell polarity to coordinate the complex events that ultimately lead to efficient humoral responses.

Immunological synapse
The interface between an antigen-presenting cell and a lymphocyte. The hallmark of this structure comprises two concentric regions: one region that is referred to as the central supramolecular activation cluster (cSMAC), where immune receptors are enriched, and another region that is referred to as the peripheral SMAC (pSMAC), which contains adhesion molecules such as lymphocyte function-associated 1 (LFA1) bound to its ligand intercellular adhesion molecule 1 (ICAM1).

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The ability of B cells to capture external antigens and to present them as peptide fragments on MHC class II molecules to $CD4^+$ T cells is a crucial step in the adaptive immune response. This communication between B cells and T cells — known as T cell–B cell cooperation[1] — is required for B cells to form germinal centres, to differentiate into high-affinity antibody-producing plasma cells and to develop into memory B cell populations. The presentation of antigens by B cells on MHC class II molecules is a complex process that involves several stages: first, external antigens are recognized and captured by B cells through their B cell receptor (BCR); second, the antigen is processed by degrading the antigen in internal compartments within the B cell and then the corresponding peptide fragments are loaded on MHC class II molecules; and third, MHC class II–peptide complexes are presented to $CD4^+$ T cells.

As described below, the recognition by B cells of antigen that is tethered at the cell surface of specialized antigen-presenting cells (APCs) leads to the formation of an immunological synapse[2]. This dynamic platform coordinates signalling with antigen extraction and processing, and is crucial for B cells to become fully activated. In this Review, we describe each of the key stages involved in the processing and presentation of antigens by B cells, and we discuss the molecular mechanisms that govern each process. Special emphasis is given to the role of cell polarity in coordinating the formation of the immunological synapses that enable B cells to efficiently respond to antigenic challenges and to carry out their immune effector functions.

Antigen encounter and the role of APCs

Encounter of B cells with antigen in SLOs. In mammals, B cells are generated in the bone marrow and, after going through several developmental checks[3], they migrate to the spleen where they differentiate into mature naive B cells. Mature B cells then recirculate through the bloodstream and migrate to secondary lymphoid organs (SLOs), such as the spleen and the lymph nodes, which provide the necessary structural and chemical microenvironment for B cells to encounter antigens and to become fully activated[4]. In the lymph nodes, B cells migrate from high endothelial venules towards the B cell zone (which is found in the lymph node cortex), where they are organized into cell aggregates known as follicles. This directed migration is mediated by a chemokine gradient of CXC-chemokine ligand 13 (CXCL13), which is produced by a network of follicular stromal cells[5]. The recognition of CXCL13 by CXC-chemokine receptor 5 (CXCR5), which is expressed by B cells, generates signals that promote the migration of B cells, thus enabling them to constantly monitor their environment for antigens.

B cells can encounter soluble or large particulate antigens that are attached to the surface of neighbouring cells, such as macrophages, follicular dendritic cells (FDCs) or dendritic cells (DCs)[6–9]. Small soluble antigens gain access to the lymph nodes through the afferent lymph vessels and might directly pass into the B cell follicle independently of cell-mediated antigen presentation[10]. The mechanisms by which low-molecular-mass

antigens access the B cell zone remain controversial, but it has been suggested that their entry is facilitated by small pores located in the subcapsular sinus region of the lymph nodes[11] or that it might occur by simple diffusion[12].

Despite the ability of follicular B cells to rapidly gain access to soluble antigens, there is convincing evidence from *in vivo* studies that larger antigens (with a molecular mass that is greater than 70 kDa) — such as viral aggregates, immune complexes or antigen-coated microspheres — which have limited access to the follicle, can reach the B cell zone and can trigger B cell activation[13]. Such antigens are found tethered to the surface of specialized APCs and are particularly efficient at inducing B cell responses, even at low densities[2]. B cells are localized in defined environments in SLOs, which favour their encounter with antigens; for instance, in the cortex of the lymph nodes, B cell follicles are strategically positioned beneath the subcapsular sinus, where B cells can continuously sample antigens that are presented by subcapsular sinus macrophages[6,7]. In addition, B cell follicles contain FDCs, which are also capable of presenting antigens to B cells[8]. The interaction of B cells with antigens that are presented on the surface of neighbouring cells in the SLOs triggers the formation of an immunological synapse that facilitates the efficient extraction and processing of membrane-tethered antigens (discussed in detail below). For a more detailed overview of the different cell types and surface molecules that are involved in antigen presentation to B cells, we refer the reader to a recent review[13].

The establishment of immunological synapses must be coupled to an arrest in B cell migration, which enables antigens to be acquired. Interestingly, the presence of CXCL13 enhances B cell activation during antigen recognition, which suggests that there is interplay between BCR and CXCR5 signalling. Indeed, CXCL13 promotes membrane ruffling and lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin)-supported adhesion during antigen recognition (the formation of kinapses) through a mechanism that requires the actomyosin network. Furthermore, the strength of BCR signalling alters CXCL13-mediated B cell migration[14], which shows how B cells couple antigen acquisition to their migratory capacity.

Formation of the B cell immunological synapse. It is well established that B cells form an immunological synapse upon engagement of their BCR with antigens that are bound to the surface of specialized APCs. This dynamic structure that B cells form shows the classical features of the immunological synapse that was originally described in T cells[15,16], where the BCR forms a central cluster surrounded by a ring of adhesion molecules, including LFA1. Integrins promote the adhesion of B cells to APCs, and their engagement can lower the threshold for B cell activation when antigen avidity is low[17].

The BCR comprises a plasma membrane immunoglobulin coupled to a signalling module that is formed by the Igα–Igβ dimer, containing immunoreceptor tyrosine-based activation motifs (ITAMs), in which tyrosine residues rapidly become phosphorylated by SRC family kinases upon antigen engagement[18,19]. This results in the recruitment and activation of SYK, followed by the induction of calcium signalling, which is involved in initiating the gene transcription required for B cell function[20]. The early events of B cell activation induce a rapid actin-dependent membrane-spreading response at the antigen-contact site, which helps to increase the number of BCR–antigen encounters and that is required for the formation of signalling microclusters[21,22]. Following this, antigens are gathered into BCR microclusters, in which co-receptors (such as CD19)[23], as well as cytosolic signalling components (such as LYN, SYK and VAV1[24,25]) are recruited to form 'microsignalosomes'. This is followed by a contraction phase in which antigen–BCR complexes converge into a central cluster. This contraction is caused by the concerted actions of rearrangements of the cortical actin cytoskeleton, which are mediated by ERM proteins (ezrin, radixin and moesin proteins; which link plasma membrane proteins to the actin cytoskeleton[26]), and the microtubule-based motor protein dynein[27]. The spreading response that is shown by B cells is tightly coupled to the signalling capacity of the cell, as cells that recruit fewer signalling molecules to microclusters show deficient spreading responses to membrane-bound antigens[23]. This has a direct effect on the amount of antigens that are accumulated at the synapse and, therefore, that are essential for efficient antigen extraction. These results show that signalling events are tightly coordinated during synapse formation and that they are crucial for B cell activation (FIG. 1).

Role of the actin cytoskeleton at the B cell synapse. As mentioned above, B cells undergo dynamic changes in their actin cytoskeletons to induce cell spreading following synapse formation, which helps to promote the gathering and the extraction of membrane-tethered antigens (FIG. 1). Several downstream BCR effectors that regulate actin cytoskeletal rearrangements have been identified. These include the RHO GTPases RAC1 and RAC2, which promote actin polymerization and are important for cell spreading upon BCR stimulation. In particular, RAC2 (REF. 28), which functions with RAP1 GTPases[29], is required for LFA1-mediated membrane spreading during synapse formation. Conversely, RAP1 GTPases also promote B cell spreading in the absence of LFA1 engagement, by generating fast rearrangements of the actin cytoskeleton through the activation of the actin-severing protein cofilin[30]. Indeed, BCR engagement was shown to initially induce a fast de-polymerization of the actin cytoskeleton, followed by a polarized re-polymerization[30,31]. Furthermore, the severing of F-actin, which is controlled by RAP GTPases, regulates the formation and the mobility of BCR microclusters, thereby affecting B cell signalling and antigen gathering[30].

It has been suggested that the severing of the cortical actin cytoskeleton might be required to remove the barriers that restrict receptor diffusion[32], thus promoting BCR microcluster formation. Such a mechanism could be coupled to the inactivation of ERM proteins

Cell polarity
The asymmetric organization of both functional and structural cell components, which are crucial to coordinate diverse biological functions ranging from directional cell migration and asymmetric cell division to the maintenance of tissue integrity.

CXC-chemokine ligand 13 (CXCL13). A chemokine belonging to the CXC-chemokine family that functions as a chemoattractant for B cells by binding to CXC-chemokine receptor 5 (CXCR5).

Lymphocyte function-associated antigen 1 (LFA1). An integrin that is formed by the α-integrin (also known as CD11a) and β-integrin (also known as CD18) chains, It is present in diverse cell types of the immune system, such as lymphocytes, macrophages and neutrophils. LFA1 binds to its ligand intercellular adhesion molecule 1 (ICAM1), which is present on the cell surface of antigen-presenting cells. In B cells, it promotes cell adhesion and antigen gathering during immunological synapse formation, thereby facilitating B cell activation.

Kinapses
Motile adhesive interactions between lymphocytes and antigen-presenting cells. They differ from synapses because the interactions can be transitory.

ERM proteins
A family of three closely related proteins formed by ezrin, radixin and moesin that connect actin filaments with the plasma membrane. They possess a FERM (protein 4.1, ezrin, radixin and moesin) domain that mediates interactions with proteins in the plasma membrane and a charged carboxyl terminus that interacts with actin filaments.

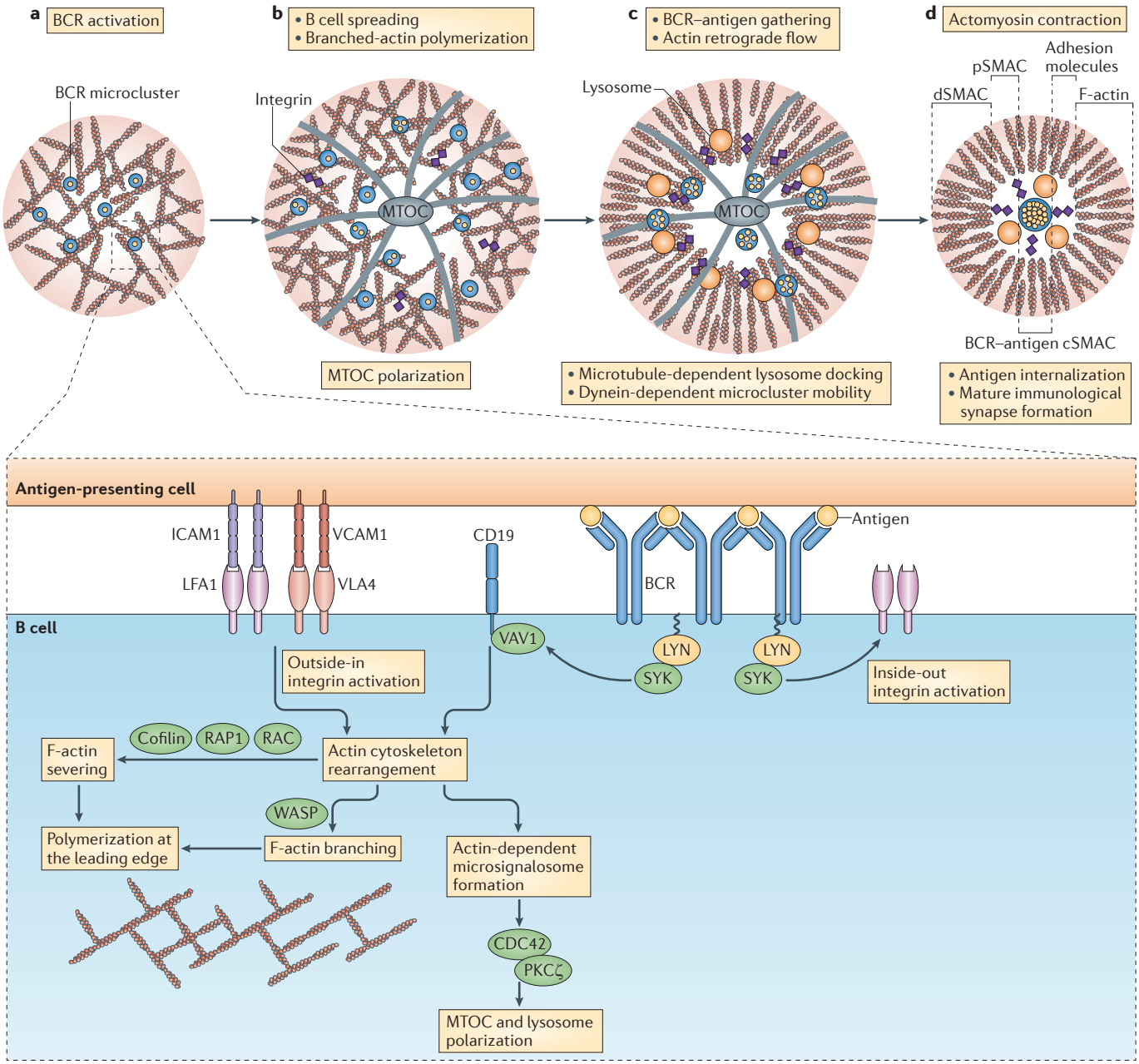


Figure 1 | **B cell immunological synapse formation.** **a** | B cell receptor (BCR) engagement by an antigen triggers the formation of BCR oligomers, which converge to form signalling platforms called microclusters. CD19 is transiently recruited to these microclusters, leading to the activation of signalling molecules such as SYK, LYN and VAV1. The activation of the integrins lymphocyte function-associated antigen 1 (LFA1) and very late antigen 4 (VLA4) promotes B cell adhesion to the surface of the antigen-presenting cell, thereby lowering the threshold of activation (shown in inset). The downstream signalling events that these integrins initiate promote RAC-dependent actin polymerization as well as F-actin severing by the activation of RAP1 and cofilin. **b** | The actin cytoskeletal rearrangements are essential for the spreading and contraction response that is required for antigen gathering by B cells. Concomitantly, the microtubule network is organized towards the immunological synapse where the minus-end molecular motor dynein drives the concentration of microclusters at the synapse. **c** | The polarization of the microtubule-organizing centre (MTOC) is also essential for the local recruitment and secretion of MHC class II$^+$ lysosomes that promote antigen extraction. **d** | Together, these dynamic events lead to the formation of a mature immunological synapse that is characterized by concentric regions: the central supramolecular activation cluster (cSMAC) in which BCRs that are engaged with antigens are concentrated, the peripheral SMAC (pSMAC), which contains adhesion molecules such as LFA1, and the distal SMAC (dSMAC) in which actin is enriched. This structure is essential for B cells to coordinate cell signalling with antigen extraction. CDC42, cell division control protein 42; ICAM1, intercellular adhesion molecule 1; PKCζ, protein kinase C ζ-type; VCAM1, vascular cell adhesion molecule 1; WASP, Wiskott–Aldrich syndrome protein.

Stochastic simulations
A system of particles can be described by its equations of motion. In a system that is subject to thermal fluctuations it is necessary to include a stochastic (random) term that accounts for these fluctuations. By using stochastic simulations a numerical integration of these equations can be generated. Quantities that are experimentally measurable are obtained by averaging several realizations of the process.

Microtubule organizing centre
(MTOC; also known as the centrosome in animal cells). A major site of microtubule nucleation that is enriched in α-tubulin. This dynamic structure organizes the mitotic and meiotic spindle and basal bodies that are associated with cilia.

Lysosomes
The central degradative compartments of the cell. The lysosomes contain an acidic pH (4.6–5.0) and they are where lysosomal hydrolases are concentrated.

— driven by BCR stimulation — which also leads to a transient increase in BCR mobility and is required for efficient microcluster formation, for membrane spreading and to gather antigens at the synapse[26]. In addition, the proteins that regulate actin cytoskeleton dynamics are essential to promote the development and the activation of B cells.

Wiskott–Aldrich syndrome protein (WASP)-deficient B cells show aberrant cell surface clustering of LFA1 downstream of BCR engagement during the formation of B cell synapses, as well as showing defects in spreading and migration[33].

Overall, the control of receptor diffusion dynamics by actin cytoskeletal rearrangements is crucial for B cells to 'tune' BCR signalling and to promote antigen uptake during B cell synapse formation.

Microtubule-dependent trafficking of molecules in synapse formation. Stochastic simulations of the dynamics of BCR and LFA1 molecules suggest that the formation of the synapse occurs only if BCR mobility is enhanced by directed motors[34–36]. Recent work has highlighted a role for the microtubule network in B cell synapse formation. It was shown[27] that dynein — a microtubule minus-end motor protein that is involved in the transport of intracellular cargo — is required to concentrate BCR microclusters at the synapse. Moreover, and similarly to observations that were originally made in immunological synapses established by natural killer (NK) and cytotoxic T cells[37], B cells were found to rapidly relocate their microtubule organizing centre (MTOC) to the site of antigen encounter[38]. In B cells, the repositioning of the MTOC at the immunological synapse occurs at the same time as the repositioning of MHC class II-containing lysosomes, which could control the local concentration of MHC class II molecules required to process incoming antigens[38]. In addition, the local secretion of MHC class II-containing lysosomes at the synapse is required to promote the extraction and the processing of tethered antigens[38].

The role of MTOC polarization in lysosome transport to the B cell synapse has been assessed by carrying out laser ablation of the MTOC and subsequently analysing the effect on lysosome distribution (FIG. 2). It was shown that lysosomes remained dispersed and that they did not cluster at the immunological synapse when the MTOC had been disrupted. This shows that the microtubule network guides lysosome trafficking to the synapse that forms upon BCR engagement with immobilized antigen. The molecular mechanisms behind MTOC repositioning have not been fully resolved, but they have been suggested to be dependent on forces generated by synapse-associated dynein in T cells[39]. This could also involve the coupling of microtubules to the actin network at the synapse by proteins such as cytoplasmic linker protein 170 (CLIP170), IQ-containing RAS GTPase-activating protein 1 (IQGAP1) and CLIP-associating protein 1 (CLASP1)[40,41]. In addition, the MTOC is reorientated in migrating fibroblasts as a result of the rearward movement of the nucleus that is generated by the actomyosin network[40]. Whether MTOC polarization to the synapse of B cells is also promoted by dynein and whether it is accompanied by nuclear movement will be interesting to investigate. Interestingly, microtubule polarization in B cells uses the conserved atypical protein kinase C ζ-type (PKCζ)–cell division control protein 42 (CDC42) polarity machinery[38], which is shared by many other biological systems and which modulates cell polarity during directed cell migration, tissue development and asymmetric cell division[41]. Accordingly, recent studies show that B cells also undergo asymmetric cell division following antigen stimulation (see below).

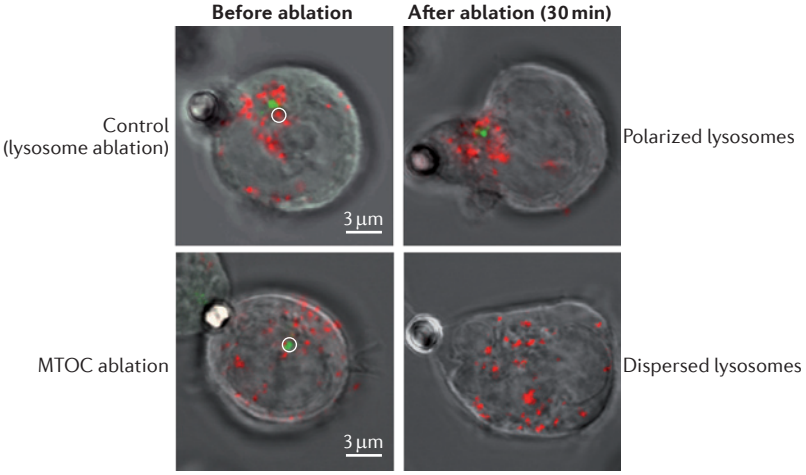


Figure 2 | **Polarization of the MTOC directs the trafficking of lysosomes towards the immunological synapse.** The figure shows microscopy images of a B lymphoma cell line expressing red fluorescent protein (RFP)-tagged cathepsin D and green fluorescent protein (GFP)-tagged centrin. The cells are engaged with an anti-IgG-coated bead before and after a two-photon laser-mediated ablation of either the microtubule organizing centre (MTOC) (lower panel) or a lysosome (upper panel), as a control. White circles show ablated regions. In the control cell there is recruitment and clustering of lysosomes towards the immunological synapse, whereas in the MTOC-ablated cells, the lysosomes remained dispersed. Scale bar represents 3 μm.

Antigen processing by B cells

Lysosome secretion at the immunological synapse. The immunological synapse is a dynamic platform where both endocytic and exocytic processes take place. Indeed, T helper and cytotoxic T cells were shown to secrete effector molecules, such as cytokines and lytic granules, through the immunological synapse[42,43], thereby providing a selective way in which to activate or to destroy interacting target cells. Exocytosis at the immunological synapse in T cells was found to occur in subdomains that have low levels of polymerized actin[44], which could facilitate the local secretion of molecules[45]. Cortical actin was shown to function as a fusion barrier that impedes exocytosis at the plasma membrane of epithelial cells[46]. In the case of B cells, the recruitment of lysosomes to the synaptic interface was shown to occur using total internal reflection fluorescence microscopy (TIRFM). The recruited lysosomes became progressively immobile, which suggests that vesicle docking at the plasma membrane was taking place. In addition, these studies showed that lysosomes were being locally secreted at the synapse, as shown by measuring the local

acidification of the extracellular synaptic space and by directly monitoring exocytic events using TIRFM. Whether lysosome exocytosis at the B cell synapse is associated with regions that have a low density of actin remains to be investigated.

The accurate docking and fusion of specialized vesicle carriers at the immunological synapse relies on complex cellular machinery that includes tethering factors from the RAB family of small G proteins, as well as factors from the SNARE (soluble *N*-ethylmaleimide-sensitive factor accessory protein receptor) family[47,48]. For instance, the delivery of recycling endosomes carrying T cell receptors at the immunological synapse is dependent on the SNARE proteins vesicle-associated membrane protein 2 (VAMP2) and VAMP3, and is accompanied by the clustering of the target SNAREs syntaxin 4 and synaptosomal-associated protein 23 (SNAP23) at the plasma membrane[49]. The exocytosis of secretory lysosomes has been well documented in many cell types[50], and in cytotoxic T cells it is regulated by RAB27A[51], which was recently shown to interact with synaptotagmin-like protein 1 (SLP1; also known as SYTL1) and SLP2 (REF. 52). The molecular regulators of lysosome exocytosis in B cells have not yet been identified. However, our unpublished data suggest that VAMP7 could be involved, as this SNARE protein is recruited with lysosome-associated membrane protein 1 (LAMP1)-expressing lysosomes to the B cell synapse following BCR engagement with immobilized antigen (A.-M.L.-D., M.-I.Y. and J. Diaz, unpublished observations).

Role of proteases in antigen extraction at the B cell synapse. Lysosomes are transported towards the site of antigen encounter, where they undergo exocytosis. This suggests that the activity of extracellular proteases that are locally released at the immunological synapse is required to free membrane-tethered antigens on antigen-bearing cells[38]. Interestingly, an analogous mechanism has been described in macrophages, in which lysosome secretion in an enclosed acidic extracellular zone is used to promote the digestion of aggregated lipoproteins by lipases and proteases[53]. Whether the B cell synapse that forms when the cell is in contact with membrane-tethered antigens is also a tightly enclosed structure is unknown. In addition, an uninvestigated aspect of B cell biology is the possibility that lysosomal lipases that are secreted at the immunological synapse have a role in promoting the acquisition of membrane-bound antigens. Alternatively, B cells could extract membrane-bound antigens through a process known as trogocytosis, which involves the intercellular exchange of membrane fragments[8,54,55]. It is probable that such a mechanism would also depend on cytoskeletal rearrangements to generate the force that would be required to extract the antigens and/or to facilitate the local secretion of hydrolases and possibly lipases.

The nature and amount of proteases that are secreted at the B cell synapse — an environment that is different from that of the lysosomal compartments — could also determine the size of the peptide antigen that will be presented to T cells. In addition, antigenic peptides that are generated in the extracellular space might also be directly loaded on cell surface MHC class II molecules, independently of the intracellular compartments. Indeed H2-DM, which is the chaperone that catalyses peptide loading on MHC class II molecules, has also been detected on the cell surface of both B cells and immature DCs[56,57], where extracellular antigen processing and peptide loading was shown to take place. Interestingly, and similarly to DCs, B cells contain much lower levels of lysosomal proteases than macrophages[58]. This favours the presentation of antigenic peptides on MHC class II molecules by limiting the complete destruction of the peptide determinants[59].

BCR internalization and signalling. Antigens that are acquired by the BCR in either a soluble or a tethered form induce receptor endocytosis and signalling events, which are both equally important for B cells to become fully activated. The local secretion of hydrolases at the immunological synapse promotes the extraction of membrane-bound antigens[38], which suggests that antigen degradation occurs, at least partially, in the extracellular space and probably at the same time as the main intracellular pathway. Indeed, BCR engagement induces the maturation of late endosomal and lysosomal compartments, into which receptors are rapidly internalized and antigens are degraded[60] and processed to form MHC class II–peptide complexes (FIG. 3). BCR internalization is clathrin-dependent[61] and relies on the ubiquitylation of the immunoglobulin α-chain[62] and the immunoglobulin heavy chain[63] of the BCR complex. Ubiquitylation of antigen–BCR complexes was recently shown to occur downstream of SYK-dependent signalling[64], which highlights that endocytic trafficking and signalling of the BCR are tightly linked. These events are also influenced by the nature of the antigen that binds to the BCR. Indeed, oligomeric antigens, such as those that are membrane-bound, were shown to trigger stronger BCR-mediated signalling and to promote more efficient endocytic trafficking of BCR–antigen complexes than monovalent antigens[65]. Interestingly, BCR signalling continues within endocytic compartments, where it leads to the sequential phosphorylation of kinases that control the transcription of genes required for B cell activation[66].

Recent work has highlighted the importance and complexity of the endomembrane machinery that is involved in this process. Beige mice, which are deficient in endosome biogenesis, show delayed transport of the endocytosed antigen–BCR complexes to their lysosomes and are consequently less efficient at presenting antigens to T cells[67]. However, these B cells show more sustained BCR signalling, which suggests that the delayed delivery of the BCR–antigen complex to the lysosomes facilitates continuous signalling from early endosomal compartments. Thus, the subcellular localization of the BCR, which changes following antigen recognition, not only facilitates the transport of antigens to be processed but also regulates the signalling effector functions that affect B cell fate *in vivo*.

Atypical protein kinase C ζ-type
(PKCζ). An atypical member of the PKC family that does not require either calcium or diacylglycerol for its activation. It associates with partitioning defective 3 (PAR3) to regulate cell polarity in diverse cell types.

Cell division control protein 42
(CDC42). A RHO-GTPase that controls diverse cellular functions including cell morphology, migration and cell division. By interacting with Wiskott–Aldrich syndrome protein (WASP), CDC42 regulates actin polymerization.

Asymmetric cell division
A cell division in which the organelles and proteins do not distribute equally, giving rise to two daughter cells with different properties and fates. This is required for cell specialization and mainly relies on asymmetry in the spindle position during the prophase stage of mitosis. Notably, stem cells can divide asymmetrically to give rise to two distinct daughter cells: one cell that is a copy of themselves and one cell that is programmed to differentiate into another cell type.

Total internal reflection fluorescence microscopy
(TIRFM). A fluorescence microscopy technique that involves illuminating and observing a thin layer of the specimen (about 200 nm) close to the cover slip using an 'evanescent wave' (which is formed when a laser encounters the glass surface above the critical angle in such a way that it is 'totally reflected'). It combines the speed and the resolution of the usual fluorescence microscopy (being an example of widefield microscopy) with the possibility of excluding excitation and emission from unwanted planes, providing a high signal to noise ratio.

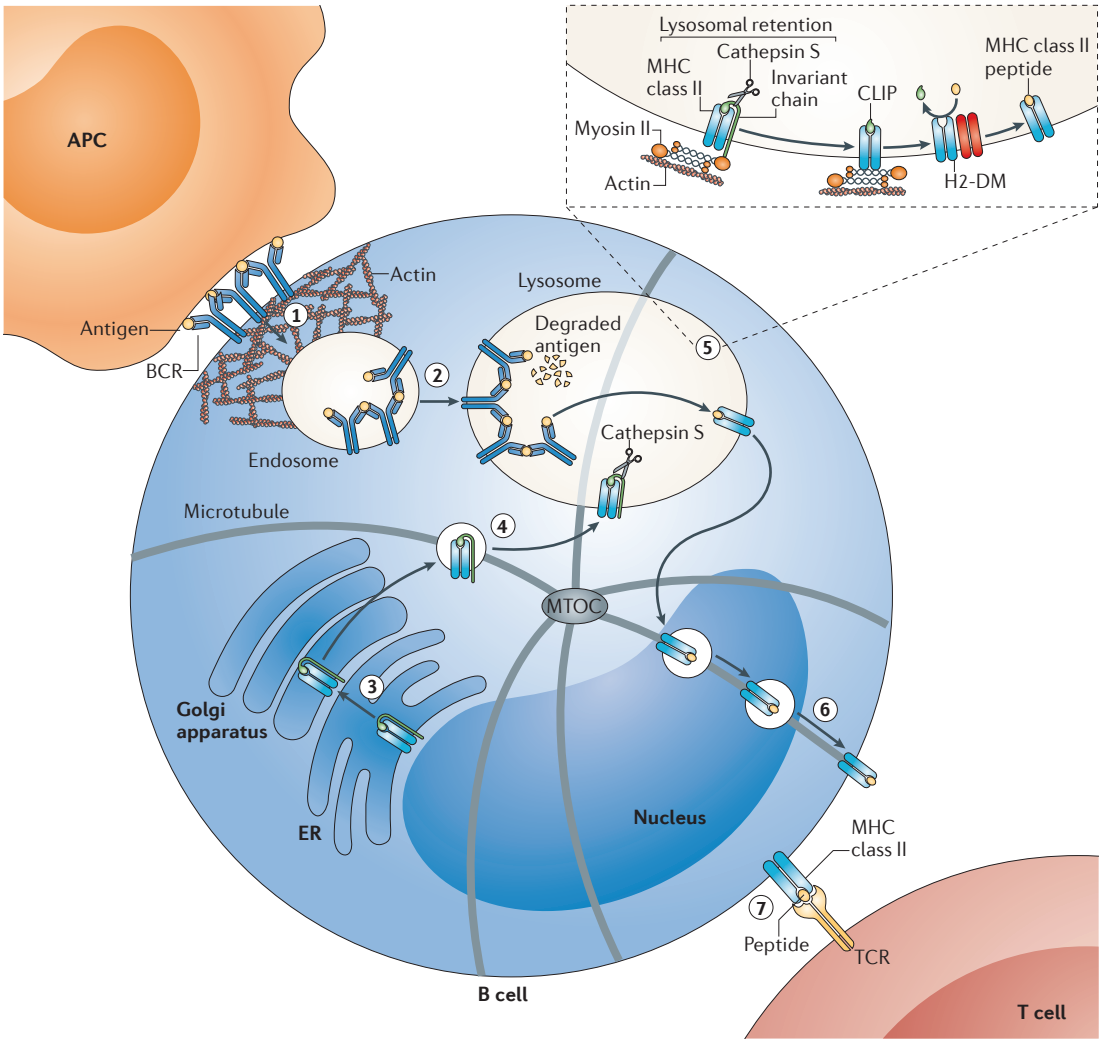


Figure 3 | **Membrane trafficking events required for antigen processing in B cells.** Antigen recognition is mediated by the B cell receptor (BCR). The engagement of antigen and BCR induces receptor oligomerization and downstream signalling that promotes dynamic actin cytoskeletal rearrangements. This enables efficient BCR–antigen internalization into late endosomal compartments (step 1). Concomitant with its endocytosis, the interaction of the BCR with antigen also promotes the biogenesis of the antigen-processing compartment in which both the antigen and the accessory molecules, such as MHC class II molecules, H2-DM and proteases, converge[60,109,110] (step 2). MHC class II molecules associate with their chaperone — the invariant chain in the endoplasmic reticulum (ER) — which has three essential functions: it assists in the folding of MHC class II molecules, it prevents early antigen binding and it directs the trafficking of MHC class II molecules through the *trans*-Golgi network to the endo-lysosomes[111] (step 3). This occurs as a result of leucine-based sorting motifs that bind the adaptor complexes AP1 and AP2 (step 4). The invariant chain also promotes the interaction with myosin II, which is required for the convergence of MHC class II molecules and BCR–antigen complexes in lysosomes that process antigens. In the antigen-processing compartment, proteolysis of both the antigen and the invariant chain takes place to produce antigenic peptides and to free the peptide-binding groove of MHC class II molecules, respectively. The last step of invariant chain cleavage requires the activity of the cysteine protease cathepsin S and is followed by peptide exchange, which is catalysed by the chaperone H2-DM[112,113] (step 5). This also removes the endosomal retention motif in the cytosolic tail of the invariant chain and enables mature MHC class II-peptide complexes to be exported to the cell surface (step 6). These complexes can then interact with their intended T cell (step 7). APC, antigen-presenting cell; CLIP, cytoplasmic linker protein; MTOC, microtubule organizing centre; TCR, T cell receptor.

Beige mice
Members of a mouse strain typified by beige hair that carry the lysosomal trafficking regulator (*Lyst*) mutation.These mice have an autosomal recessive disorder that is characterized by hypopigmentation and immune cell dysfunction. Beige mouse abnormalities result from aberrant lysosomal trafficking and are similar to those of patients with Chediak–Higashi syndrome.

Role of the actin cytoskeleton in the trafficking of BCR–antigen complexes. The molecular mechanisms responsible for the reorganization of the endocytic pathway that is triggered by BCR stimulation are not completely understood. Several lines of evidence suggest that a tight coupling of both BCR signalling and dynamic actin cytoskeletal reorganization is required. Indeed, BCR engagement triggers tyrosine phosphorylation of actin-binding protein (ABP1). This, in turn, promotes dynamin recruitment and actin rearrangements that enable efficient receptor internalization into late endosome compartments, where antigen processing takes place[68]. Other downstream BCR molecules that link receptor signalling to the actin cytoskeleton include

Bruton tyrosine kinase (BTK), which was recently shown to control the WASP-dependent actin dynamics that are required for the internalization and the processing of BCR–antigen complexes[69]. In addition, the tyrosine kinase SYK regulates actin remodelling, on which the transport of MHC class II$^+$ lysosomes towards incoming antigens depends[70]. Actin remodelling by SYK could involve its downstream effectors protein tyrosine kinase 2β (PYK2; also known as PTK2B) — which regulates the activity of the actin severing and capping protein gelsolin[71] — or VAV1 (which is a GTP exchange factor for RHO GTPases); both are known to be modulators of actin dynamics[72]. Indeed, VAV1 is recruited to BCR-signalling microclusters and is crucial for the propagation of B cell spreading in response to antigen stimulation[73].

Furthermore, through a mechanism that also requires the actin cytoskeleton, internalized BCR–antigen complexes are stored within non-terminal lysosomal compartments in which antigen degradation is limited. This therefore facilitates the prolonged production and the cell surface expression of antigenic peptide–MHC class II complexes, thereby favouring the presentation of antigens to T cells[74]. Finally, the actin-associated motor myosin II — which is activated following BCR engagement — was shown to regulate the polarized transport of MHC class II molecules towards internalized antigens[75]. This highlights that BCR-dependent actin remodelling also controls the trafficking of MHC class II molecules, promoting the formation of the antigen-processing compartment (FIG. 3).

Antigen presentation to T cells: T cell–B cell cooperation. A pivotal step in B cell activation is the presentation of processed antigens to CD4$^+$ T cells, which enables B cells to receive the necessary stimuli to become fully activated[1]. After antigen encounter, B cells must migrate towards the T cell boundary, where contacts with cognate T helper cells can be established[76]. The receptors that support this directed B cell migration are CC-chemokine receptor 7 (CCR7; which recognizes CC-chemokine ligand 19 (CCL19) and CCL21 that are produced by T stromal cells[77]) and Epstein–Barr virus-induced G protein-coupled receptor 2 (EBI2; also known as G protein-coupled receptor 183), which promote the migration of B cells to the outer follicle during the early stages of activation[78]. Importantly, the interaction between B cells and T cells also leads to the formation of an immunological synapse, where bidirectional activation signals are exchanged between the cells. Local secretion was shown to be promoted at the immunological synapse in both cell types by the generation of a polarized phenotype and the re-orientation of their MTOC, together with their Golgi apparatus, towards the synaptic interface[79]. In addition, in the same study it was shown that B cells also polarize MHC class II compartments towards the contact site with T cells, which the authors suggest could facilitate the local delivery of antigenic ligands for TCR stimulation[79]. After interacting with T cells, B cells migrate to the interfollicular region where they proliferate and differentiate into short-lived plasmablasts. These cells contribute to the primary immune response by generating antibodies that are of a relatively low affinity[80]. Another group of activated B cells, expressing high levels of CXCR5, concomitantly migrates into the B cell follicle, where they continue to proliferate and to form germinal centres. In the B cell follicle, B cells undergo affinity maturation and differentiate into antibody-producing plasma cells or long-lived memory B cells[81,82]. The signals that determine the differentiation of B cells are poorly understood, but the context of the early activation events, such as antigen recognition and presentation to T follicular helper cells[83] (see below), probably influences their final outcome.

Cell polarity in B cell activation

Cell polarity has been extensively studied in a wide range of systems from budding yeast to immune cells (BOX 1), in which the interpretation of spatial cues initiates the formation of landmarks that are progressively reinforced to form a specialized domain[84]. In this section, we review the role of B cell polarity during the key stages of B cell activation: antigen encounter and differentiation in germinal centres.

B cell polarity and antigen acquisition. The cellular contacts that occur between B cells and antigen-bearing cells, such as FDCs or macrophages, can generally last between 20 and 30 minutes[6,8]. Therefore, the immediate establishment of cell polarity could be a mechanism that is used by B cells to rapidly acquire antigen and to optimize their activation during these short encounters. Indeed, it has been shown that B cells rapidly polarize their MTOC, together with MHC class II$^+$ lysosomes, towards the antigen contact site — a process that relies on the small GTPase CDC42 and its downstream effector protein PKCζ. Impairment of MTOC and lysosome polarization by silencing CDC42 or PKCζ compromises the ability of B cells to extract, process and present immobilized antigen to T cells[38]. Importantly, genetic evidence also shows the involvement of these polarity proteins in B cell functions. Mice with a conditional deletion of CDC42 show defects in B cell lymphogenesis, whereas PKCζ-deficient mice show impaired humoral immune responses[85,86].

Studies *in vivo* using two-photon microscopy to observe where and how B cells acquire particulate antigens, have shown that internalized antigens are frequently concentrated at the uropod of B cells that are migrating to the T cell zone[6]. This suggests that following antigen acquisition, B cells continue to show a polarized phenotype. Furthermore, recent studies have shown that this asymmetric distribution of antigens within B cells is maintained throughout cell division, which leads to asymmetric antigen segregation among daughter cells; this, consequently, provides the daughter cells with differential capacities for antigen presentation[87]. The daughter cells that have inherited larger amounts of antigens are more effective at stimulating cognate T cells, which might give them an advantage in competing for the limited T cell help that is available. Whether the generation of these unequal populations ultimately changes the effector fates of B cells *in vivo* remains to be elucidated.

Uropod
Protrusion of the plasma membrane that forms at the rear end of migrating cells.

B cell polarity in germinal centres. The mechanisms that determine the differentiation of B cells into long-lived memory cells or plasma cells in germinal centres have not yet been resolved, but they are probably influenced by antigen affinity, by extracellular cues that are produced by other cell types and by the time of activation[88]. The selection of high-affinity B cell clones in the germinal centres is thought to be dependent on signals generated either by BCR crosslinking with antigens that are tethered to the surface of FDCs or by germinal centre T helper cells that have been stimulated by high-affinity B cell clones presenting uptaken antigen on MHC class II molecules[89]. Recent studies using intravital microscopy imaging combined with *in situ* photoactivation to label germinal centre B cells have provided strong evidence that T helper cells in the germinal centre are the limiting factor in affinity-based selection[90].

The ability of B cells to rapidly polarize their antigen-processing machinery in response to defined extracellular cues could determine their different cellular fates. It has been shown that mutations in the RHO or RAC guanine nucleotide exchange factor dedicator of cytokinesis 8 (DOCK8) leads to deficient intercellular adhesion molecule 1 (ICAM1) clustering at the B cell synaptic interface, thereby impairing peripheral supramolecular activation cluster (pSMAC) formation. This defect results in impaired affinity maturation of B cells and deficiencies in the mature antibody responses[91]. Importantly, DOCK8 is a CDC42-specific guanine-nucleotide exchange factor and was recently shown to control the activity of CDC42 that is present at the leading edge of migrating DCs. DOCK8-deficient DCs show impaired CDC42 activation at the leading-edge membrane, which results in an inability to extend long protrusions in the direction of migration[92]. Whether the DOCK8-dependent defects that are observed in B cells also result from a failure to locally activate CDC42 and, consequently, to efficiently polarize the cell during synapse formation is an interesting possibility to investigate.

Activated B cells move with a highly polarized morphology within germinal centres, forming extensions such as filopodia at the leading edge and uropods at the trailing edge. In addition, stationary B cells have extending protrusions to contact FDCs in order to continue to sample their microenvironment[93]. Whether B cells also use conserved polarity proteins to establish this specialized morphology when scanning for antigens remains to be addressed. The recent findings about the influence of polarity in B cell asymmetric cell division[94] suggest that the first antigen encounter could provide the extracellular cue that the cell uses to successfully carry out its differentiation programme through a highly conserved polarity mechanism. Therefore, it is essential to define the molecules that regulate early B cell polarization.

In addition to its role in promoting efficient antigen processing and presentation, PKCζ-dependent B cell polarization is also required for the later stages of B cell activation. In germinal centres, activated B cells asymmetrically segregate PKCζ, along with the transcriptional regulator B cell lymphoma 6 (BCL-6) and the interleukin-21 receptor (IL-21R), which are unequally distributed among daughter cells. This promotes diversity among the daughter cells and could ensure that two cells with different fates are generated, which are destined either to accomplish effector functions or to develop into memory cells, thereby providing a mechanism for self-renewal[94]. However, the fate of daughter cells *in vivo* remains to be determined. Interestingly, this asymmetric cell division requires polarity cues from the microenvironment, as B cells that have defects in the adhesion molecule ICAM1 fail to divide asymmetrically[94].

Models of cell polarity: perspectives and analogies. From a physical point of view, cell polarity is an interesting example of symmetry breaking[95], in which the system starts with a homogeneous distribution of molecules and finishes by forming a defined pattern, such as a single patch of molecules at the pole of the cell[96]. Although cell polarity in B cells is not as well studied as cell polarity in yeast and in the model organism *Caenorhabditis elegans*, several key features of cell polarity that are specific to B cells are beginning to be identified, and some computational models have been created[97,98]. First, the synapse comprises a signalling platform that breaks cell symmetry and guides the formation of a polarized phenotype (BOX 1). At the immunological synapse, microtubule molecular motors such as dynein are required for the coalescence but not for the formation of small signalling BCR microclusters[27]. Second, the activation of CDC42 and its downstream effector PKCζ are required to establish and to maintain B cell polarity[38]. Third, B cells develop actomyosin contractions that might have a number of functions: they might create an intracellular flow towards the synapse as in *C. elegans*[99]; they might gather BCR–antigen complexes at the central SMAC (cSMAC)[21,75]; or they might generate the forces that are required for antigen internalization. Fourth, integrins such as LFA1 have been shown to enhance the signal that is delivered following BCR engagement[17]. Whether integrin-mediated mechanosensing affects cell polarity remains to be established. Interestingly, it has recently been shown that B cells discriminate between antigens that are associated with substrates of different rigidities, which indicates that they are competent for mechanotransduction[100].

Data that have been gathered from theoretical models and genetics tools that are available in yeast are consistent with the idea that there is a general mechanism for cell polarity, which consists of a combination of positive and negative feedback loops[101–103]; the positive feedback loops function swiftly and amplify small fluctuations in signalling, whereas the negative feedback loops confine the actions of the initial positive feedback loops using a long-range inhibitor mechanism. In the case of B cells, positive feedback loops could correspond to the positive signals that control proteins involved in the actomyosin dynamics downstream of BCR, such as RAC[28], RAP1 (REF. 29) or BTK, which activate the actin nucleator WASP[69], or to pathways that are induced by co-stimulatory receptors such as CD19 (REF. 23). The inhibitory mechanisms might simply arise from the limited levels of proteins, or from the involvement

Filopodia
Dynamic actin-rich filamentous protrusions that extend from cells.

Symmetry breaking
The process by which a system switches from a disordered or a uniform state to a state in which an ordered shape, direction or pattern is established; for example, proteins that are normally uniformly distributed will concentrate at a single spot after receiving a certain stimulus.

Mechanosensing
The capacity of cells to sense mechanical stimuli, such as deformation of the membrane, the cortex, the nucleus and other structures, or to sense changes in the adhesive properties of the substrate.

Box 1 | Establishing polarity: the roles of PAR, actin and microtubules

Cell polarity is fundamental for the development of multicellular organisms as well as for the functional responses of individual cells. Cell polarity is coordinated by conserved proteins belonging to the partitioning defective (PAR) polarity complex, namely Scribble and Crumbs. PAR proteins function downstream of cell division control protein 42 (CDC42) to regulate actin polymerization and they are dynamically associated with the cell cortex and the cytoskeleton. Relevant models of cell polarity are described below.

Yeast

In the budding yeast *Saccharomyces cerevisiae*, Cdc42 is the main polarity activator that functions downstream of Bud1 (which is the yeast homologue of Rap1). In its active GTP-bound form, Cdc42 localizes to sites of active growth (the bud) where it regulates actin polymerization via formins and Wiskott-Aldrich syndrome protein (Wasp). Actin cytoskeletal rearrangements and the microtubule network coordinate the delivery of proteins to the polarized bud (see the figure).

Caenorhabditis elegans oocyte

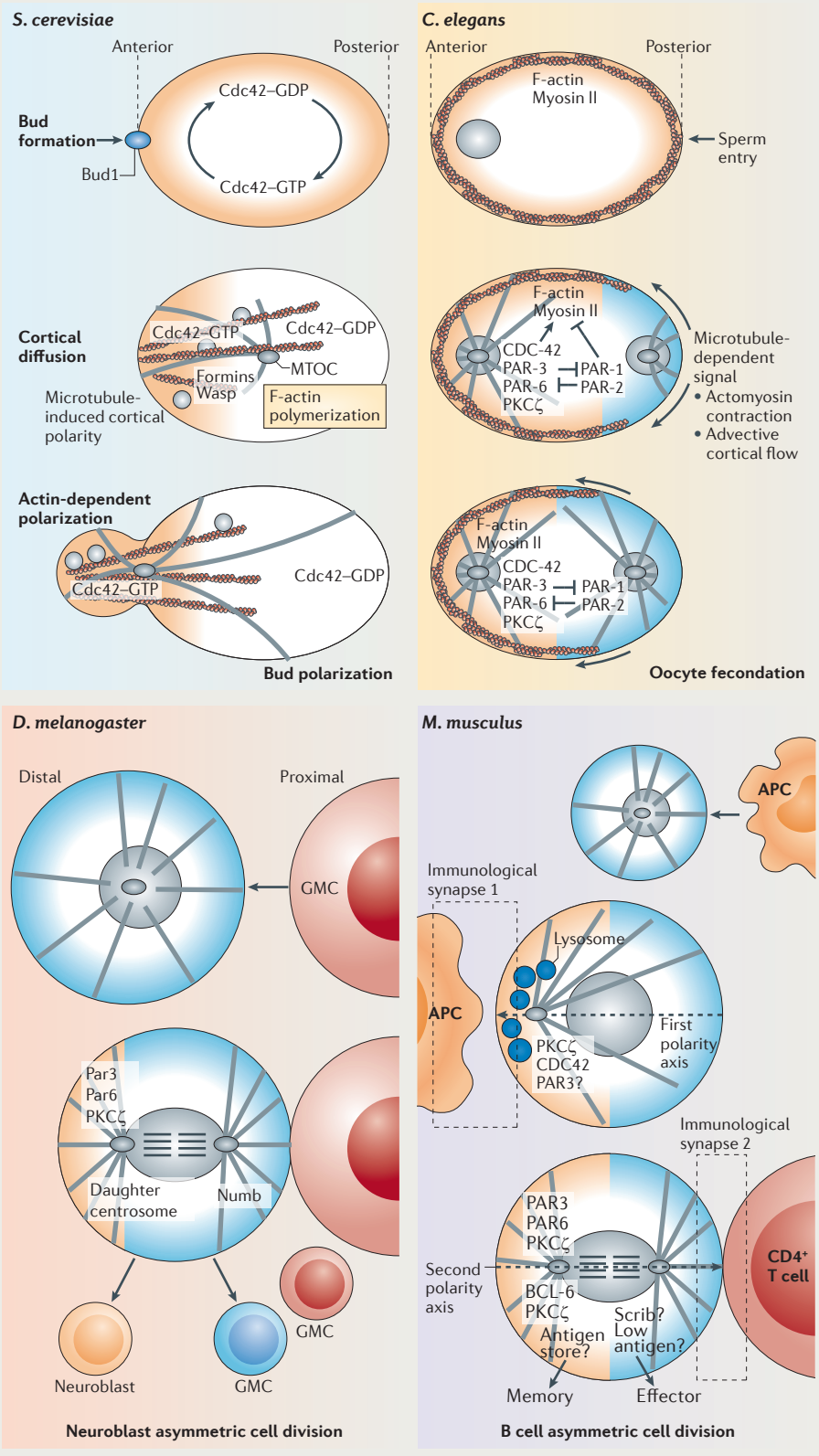
PAR is crucial for generating the anterior–posterior body axis in *C. elegans* embryos. The PAR-3–PAR-6– protein kinase C ζ-type (PKCζ) domain forms at the anterior pole, opposite to the entrance of the sperm. Depletion of these proteins on the posterior side causes an accumulation of PAR-1 and PAR-2 via cross-inhibition mechanisms. Anterior PAR proteins promote actomyosin contraction, which is inhibited on the opposite side by PAR-1. In addition, there is a diffusive signal from the microtubule organizing centre (MTOC) to the local cortex that inhibits myosin II contraction. As a result CDC-42 and anterior PAR accumulate away from the sperm MTOC (see the figure).

Neuroblast progenitor cells in *Drosophila melanogaster*

The mitotic spindle orientates following contact with the ganglion mother cell (GMC) and the daughter centrosome is positioned at the distal pole. During this process, Par proteins are sequestered at the distal pole, whereas the fate determinant Numb remains at the proximal pole. This induces the formation of two different daughter cells: a neuroblast and a GMC (see the figure).

Polarity in B cells in *Mus musculus*

During B cell activation, several axes of polarity are established. First, interaction with antigen-presenting cells (APCs) induces MTOC polarization to the immunological synapse by a mechanism that is dependent on CDC42 and PKCζ. This promotes the polarized recruitment and secretion of lysosomes at the immunological synapse, which is required for antigen extraction. Second, when B cells present antigens to T helper cells they receive a polarity cue that leads to asymmetric cell division in germinal centres. B cells asymmetrically segregate PKCζ, the transcriptional regulator B cell lymphoma 6 (BCL-6) and the interleukin-21 receptor, which are unequally distributed among daughter cells (see the figure). Question marks indicate proteins that are thought to be involved but for which further evidence is required.



Membrane tension
A measure of how stretched the cell membrane is to compensate for the osmotic pressure of the cytoplasm. It can be modified by changing the osmotic pressure of the medium and can be measured by micromanipulation techniques measuring the force necessary to pull tubes of membrane.

Partitioning defective 3
(PAR3). Together with PAR6 these proteins form the PAR polarity complex and are both scaffolding proteins that are implicated in cell polarity. PAR3 and PAR6 bind to each other via their PDZ (PSD95, DLGA and ZO1 homology) domains. They localize at the plasma membrane via atypical protein kinase C ζ-type, cell division control protein 42 (bound to PAR6) or via their PDZ domains.

Table 1 | **Effect of polarity-related proteins on B cell functions**

Protein	Functions shown *in vitro*	Functions shown *in vivo*	Refs
DOCK8	Required for spreading and synapse formation	Required for mature antibody responses and germinal centre formation	91
CDC42	Required for antigen extraction, processing and presentation	Required for early and late B cell development, and B cell proliferation, survival, and signalling. Absence of CDC42 leads to impaired antibody production	38,85
PKCζ	Required for antigen extraction, processing and presentation	Required for BCR signalling, cell proliferation and survival; knockout mice show defects in immune responses	38,86
Dynein	Involved in the gathering of antigen during immunological synapse formation	Data not available	27
RAP1	Required for membrane spreading and synapse formation, and for BCR signalling in response to particulate antigen	RAP1B-knockout mice have defects in the development of marginal zone B cells and reduced homing of B cells to the lymph nodes	29,114,115
RAC	RAC1 and RAC2 regulate B cell spreading during immunological synapse formation	RAC1 and RAC2 are required for B cell development, signalling and survival	28,114,115
WASP	Required for B cell spreading and migration	WASP deficiency leads to a reduction in mature peripheral B cell subsets. Mice that are double-deficient for both WASP and NWASP have reduced B cell immune responses to T cell-independent and T cell-dependent antigens	33,116

BCR, B cell receptor; CDC42, cell division control protein 42; DOCK8, dedicator of cytokinesis 8; NWASP, neural Wiskott–Aldrich syndrome protein; PKCζ, protein kinase C type-ζ.

of negative regulators, such as phosphatase and tensin homologue (PTEN)[104] or SH2 domain-containing protein tyrosine phosphatase 1 (SHP1; also known as PTPN6)[105]. In addition, the mechanical properties of the membrane, such as membrane tension, might help to determine a polarity axis by restricting the diffusion of positive signals[106].

It is worth noting that the process of polarization should be understood in a stochastic manner; in many cases the axis of polarity is randomly established and, for the most part, it is only stabilized following signalling that induces cytoskeletal rearrangements[107] (BOX 1). This supports the idea that, in order to quickly respond to a signal, cells can maintain an unstable state. It is tempting to speculate that, in B cells, contact with immobilized antigens breaks the symmetry of the cell, and that ancestral polarity proteins, such as CDC42, PKCζ and partitioning defective 3 (PAR3), amplify the initial signals, thereby reinforcing the selected polarity axis. Testing the robustness of B cell polarity by activating the BCR at two different cell poles should help to determine whether this hypothesis is correct. Super resolution microscopy, which has so far been implemented to study the static synapse substructures[108], coupled with physical modelling, should shed light on the dynamics and the spatial regulation of polarity proteins.

Conclusions

Recent work has provided new insights into how B cells coordinate complex cellular pathways in order to achieve their immune functions. Studies that have been carried out both *in vitro* and *in vivo* have highlighted the dynamic cellular changes that B cells undergo while they are scanning for antigens in the lymph nodes, as well as following antigen extraction, processing and presentation. It is becoming increasingly clear that B cells are dependent on cell polarity as a central mechanism to coordinate these functions and that alterations in key polarity proteins can affect the outcome of B cell immune responses (TABLE 1). Therefore, the identification of new intracellular proteins that regulate B cell polarity during synapse formation and during their directed cell migration in germinal centres could provide insights into how B cells efficiently achieve these immune functions. Special focus should be given to extracellular proteins in the lymphoid microenvironment, such as chemokines, extracellular matrix proteins and galectins. These proteins could modulate different aspects of cell polarity, such as cytoskeletal rearrangements and lysosome trafficking, and could thereby affect the ability of B cells to respond to antigens. Proteins that modulate B cell polarity could represent valuable candidates to modulate B cell responses *in vivo*.

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References 6 and 7 highlight roles for subcapsular macrophages in the lymph nodes in the presentation of large antigens to B cells *in vivo*.

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Germinal center B and follicular helper T cells: siblings, cousins or just good friends?

Stephen L Nutt[1,2] & David M Tarlinton[1,2]

Humoral immunity requires interaction between specialized populations of B cells and CD4$^+$ T cells, called follicular helper T cells (T_{FH} cells), in the germinal center (GC) to produce memory B cells and long-lived plasma cells. Molecular crosstalk between GC B cells and T_{FH} cells influences the survival, proliferation and differentiation of each cell type. This pairing of GC B cells and T_{FH} cells also occurs at the transcriptional level as the Bcl-6–IRF4–Blimp-1 axis, which is crucial for B cell differentiation, is also essential for the T_{FH} cell identity. Less is known about the memory B cells that arise from the GC pool, as they seem to be distinctly 'programmed' on the basis of their antigen receptor affinity to enter the long-lived memory pool.

The ability to remember past experiences and to 'recall the troops' after repeated challenge with the same antigen is the defining feature of the adaptive immune system. A typical immune response results in the production of a high-affinity, predominantly isotype-switched antibody that is essential for the clearance of many infectious pathogens and provides the basis for humoral immunity and vaccine efficacy[1]. This protective antibody is maintained by the combination of a relatively long intrinsic lifespan in serum and by the continuous secretion of new antibodies by long-lived plasma cells[2]. The second key outcome of the humoral immune response is the formation of memory B cells. Memory B cells often have isotype-switched and somatically mutated antigen receptors, have extremely long lives (years) and maintain the ability to respond rapidly to antigen re-exposure by differentiating into plasma cells[3,4]. Canonical B cell memory requires direct contact between activated B cells and CD4$^+$ T cells in a specialized structure known as the germinal center (GC)[5].

In secondary lymphoid organs, lymphocytes are segregated on the basis of chemokine sensitivity into discrete B cell zones (follicles) and T cell zones (**Fig. 1**). Localization to the B cell zones and T cell zones depends on the chemokine receptors CXCR5 and CCR7, respectively[6]. After being activated by antigen, B cells increase their expression of CCR7 and migrate to the interface between the zones, where they encounter cognate T cells. This encounter results in a burst of proliferation in the outer follicle[7,8]. It seems that these cells have three potential fates. They can become plasmablasts and migrate to extrafollicular sites under the influence of the orphan G protein–coupled receptor EBI-2 (refs. 9,10), producing a transient wave of relatively low-affinity, antigen-specific antibodies. Some activated B cells can, by a poorly understood process, return to the center of the B cell follicle and proliferate further to form a GC[5]. Finally, some of these expanded clones assume a memory phenotype, forming what is referred to as early memory B cells that enter the circulation[11]. Proliferating B cells in a GC, called centroblasts, initiate somatic hypermutation (SHM) and thereby diversify their antigen receptors to enable further clonal selection on the basis of affinity. Germinal centers also contain a specialized type of CD4$^+$ T cell, the follicular helper T cell (T_{FH} cell), that upregulates CXCR5 expression to enable its follicular localization[12,13], and antigen-presenting cells such as dendritic cells, macrophages and a specialized stromal cell known as a follicular dendritic cell (FDC)[14]. Germinal center B cells with potentially altered affinity for antigens then stop proliferating and become centrocytes that are selected according to their ability to bind antigen complexes on FDCs and to elicit help from cognate T_{FH} cells. The interaction between GC B cells and T_{FH} cells is under normal circumstances essential for the GC response, but the requirement of B cells for T_{FH} cell differentiation can be overcome by large amounts of antigen, which suggests that it is the antigen-presenting function of B cells and not unique B cell–derived signals that promotes the development of T_{FH} cells[15].

However, all good things must come to an end, and for GC B cells, this results in emigration from the follicle and differentiation into long-lived plasma cells and memory B cells. This seems to be a continuous process, as both cell types appear in the blood early after the initiation of the GC, with high-affinity variants dominating later in the response[16]. Once it is formed, the memory B cell pool has a relatively stable size, although it is unclear how such homeostatic regulation is achieved. The crosstalk between the products of the GC response also continues at this final stage, as antigen presentation by plasma cells has been proposed to negatively regulate T_{FH} cell function[17].

Although the basic anatomy of the adaptive immune response is well defined, the nature and consequences of the many signals that mediate the interactions between B cells and T cells in the GC are only now emerging. The intimate pairing of the GC B cells and T_{FH} cells is also apparent at the molecular level, at which both cell types require the transcription factors Bcl-6 and IRF4 and the absence of the plasma cell–differentiation factor Blimp-1, as well as the ability to

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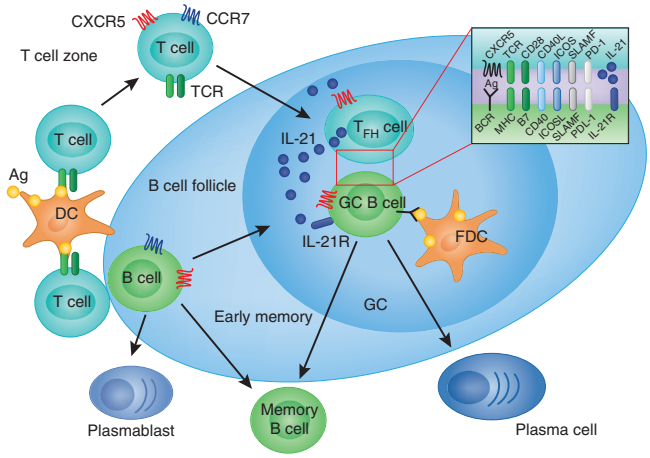

Figure 1 Multiple signals control the GC output. Antigen-specific T cells, primed on dendritic cells (DC) in the T cell zone, upregulate ICOS, PD-1 and CXCR5 and migrate toward the B cell follicles. After interacting with their cognate B cells, these T cells mature into T_{FH} cells. When follicular B cells encounter antigen (Ag), they move to the border of the T cell zone and can further differentiate into extrafollicular plasmablasts, give rise to early memory B cells or return to the follicle and undergo rapid proliferation to form a GC. In the GC, T_{FH} cells interact with GC B cells through an array of molecular pairings, including those between T cell antigen receptor and major histocompatibility complex class II (TCR-MHC); CD28–B7 family members; CD40 and its ligand CD40L; ICOS and its ligand ICOSL; signaling lymphocytic-activation molecule family members (SLAMF) on both cell types; PD-1 and its ligand PDL-1; and IL-21 and its receptor IL-21R. These interactions culminate in the T cell–secreting cytokines, particularly IL-4 and IL-21, which are received by the B cells to influence the output of the GC in the form of affinity-matured memory B cells and long-lived plasma cells.

respond to the cytokine interleukin 21 (IL-21). We discuss how these interactions in the GC shape the immune response and the formation and maintenance of B cell memory.

Humoral immune responses: early developmental decisions

The peripheral maturation and differentiation of B cells and T cells into effector cells has traditionally been thought of as being programmed by distinct factors. Pax5 is a master regulator of many aspects of B cell life, as it regulates genes that are required for early development, antigen-receptor recombination and signaling and adhesion as well as the repression of the transcription of genes that are required for the development of other hematopoietic lineages and plasma cells[18,19]. In contrast, peripheral $CD4^+$ T cells seem to lack such a 'pan-lineage' master regulator and instead rely on subset- and context-specific factors such as Foxp3 (regulatory T cells), T-bet (T helper type 1 (T_H1) cells), GATA-3 (T helper type 2 (T_H2) cells) and RORγt (IL-17-producing helper T (T_H17) cells)[20]. Although it has been known for more than a decade that GC B cells express a unique transcriptome to facilitate clonal expansion, class-switch recombination (CSR) and SHM that is layered on top of the basic B cell program, it has only recently become apparent that key components of this process are shared between GC B cells and T_{FH} cells (**Fig. 2**).

The gene-expression changes required for an activated B cell to differentiate into a plasma cell are regulated by the coordinated activity of a small group of transcription factors. These factors can be divided into those such as Pax5, Bcl-6 and Bach2, which promote the B cell program, and others such as Blimp-1 and Xbp1, which control plasma cell differentiation[21]. A final factor, IRF4, sits uneasily in this model, as it is required for the differentiation of both activated B cells and plasma cells, potentially in a concentration-dependent manner[22–24].

Bcl-6 is a transcriptional repressor that has been extensively studied in the context of its role in diffuse large B cell lymphoma. The regulation of Bcl-6 is complex, with the mRNA being transcribed in many lymphocytes, and several post-transcriptional and translational mechanisms restrict the expression of Bcl-6 protein[25]. Bcl-6 has high expression in GC B cells and is essential for their formation[26–28]. In keeping with this broad function in GC B cells, Bcl-6 binds to the regulatory regions of thousands of genes in GC B cells, inhibiting the DNA-damage response (which is crucial for SHM) and promoting rapid B cell proliferation[29–31]. Another proposed target of Bcl-6 is the gene encoding Blimp-1 (*Prdm1*; called *Blimp1* here), whose suppression inhibits plasma cell differentiation in the GC[32] (**Fig. 2**). The expression of *Bcl6* in GC B cells requires IL-21 (refs. 33,34) and is repressed by Blimp-1 (ref. 35). IRF4 also represses *Bcl6* expression in human lymphoma cell lines, although this interaction remains to be demonstrated in primary B cells[36]. Bach2 is another transcriptional repressor that is essential for GC formation and CSR[37]. *Bach2* seems to be a direct target of Pax5 in B cells[38], and the Bach2 protein functions mainly to repress *Blimp1*, thereby allowing proliferation and CSR to occur in GC B cells before these processes are shut off in plasma cells[39]. IRF4 is a multifunctional transcriptional regulator that is required for many aspects of the differentiation of cells of the adaptive immune response, including CSR, GC formation and plasma cell differentiation[22–24]. IRF4 seems to function as a dose-dependent 'rheostat', with relatively lower concentrations promoting GC fate and CSR but higher concentrations seeming to repress *Bcl6* and activate *Blimp1* (refs. 24,36). The direct regulation of *Blimp1* by IRF4, which was initially controversial, has now been confirmed in the context of IL-21 stimulation. IL-21 induces phosphorylation of the transcription factor STAT3, which in turn binds to a regulatory region 3′ of *Blimp1* in an IRF4-dependent manner[40] (**Fig. 2**).

Although the studies outlined above neatly describe the transcriptional network that controls late B cell differentiation in the GC, many questions remain. A crucial issue is how *Bcl6* is regulated and at what point in B cell activation Bcl-6 protein is induced. This question is particularly pertinent at the onset of the GC reaction, when an activated B cell must make a differentiation decision among a Blimp-1-dependent extrafollicular plasmablast fate, entering the GC or the early adoption of a memory phenotype. Similarly, the signals that turn off *Bcl6* to allow differentiation into either a memory B cell (Blimp-1^-Pax5^+) or a plasma cell (Blimp-1^+Pax5^-) are unclear, beyond a potential involvement of IRF4 (refs. 36,40). This uncertainty is highlighted by the finding that the expression of both *Bcl6* and *Pax5* is silenced in *Blimp1*-expressing plasma cells in the absence of functional Blimp-1 protein (ref. 41 and unpublished observations). Although the interacton of this small group of transcription factors has been the most studied aspect of this gene-regulatory network, it is also noteworthy that many other factors, including Oct2, Obf1 (ref. 42), IRF8 (ref. 43) and epigenetic factors such as Ezh2 (ref. 44), are also involved in the biology of GC B cells.

Given their prominent role in GC B cells, it is surprising that no transcription factor has been proven to be required for memory cell formation independently of a more general role in B cells. Bcl-6-deficient mice produce memory cells that, despite not being derived from the GC and lacking SHM, are similar in most other aspects to wild-type memory B cells[45]. This is consistent with the findings that *Bcl6* has weak expression in memory B cells and that ectopic expression of *Bcl6* blocks memory formation in an *in vitro* model of human memory cell development[46]. Although memory-specific factors may

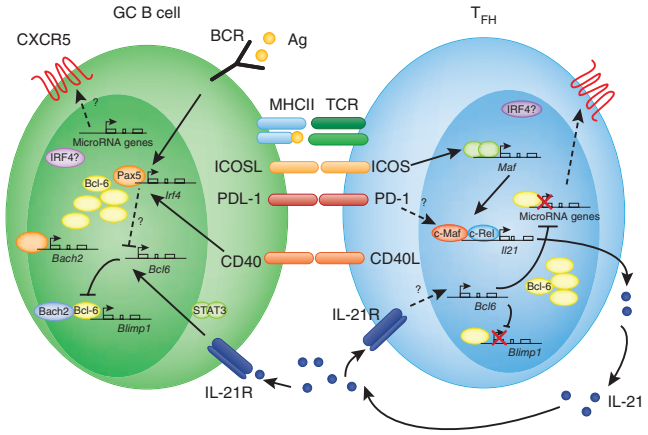

Figure 2 Molecular interactions in the GC. Some of the ligand-receptor interactions between T_{FH} cells and GC B cells are presented here. Pax5 and Bcl-6 are required for GC B cell formation. Bcl-6 represses expression of the gene encoding the plasma cell factor Blimp-1, whereas Pax5 activates the gene encoding IRF4, which is essential for GC B cells, and the gene encoding Bach2, another repressor of *Blimp1*. In GC B cells, *Bcl6* expression is sustained by IL-21, which also activates *Blimp1* in an IRF4-dependent manner. Bcl-6 contributes to GC B cell identity by regulating the expression of genes involved in cell-cycle control and DNA-damage response (not presented here). Signals from the BCR and CD40 activate *Irf4* expression, repress *Bcl6* and ultimately induce *Blimp1* to terminate the GC phase. There is evidence for an analogous gene-regulatory network in T_{FH} cells mediated by Bcl-6–IRF4–Blimp-1. A crucial function of T_{FH} cells is IL-21 production. In this pathway, ICOS signaling activates the gene encoding c-Maf (*Maf*) in a STAT3-dependent manner. Then, c-Maf and c-Rel directly regulate *Il21* transcription. As in GC B cells, Bcl-6 is central to T_{FH} cell identity, repressing the expression of genes encoding microRNAs that ultimately control the expression of CXCR5 and thus the localization of T_{FH} cells in the GC. Bcl-6 is also proposed to repress the genes that encode T-bet and RORγt (not presented here) and thus to suppress alternative $CD4^+$ T cell lineages. Arrows indicate positive interactions; bars indicate negative events; and '?' indicates a putative interaction.

yet be identified, a plausible alternative is that the distinctive nature of memory B cells is maintained through epigenetic modifications of the genome that are laid down during the GC phase.

T_{FH} cells are defined by follicular location and high expression of CXCR5, the T cell inhibitory receptor PD-1 and IL-21 (ref. 47; **Fig. 1**). However, their developmental origin and gene expression program were until recently largely unknown. Early microarray studies showed that the gene-expression profile of T_{FH} cells was distinct from that of T_H1 or T_H2 cells, which suggested that they represent a distinct lineage or maturation state[48,49]. Three studies have reported that Bcl-6 is a crucial regulator of T_{FH} cell fate[50–52]. Loss-of-function studies have shown that Bcl-6 is an intrinsic regulator of the T_{FH} cell program, being essential for CXCR5 expression and follicular homing. Bcl-6 seems to function by repressing the expression of a collection of microRNAs that target several key T_{FH} genes, such as *Cxcr5* (ref. 52). Overexpression of *Bcl6* in activated T cells is sufficient to upregulate CXCR5 and PD-1 while apparently concurrently repressing the expression of the genes that encode T-bet, RORγt and GATA-3—crucial regulators of other helper T cell lineages[50,52,53]. The essential role of Bcl-6 in T_{FH} differentiation provides an attractive parallel to GC B cells. The similarity between these cell types is further highlighted by the finding that *Blimp1* expression is specifically excluded from T_{FH} cells[51], despite its expression in most other activated T cell subsets[54,55]. Ectopic Blimp-1 blocks *Bcl6* expression and the formation of T_{FH} cells, whereas loss of Blimp-1 results in more T_{FH} cells[51]. IRF4, the other determinant of GC B cells, is also required for the development of T_{FH} cells, in which it is again probably involved in the STAT3-dependent IL-21 response[40]. How IRF4, which represses *Bcl6* and induces *Blimp1* in B cells, acts differently in T_{FH} cells remains to be determined. Nevertheless, the striking parallels between the genetic programs and the cellular interdependence of GC B cells and T_{FH} cells suggests that the two cell types have coevolved to fulfill this highly specialized function (**Fig. 2**).

IL-21, the signature cytokine product of T_{FH} cells, was initially proposed to be an essential autocrine regulator of T_{FH} lineage fate[56,57]. However, subsequent studies have shown that it acts predominantly as a paracrine factor for GC B cells, with only limited autocrine function[33,34,58,59]. The regulation of *Il21* has not been characterized in detail, although c-Maf (a T_H2-promoting factor) and c-Rel are involved in both *Il21* expression and T_{FH} cell differentiation[60,61].

The differentiation path from naive $CD4^+$ T cell to T_{FH} cell is the subject of intense interest, and two alternative models have been proposed. In one scenario, T_{FH} cells are a separate $CD4^+$ helper T cell lineage, akin to T_H1 and T_H2 cells, that use Bcl-6 as their master regulator. The second model proposes that T_{FH} cells derive from partially differentiated helper T cells that, in a manner analogous to the differentiation of extrafollicular versus follicular B cells, choose between a Blimp-1^+ effector cell path and a Bcl-6^+ T_{FH} cell path[12,47]. Regardless of their developmental origin, it is clear that the correct localization of activated $CD4^+$ T cells with a T_{FH} phenotype is an essential step in generating a protective humoral response.

T cell–B cell interactions in the GC

The signals delivered by T_{FH} cells to B cells that lead to and then occur in the GC have been intensively examined. One way of delineating the interactions between B cells and T cells is to analyze the immune responses that develop in the absence of specific molecules from either cell type or both cell types[4,62]. Such an approach has identified the ligand for the costimulatory molecule CD40 and the inducible costimulator ICOS as being absolutely required for T_{FH} cells and thus for GC development. CXCR5, the adaptor SAP, IL-21, IL-4 and various members of the signaling lymphocytic-activation molecule family are partially required in T_{FH} cells in that GCs develop in their absence but are defective in varying ways and to varying extents[4,63,64] (**Fig. 1**). The absence of IL-27 also has a detrimental effect on T_{FH} cell development in that IL-27-deficient cells do not expand their populations normally and produce less IL-21 (ref. 65). Perhaps not surprisingly, abnormalities in the immune response of mice lacking the IL-27 receptor α-chain are similar to those of mice lacking IL-21 (ref. 65).

In the context of T_{FH} differentiation, there may be additional complexity that is yet to be fully appreciated. T_{FH} cells can be subcategorized into cytokine-producing and cytokine-nonproducing fractions by their expression of GL7 (a marker of GC B cells), with both IL-21 and IL-4 being secreted by $GL7^{hi}$ cells[66]. Other studies have also noted the production of IL-4 or interferon-γ by T_{FH} cells, depending on the immunization strategy[67,68], which raises the possibility that the ability of T_{FH} cells to secrete cytokines rests with a particular subset rather than being a universal property of $CD4^+CXCR5^+PD\text{-}1^+$ cells.

Comparisons among the consequences of mutations that affect GC outcomes raise the interesting possibility that the molecules that mediate interactions between T_{FH} cells and B cells have unique functions in modulating B cell differentiation[4]. In mice immunized with an alum-based adjuvant, the kinetics of the GC are affected by loss of PD-1 (ref. 69) and IL-21 (refs. 33,34) in superficially similar ways.

However, closer examination of the output of these responses shows there are substantial differences. Memory B cells and plasma cells made in the absence of IL-21 are markedly undermutated and underselected[33,34], whereas PD-1 deficiency leads to more stringent selection in plasma cells and normal mutation in memory B cells[69]. Such differences may reflect differences in putative modes of action: IL-21 is thought to sustain Bcl-6 expression by GC B cells[33,34], whereas PD-1 signals enhance B cell survival[69]. However, loss of either IL-21 or PD-1 results in premature dissolution of the GC, so their memory phenotypes exclude the possibility that this is a single cause and suggest more subtle changes within GCs themselves. Mutations in the genes encoding the adaptor Dock8 (ref. 70) or the adhesion molecule CD84 (ref. 71) also generate remarkably similar GC dynamics, with the initially normal population expansion of GC B cells being curtailed at later time points. Although the effects on memory are unreported, mutation of the genes encoding Dock8 or CD84 results in loss of plasma cells. Dock8 deficiency does not alter the overall frequency of mutations in the variable heavy-chain region but does diminish selection by affinity[70]. CD84 deficiency affects the length of B cell–T cell conjugation in the GC[71], whereas Dock8 deficiency in B cells prevents their appropriate segregation of the adhesion molecule ICAM-1 in the membrane of the antigen-presenting cell to the periphery of the immunological synapse[70]. ICAM-1 has high expression on FDCs, and the amount of expression is crucial for GC size and affinity maturation[72]. The similarity in outcome of these two cases may reflect the ability of B cells to engage T cells in the GC, which reinforces the idea that the driving force behind affinity maturation is competition among GC B cells for T cell help.

Refining ideas on affinity maturation

The starting point for discussions about the mechanism of affinity maturation has been a model in which proliferation and SHM occur in the dark zone, whereas selection, differentiation and apoptosis occur in the light zone, which is rich in FDCs and T_{FH} cells, so interzonal migration is a prerequisite for selection[73]. Initial intravital studies of GC dynamics[74–76] have reported diverse patterns of interzonal and intrazonal B cell migration and have emphasized the role of T cells in mediating selection, as B cells have only brief contact with antigen-laden FDCs but have much longer contact with T_{FH} cells[5]. The use of an inducible label to paint B cells in only one GC zone has allowed to quantification of the migration between zones[77], leading to the conclusion that migration is predominantly from the dark zone to the light zone and is sufficient for full replacement within 6 h. Additional experiments have provided compelling evidence indicating that access to T cell help is the main force that drives differences in clonal expansion[77]. These experiments separate GC B cell affinity maturation from clonal expansion, which suggests that if the machinery that recruits T cell help is saturated, then differences in signaling by the B cell antigen receptor (BCR) provided by enhanced affinity for antigens are inconsequential. However, the role of FDCs in affinity maturation should not be completely dismissed; expression on FDCs of adhesion molecules, whose ligands are on GC B cells, influences affinity maturation[72], as do mutations that influence the ability of B cells to interact correctly with such molecules through their BCRs[70].

Formation of memory B cells

After immunization, antigen-specific B cells and T cells migrate toward the boundary that separates their respective areas in secondary lymphoid organs[78]. This B cell–T cell conjugation results in a period of B cell population expansion in the outer follicles that precedes the appearance of the GC[8,79]. The B cell population that results from this early expansion then apparently segregates into a GC fraction, another that eventually constitutes the extrafollicular foci of antibody-secreting cells and a third population that produces early memory B cells[7,11,80] (**Fig. 1**).

Such pre-GC B cell memory was presaged by the discovery of memory B cells in mice whose hematopoietic cells lacked Bcl-6 and thus GCs[45]. These mice have a phenotypically normal antigen-specific B cell memory compartment that differs only in having never been in a GC and thus having no SHM. One fascinating aspect of this work when viewed from today's perspective is that the $CD4^+$ cells, which lack Bcl-6 and are thus incapable of producing T_{FH} cells[50–52], provide sufficient help to generate a substantial isotype-switched population of antigen-specific B cells[45]. Inhibition of the development of the GC identified early memory B cells and led to the proposal of two waves of memory B cell development, one preceding and independent of the GC and the other being the traditional GC-derived variety[80]. The formation of an early memory compartment has been studied during the early stages of the B cell response with an elegant system that involves transferring transgenic B cells with simultaneous immunization[7]. This demonstrated the appearance of antigen-specific B cells that had undergone several rounds of division but were not in the cell cycle, did not express GC or plasma cell markers but did express CD38—a marker consistent with memory—and contained both isotype-switched B cells and B cells expressing immunoglobulin M (IgM), all of which lacked mutations. Earlier studies had reported the appearance of similar memory B cells at early time points in wild-type mice but could not determine with confidence that these cells were of GC origin. However, they did note that these cells made a diminishing contribution to the memory pool as the response progressed[11], which suggested either that they were recycled through the GC or that they showed diminished survival in the face of competition from GC-derived memory[3,81]. If competition between early and late memory B cells were to occur, its possible basis is an interesting issue to consider, whether it relies on differences in the expression of prosurvival proteins or represents an improved ability to compete for an undefined limiting resource. Closer examination of this question may provide insight into the mechanics of memory B cell persistence.

In considering the nature of the signals delivered to B cells that proliferate in the outer follicle, signals that presumably influence the differentiation of these B cells, the circumstances in which early memory B cell populations seem to be expanded abnormally are an interesting issue. One such circumstance is in mice with deficient IL-21 signaling, in which a substantial fraction of the memory compartment is unmutated but isotype switched[34]. In this case, the absence of IL-21 does not affect GC formation early but does result in less extrafollicular plasmablast formation[33,34]. It may be that IL-21 directs B cells in the outer follicle into the extrafollicular plasmablast pathway at the expense of the early memory pathway (but not the GC pathway, which is initially relatively normal). Another example is in mice transgenic for *Bcl2*, in which the expansion of the memory B cell compartment is due in large part to the retention of unmutated, isotype-switched B cells[82]. This suggests that access to the signals that maintain the expression of prosurvival proteins is the limiting determinant in the persistence of early memory B cells under normal circumstances.

Memory B cell survival

Studies have provided further insight into the means by which memory B cells survive. One such study has examined the sensitivity of an immune response to the compound ABT-737, which specifically

inhibits three of the five prosurvival members of the Bcl-2 family[83]. Although GC B cells and established plasma cells are resistant to ABT-737, memory B cells are not[84]. As ABT-737 inhibits Bcl-2, Bcl-x_L and Bcl-w but leaves the other Bcl-2 family members, Mcl-1 and A1, unaffected, this suggests that the reliance on prosurvival proteins changes as B cells emigrate from the GC. Although the involvement of Bcl-2 and Bcl-x_L in the survival of memory B cells is not unexpected, the apparent independence of GC B cells is. The subsequent finding that the formation and maintenance of GC B cells depends entirely on Mcl-1 supported the idea that there are different survival mechanisms during the development of memory[85]. Identification of the conditions that sustain the expression of the distinct prosurvival genes will provide further insight into the persistence of memory and GC B cells.

The contribution of isotype

Whether immunoglobulin isotype has an effect on the function of memory B cells distinct from the effector functions of the secreted antibodies has been a longstanding question in immunology. This issue has become more prominent with the clear identification of IgM memory B cells in humans[86] and mice[86–88], which has rendered obsolete the convention whereby memory is considered to be isotype switched. This is especially pertinent given the proposal that IgM memory is weighted toward reformation of the GC, whereas IgG memory favors plasma cell differentiation[87–90]. Analysis of the biochemistry of IgM and non-IgM receptors has provided some interesting, albeit contrasting, suggestions about how this functional separation might occur. The adaptor protein Grb2 binds to a conserved phosphorylated tyrosine residue in the cytoplasmic tail of the IgG or IgE BCR and thereby enhances several signaling pathways[91]. An earlier study, having found that the greater release of intracellular calcium is the only measurable consequence of signaling through the IgG cytoplasmic tail, hypothesized that the enhanced plasma cell differentiation of IgG B cells is due to the ability of IgG signaling to alter the magnitude of the gene-expression changes that flow from IgM BCR signaling[92]. Subsequent analyses have looked at the movement of individual BCR molecules in the plasma membrane before and immediately after antigen binding and have compared the outcome for BCRs that differ only in whether the cytoplasmic domain is from IgM or IgG1 (ref. 93). The IgG1 tail confers more rapid movement in the absence of antigen, together with enhanced oligomerization and cluster formation after the addition of antigen. These improvements, which are independent of affinity, suggest that there is an intrinsic advantage to IgG class-switched B cells in the immune response. Exactly how the IgG1 tail mediates this effect is uncertain, although the Grb2-binding tyrosine residue is not important in this[93]. The answer may instead lie in the mechanism by which BCR movement in the plasma membrane is regulated[94], in which the attachment of the BCR to the cytoskeleton through the immunoglobulin β-chain is crucial in regulating motility and thus signaling. It is not impossible that the immunoglobulin isotypes have different properties of movement and thus of signaling both at rest and in response to antigen. Investigation of these biophysical properties of BCRs in primary memory B cells will be an interesting area of future research.

Concluding remarks

Much is already known about the anatomy, ligand-receptor interactions, transcriptional wiring and survival mechanisms that are associated with productive humoral immune responses, but many areas of uncertainty remain. Issues such as the developmental origin and plasticity of T_{FH} cells, how memory B cells are transcriptionally programmed, and how antigen affinity influences the survival of GC and memory B cells remain to be resolved. Moreover, little is known of the rules that govern the homeostasis of memory B cells and long-lived plasma cells, a crucial determinant of the quality of an immune response. Finally, given the large number of molecules that are apparently involved in the interactions between T cells and B cells, why is it that some are crucial whereas others refine the basic response? One answer may be that each molecular pairing has a specialized function and that research using immunizations designed to generate maximal responses misses important subtleties and perhaps unique functions.

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Regulation of immune responses by extracellular vesicles

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Abstract | Extracellular vesicles, including exosomes, are small membrane vesicles derived from multivesicular bodies or from the plasma membrane. Most, if not all, cell types release extracellular vesicles, which then enter the bodily fluids. These vesicles contain a subset of proteins, lipids and nucleic acids that are derived from the parent cell. It is thought that extracellular vesicles have important roles in intercellular communication, both locally and systemically, as they transfer their contents, including proteins, lipids and RNAs, between cells. Extracellular vesicles are involved in numerous physiological processes, and vesicles from both non-immune and immune cells have important roles in immune regulation. Moreover, extracellular vesicle-based therapeutics are being developed and clinically tested for the treatment of inflammatory diseases, autoimmune disorders and cancer. Given the tremendous therapeutic potential of extracellular vesicles, this Review focuses on their role in modulating immune responses, as well as their potential therapeutic applications.

Almost all cells release different types of membrane microvesicle and nanovesicle, which have a variety of important physiological effects. Microvesicles mainly differ from nanovesicles in terms of their size and their mechanism of generation[1–4]. Microvesicles are released from the plasma membrane by shedding or budding, they are usually larger than 0.2 μm in size and have been referred to as microparticles or ectosomes. By contrast, nanovesicles, including exosomes, are between 30–100 nm in diameter, are characterized by an endocytic origin and are formed by the reverse budding of the peripheral membrane of multivesicular bodies (MVBs) or late endosomes (BOX 1). However, certain nanovesicles seem to be derived from the plasma membrane[5].

The protein content of different types of extracellular vesicle mostly reflects that of the parent cells and vesicles are enriched in certain molecules, including adhesion molecules, membrane trafficking molecules, cytoskeleton molecules, heat shock proteins, cytoplasmic enzymes, signal transduction proteins, cytokines, chemokines, proteinases and cell-specific antigens. Moreover, extracellular vesicles contain mRNAs, non-coding RNAs (ncRNAs), including microRNAs (miRNAs), and even extra-chromosomal DNA, such as amplified *MYC*[6]. Almost all cell types release extracellular vesicles that are found in the plasma and other bodily fluids, including breast milk, semen, saliva, urine and sputum. Extracellular vesicles participate in important biological functions, acting as a mode of communication between cells. This intercellular communication can be conferred by mediators that are expressed on the surface of the extracellular vesicles or that are contained within the extracellular vesicle lumen.

Extracellular vesicles that are produced by both immune and non-immune cells have important roles in the regulation of immunity. They can mediate immune stimulation or suppression and they can drive inflammatory, autoimmune and infectious disease pathology. Thus, extracellular vesicles have the potential to be used as therapeutic agents to modulate the immune system. In this Review, we focus on the biology of the 30–100 nm extracellular vesicles known as exosomes and the mechanisms through which they regulate the immune response. Moreover, we discuss the potential application of these extracellular vesicles in the treatment of inflammatory and autoimmune diseases as well as cancer.

Biogenesis and trafficking of exosomes

Exosomes are generated by the inward budding of an endosomal membrane. The resulting invaginations are pinched off and released as intraluminal vesicles (ILVs) inside the endosomes, which are then termed MVBs[1,3] (FIG. 1). MVBs can then follow either the secretory pathway or the lysosomal pathway. In the secretory pathway,

microRNAs
(miRNAs). Small non-coding RNAs (~22 nucleotides in length) that post-transcriptionally regulate gene expression by binding to seed sequences on target mRNAs, which results in mRNA degradation or inhibition of mRNA translation.

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Box 1 | **General features of exosomes**

- Exosomes are extracellular vesicles (<100 nm in size) that are derived from the endocytic compartment of the cell. They are released by most, if not all, nucleated cells, reticulocytes and platelets, and are present in most bodily fluids.
- They are generated by the reverse budding of the limiting membrane of late endosomes, which then become multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs).
- The formation of ILVs through the endosomal sorting complex for transport (ESCRT) machinery requires ESCRT0, ESCRTI, ESCRTII and ESCRTIII, each of which is composed of different subunits and accessory molecules. The lipid phosphatidylinositol 3-phosphate on the endosomal membrane recruits the ESCRT0 complex that binds ubiquitylated proteins. ESCRT0 recruits the ESCRTI components, which, in turn, incorporate ESCRTII subunits. ESCRTI and ESCRTII initiate the reverse budding of the MVB membrane. Inside the neck of the nascent ILVs, ESCRTII recruits the components of ESCRTIII, which catalyse the vesicle cleavage. Although ubiquitin and the ESCRT subunits are removed for recycling from the ILVs, some ESCRT components and accessory proteins (such as tumour susceptibility gene 101 protein (TSG101), hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and ALG2-interacting protein X (ALIX)) are retained within the secreted exosomes. However, not all proteins require ubiquitylation for sorting in exosomes.
- Exosomes are released into the extracellular milieu by fusion of the peripheral membrane of MVBs with the plasma membrane.
- Exosomes float at a density of 1.15–1.19 g per ml. As a result of their characteristic floating density and size, exosomes can be isolated in the laboratory by methods of continuous or discontinuous ultracentrifugation and/or ultrafiltration.
- Isolated exosomes are round-shaped vesicles. However, they acquire a typical cup-shaped morphology on electron microscopy preparations of whole vesicles because of dehydration during processing for ultrastructural analysis.
- Exosomes have a protein and lipid composition that is different from that of other types of extracellular vesicle released from the plasma membrane by shedding from living cells (that is, ectosomes and particles) or by blebbing from cells undergoing apoptosis (that is, apoptotic cell blebs).
- The protein composition of exosomes partly depends on the lineage and the state of activation, infection and/or transformation of the parent cells. Exosomes are enriched in certain proteins and lipids that, following isolation of the vesicles in the laboratory, help to differentiate exosomes from other types of extracellular vesicle, organelle or cellular debris.
- Proteins that are enriched in exosomes are likely to be involved in vesicle genesis or traffic (for example, TSG101, ALIX, annexins and RAB proteins), signal transduction (for example, kinases and G proteins), cytoskeleton organization (for example, actin and tubulin), antigen presentation or transport (for example, MHC class I and II molecules and heat shock proteins), vesicle targeting to acceptor cells or to the extracellular matrix (for example, integrins and MFGE8), protein organization in membrane microdomains (for example, the tetraspanin family proteins, including CD9, CD63 and CD81), and protection from lysis by complement (for example, CD55 and CD59). Exosomes also contain enzymes, factors required for protein synthesis and RNAs.
- As a result of their immunoregulatory properties and their ability to carry cell-derived antigens, exosomes have been used for positive or negative vaccination in experimental models. Exosomes and other extracellular vesicles could also be useful as biomarkers for the detection or staging of cancer, and probably for inflammatory, immunological or metabolic disorders.

Tetraspanin
An evolutionarily conserved four transmembrane domain protein that associates with other tetraspanins, integrins, MHC molecules or signalling receptors in tetraspanin-enriched domains, and this leads to the regulation of multiple cell functions including cell adhesion, morphogenesis, proliferation, differentiation, synapse formation and tumour invasion.

MVBs fuse with the plasma membrane, which results in the release of ILVs as exosomes and the incorporation of the peripheral membrane of the MVBs into the plasma membrane. In the lysosomal pathway, MVBs fuse with lysosomes, releasing ILVs into the lysosomal lumen for degradation.

The generation of exosomes requires the sorting of the exosome-targeted proteins and lipids into the endosomal membrane, the delivery of the exosome cargo into nascent ILVs and the excision of ILVs. The monoubiquitylation of cytosolic domains of transmembrane proteins that have been internalized from the cell surface or transported from the *trans*-Golgi network functions as a sorting signal that directs proteins to ILVs (FIG. 1). The ubiquitylated proteins are captured by the endosomal sorting complex for transport (ESCRT) machinery[7,8]. However, MVBs can still be generated in the absence of key subunits of ESCRTs, which indicates the existence of alternative mechanisms of exosome biogenesis[9]. In addition, the sorting of certain proteins, such as MHC class II molecules, into ILVs is independent of ubiquitylation. Indeed, in oligodendrocytes, the sorting of proteolipid proteins into ILVs is ESCRT independent[10] (FIG. 1); instead, it requires segregation in sphingolipid-containing lipid rafts in the MVB membrane. Sphingomyelinases then break down the sphingolipids to release ceramide, which is thought to have a role in ILV budding[10]. Indeed, exosomes are enriched in sphingolipids, ceramide and the lipid raft component cholesterol[10].

Stoorvogel and colleagues[11,12] have suggested the existence of two pools of MVBs: one that is high in tetraspanin-enriched microdomains and associated lipids (cholesterol, sphingomyelin and ganglioside GM3), that is detergent-resistant, that is low in lysobisphosphatidic acid and that follows the exosome secretory pathway; and another that is low in cholesterol, high in lysobisphosphatidic acid and that is targeted to the lysosomal pathway. Indeed, these authors showed that in immature dendritic cells (DCs) MHC molecules are sorted into ILVs in MVBs and are targeted for lysosomal degradation, whereas in mature DCs MHC molecules are sorted into ILVs in MVBs that are enriched in the tetraspanin CD9 and are targeted for secretion[11,12].

The mechanisms by which MVBs traffic to the cell periphery, fuse with the cell membrane and release their ILV cargo, require the coordinated action of the cytoskeleton, the molecular motors and the vesicle fusion machinery (FIG. 1). Similarly to most intracellular transport pathways, MVB trafficking is controlled by the RAB family of small GTPases. Knock down of expression of RAB2B, RAB9A, RAB5A, RAB27A or RAB27B inhibits exosome secretion in tumour cells[13,14]. RAB27A functions in the docking and the fusion of MVBs to the cell membrane, whereas RAB27B participates in the transfer of vesicles from the Golgi to MVBs and in the mobilization of MVBs to the actin-rich cortex under the plasma membrane[13]. In the K562 erythroleukaemic cell line, RAB11 promotes the fusion of MVBs to the surface membrane in response to increased cytosolic calcium[15]. Although the mechanism by which this occurs is poorly characterized, the fusion of MVBs to the plasma membrane seems to depend on SNAP receptors (SNAREs), which are a superfamily of proteins that regulate the fusion and the target specificity in intracellular vesicle trafficking[16].

Once secreted, extracellular vesicles bind to neighbouring cells or to the extracellular matrix, or passively traffic through the bloodstream or through other bodily fluids. Certain blood-borne extracellular vesicles are rapidly captured by marginal zone phagocytes in the spleen,

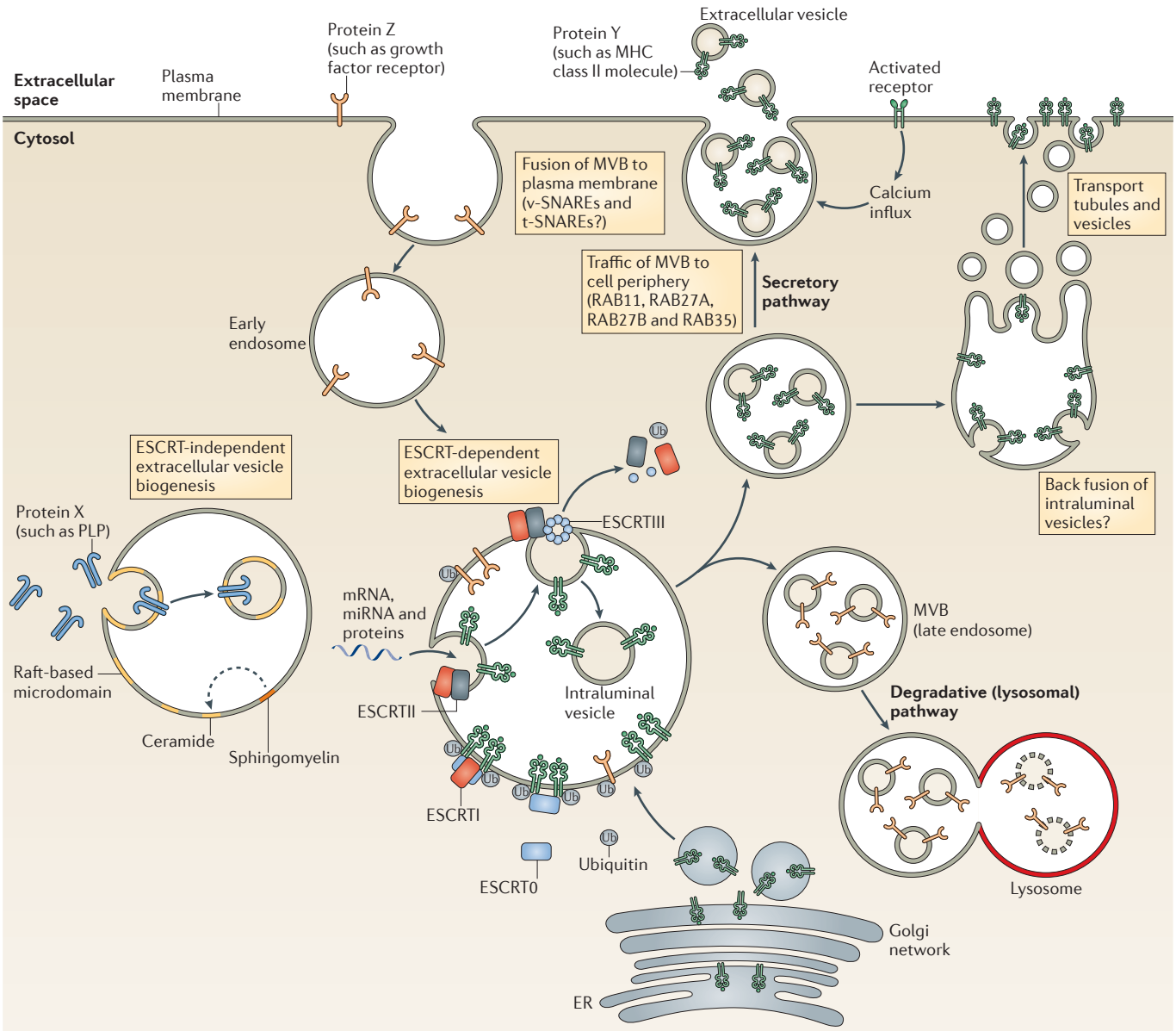

Figure 1 | **Biogenesis of extracellular vesicles.** Extracellular vesicles are generated as intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) by mechanisms that are endosomal sorting complex for transport (ESCRT) machinery dependent or independent. Proteins that are transported from the Golgi (for example, MHC class II molecules) or that are internalized from the cell surface (for example, activated growth factor receptors) are ubiquitylated on their cytosolic domains. However, not all proteins, such as MHC class II molecules, require ubiquitylation for targeting to vesicles. The ESCRT0 complex recognizes the ubiquitylated proteins on the cytosolic side of the endosome or MVB membrane, segregates the proteins into microdomains and binds the ESCRTI complex, which in turn recruits ESCRTII subunits. ESCRTI and ESCRTII initiate the reverse budding of the nascent ILVs within MVBs. Following this, cytosolic RNAs and proteins have direct access into the interior of the forming vesicles. Next, the ESCRTII complex recruits ESCRTIII subunits inside the neck of the nascent ILVs, which results in their cleavage into free vesicles. The free ubiquitin (Ub) molecules and ESCRT subunits are released into the cytosol for recycling. Certain proteins (for example, proteolipid protein (PLP)) are sorted into ILVs independently of the ESCRT machinery through raft-based microdomains that are rich in sphingolipids, from which ceramides are formed by sphingomyelinases. Ceramide induces coalescence of the microdomains and triggers ILV formation. The dashed arrow indicates that the role of ceramide in ILV formation is still controversial. The MVBs then follow either the secretory or the degradative pathway. In the secretory pathway, MVBs traffic to the cell periphery and fuse with the plasma membrane, constitutively releasing the ILVs (now termed extracellular vesicles), or following activation of surface receptors that trigger calcium influx. In the degradative route, MVBs release the ILVs into lysosomes. The lysosomal pathway is crucial for limiting the signalling of activated growth factor receptors. It is likely that differences in the MVBs confer the route of traffic. ER, endoplasmic reticulum; miRNA, microRNA; t-SNARE, target SNAP receptor; v-SNARE, vesicle SNARE.

by Kupffer cells in the liver and by DCs and macrophages in the lungs[17]. The rapid clearance of extracellular vesicles by phagocytic leukocytes could be a problem for their therapeutic use.

Exosome secretion is modulated by cell context. For example, DCs and B cells increase exosome release following cognate T cell interactions[11,18,19], T cells secrete extracellular vesicles on T cell receptor (TCR) activation[20] and mast cells augment extracellular vesicle release following crosslinking of the high-affinity Fc receptor for IgE (FcεRI) or following incubation with calcium ionophores[21]. In tumour cells, genotoxic stress upregulates the expression of the tumour suppressor p53, which indirectly augments extracellular vesicle secretion[22].

Extracellular vesicles in presentation to T cells

Direct antigen presentation. The finding that extracellular vesicles released by B cell lines carry MHC class II, co-stimulatory and adhesion molecules indicated that such vesicles could directly stimulate $CD4^+$ T cell clones[23]. This idea received further support following the observation that the vaccination of mice with exosomes derived from tumour peptide-pulsed DCs primes tumour-specific cytotoxic T lymphocytes (CTLs) and suppresses tumour growth in a T cell-dependent manner[24] (see below). Following these observations, numerous studies have shown the direct effects of extracellular vesicles in T cell activation.

Extracellular vesicles maintain the topology of the antigen-presenting cell (APC) of origin, exposing the extracellular domain of MHC molecules at the vesicle surface. Extracellular vesicles that are released by APCs carry surface MHC class I and class II molecules and, therefore, potentially can directly stimulate $CD8^+$ and $CD4^+$ T cells, respectively. At fairly high concentrations, APC-derived extracellular vesicles that bear peptide–MHC complexes function as antigen-presenting vesicles for T cell clones, lines and hybrids, and for primed T cells[18,19,23,25]. Similarly, extracellular vesicles that have been pulsed *in vitro* with peptides from Epstein–Barr virus (EBV), cytomegalovirus (CMV) and influenza virus have been shown to directly trigger interferon-γ (IFNγ) secretion by a small percentage of human peripheral $CD8^+$ T cells, which are probably memory T cells[26]. However, the T cell stimulatory activity of free extracellular vesicles seems to be 10–20-fold less efficient than that of the parent APCs[21,23]. This might explain the poor ability of free APC-derived extracellular vesicles to stimulate naive T cells *in vitro*[19,27] — which require higher levels of TCR crosslinking and co-stimulation than T cell clones, T cell lines, activated T cells and memory T cells. The weak T cell stimulatory ability of free extracellular vesicles *in vitro* is probably also due to their small size and the vesicle dispersion that is caused by Brownian motion. Indeed, when APC-derived extracellular vesicles are immobilized at high concentration on latex beads, or when the number of peptide–MHC complexes per vesicle is enhanced by the direct loading of the vesicles with peptides, extracellular vesicles substantially increase their T cell stimulatory capacity in culture[21,28,29].

Indirect antigen presentation through transfer of antigenic peptides to APCs. The capacity of free extracellular vesicles to stimulate T cells, including naive T cells, can be enhanced by the interaction of the vesicles with DCs[17,21,27,30–34]. Real-time microscopy showed that extracellular vesicles that are attached to cells move in a slow drifted mode, which is similar to the motion of integral membrane proteins that are connected to the underlying cytoskeleton; this suggests that extracellular vesicles bind to surface receptors on target cells[35]. Indeed, extracellular vesicles have surface ligands that can bind to target cells[36,37] or to extracellular matrix proteins[38,39].

The presence of certain adhesion molecules that are involved in exosome binding to DCs[40], such as integrins and intercellular adhesion molecule 1 (ICAM1; also known as CD54), depends on the lineage and the activation stage of the parent cells. Indeed, exosomes that are released by lipopolysaccharide-treated DCs (that is, mature exosomes) have more surface MHC class II, CD86 and ICAM1 molecules and have a greater T cell-stimulatory capacity than exosomes that are secreted by immature DCs[17,29,31,41]. The increased stimulatory function of mature DC-derived exosomes is at least partly due to their higher ICAM1 content, which could increase binding of the vesicles to APCs or could enhance T cell binding and/or activation during APC–T cell interactions. Indeed, DC-derived mature exosomes transfer the ability to activate naive T cells to non-professional APCs[31]. In addition, T cell activation induces a conformational change in the integrin lymphocyte function-associated antigen 1 (LFA1; also known as β2 integrin and CD11a), which augments its affinity for ICAM1 on DC-derived exosomes[18].

Other ligands, including milk fat globule E8 (MFGE8; also known as lactadherin), tetraspanins and externalized phosphatidylserine, are constitutively present on extracellular vesicles. These ligands and adhesion molecules participate either directly or indirectly in the binding of extracellular vesicles to DCs[36,37,42]. For example, MFGE8, which is a secreted glycoprotein, binds to externalized phosphatidylserine on extracellular vesicles[1,43] and can function as a bridge between the vesicles and the target cells by binding to αVβ3 or αVβ5 integrins on DCs and macrophages. However, extracellular vesicles that arise from MFGE8-deficient DCs can still transfer peptide–MHC complexes to DCs, which suggests that there is redundancy in the mechanisms by which extracellular vesicles bind to DCs[44]. Phosphatidylserine is also recognized by T cell immunoglobulin mucin receptor 1 (TIM1; also known as HAVCR1) and TIM4 on target leukocytes[45,46]. Carbohydrates on the vesicle surface also participate in the recognition and the uptake of the vesicles by phagocytes. The lectin galectin 5 impairs the uptake of reticulocyte-derived exosomes by macrophages by binding to β-galactosides on the vesicles and thereby inhibiting their recognition by macrophage lectin receptors[47].

A proportion of the acquired extracellular vesicles remains on the surface of the target cell, whereas the rest are internalized by phagocytosis or macropinocytosis[17,36,48,49]. Immature DCs internalize extracellular

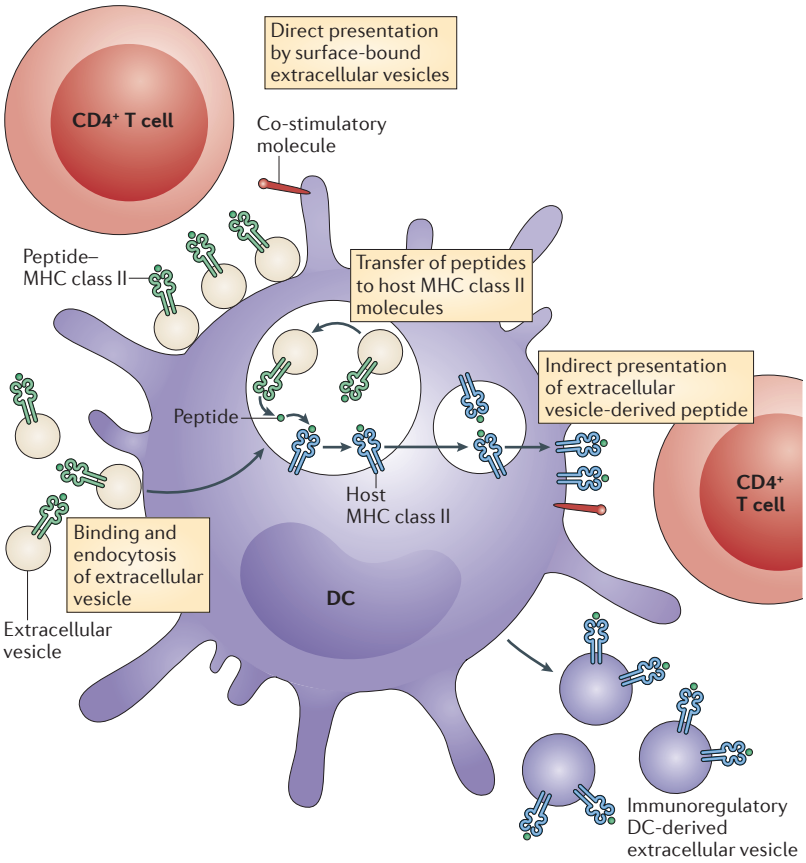

Figure 2 | **Role of extracellular vesicles in antigen presentation.** Professional antigen-presenting cells (APCs) (that is, dendritic cells (DCs)) present peptide–MHC complexes that are derived from captured exosomes. Extracellular vesicles that are retained on the APC surface present their peptide–MHC complexes directly to T cells, although the co-stimulatory molecules are provided by the APC. Alternatively, internalized extracellular vesicles transfer their antigenic peptides to the MHC molecules of the host APCs. The host MHC molecules that are loaded with the exosome-derived peptide are then transported to the APC surface for presentation to T cells. The APCs also release extracellular vesicles that can regulate antigen-specific immune responses. Although only MHC class II complexes are shown, a similar process occurs for exosomal MHC class I molecules in the regulation of $CD8^+$ T cells.

vesicles more efficiently than mature DCs, whereas mature DCs retain more extracellular vesicles on the cell surface[17]. Once exosomes are internalized by DCs, the exosome-borne peptide–MHC complexes can be degraded by the APCs and can be used as a source of peptides to indirectly interact with T cells[17,32] (FIG. 2). For example, exosomes that are released by human intestinal epithelial cells and that bear the MHC class II molecule HLA-DR4 loaded with the human serum albumin peptide only activate specific T cell hybrids in the presence of $HLA\text{-}DR4^+$ DCs, which suggests that the extracellular vesicles transfer the peptide from their HLA-DR4 molecules to HLA-DR4 molecules on the DCs[32]. Similar results have been reported *in vivo*: extracellular vesicles that carry the mouse MHC class II molecule IA^b loaded with an allopeptide (from MHC class II IE α-chain) triggered the proliferation of specific $CD4^+$ T cells in wild-type mice, but not in MHC class-II-deficient hosts[17].

Cross-dressing
The passage of intact preformed peptide–MHC class I or MHC class II complexes from a donor cell to the surface membrane of an acceptor antigen-presenting cell or another type of target cell. The transferred peptide–MHC complexes are recognized by T cells without the need of further antigen processing.

Indirect antigen presentation by cross-dressing APCs. Peptide–MHC complexes of extracellular vesicles that are attached (or fused) to APC surfaces can also be directly presented to T cells without the need for peptide–MHC reprocessing — through a mechanism known as cross-dressing (FIG. 2). The finding that optimal T cell stimulation occurs when exosomes transfer peptide–MHC complexes to mature DCs indicates that the acceptor APCs provide the required co-stimulatory molecules that are absent in the extracellular vesicles[17,27,40]. Mouse DCs deficient for the MHC class II molecule IA^b that have been incubated with exosomes bearing peptide–IA^b complexes stimulate the proliferation of cognate $CD4^+$ T cells, which shows that the peptide–MHC complexes are derived from the extracellular vesicles and not the DCs[27]. However, the maximal T cell stimulation that was triggered by extracellular vesicles in the presence of MHC class II-deficient DCs was less than that elicited in the presence of wild-type DCs, which suggests that part of the T cell response is triggered by the indirect presentation of the exosome-carried peptide by DC-derived IA^b molecules[27]. Finally, there are several lines of evidence suggesting that, following allograft transplantation, the cross-dressing of the recipient's APCs with donor MHC molecules could be mediated through exosome transfer[50]. By contrast, exosomes do not seem to participate in the cross-dressing of APCs during the priming of T cells after viral infections[34,51].

Extracellular vesicles as carriers of antigens

Tumour antigens. Extracellular vesicles also transfer native antigens to APCs. Tumour-derived extracellular vesicles containing native tumour antigens can be efficiently taken up by DCs for antigen processing and cross-presentation to tumour-specific CTLs[52,53] (FIG. 3). Moreover, vaccination of mice with tumour-derived exosomes was shown to induce a potent $CD8^+$ T cell-mediated antitumour effect not only on the autologous tumour but also against other related tumours that express the same tumour rejection antigen or antigens[52]. Importantly, vaccination of mice with syngeneic or allogeneic tumour-derived extracellular vesicles is equally effective, as cross-protection against tumours is mediated by the processing of exosome-derived tumour antigens by host DCs, which is independent of the MHC molecules of the donor extracellular vesicles[52]. Indeed, human tumour exosomes bearing the MART1 tumour antigen and a mismatched HLA haplotype promoted the activation of an HLA-A2-restricted CTL clone that was specific for MART1 when extracellular vesicles were taken up by $HLA\text{-}A2^+$ DCs[53]. The possibility that tumour extracellular vesicles efficiently deliver unknown tumour antigens to DCs for CTL cross-priming makes exosome-based vaccines an attractive prophylactic or therapeutic approach against cancer.

Pathogenic antigens. Certain microbial components can also be present in extracellular vesicles (FIG. 3). Macrophages infected with *Mycobacterium bovis* bacillus Calmette–Guérin release extracellular vesicles that contain mycobacterial antigens that, in the presence of

Cross-presentation
The transfer of biosynthesized antigen in a complex form (for example, as apoptotic cell fragments or associated with heat shock protein) from donor (infected or tumour) cells to the cytosol of acceptor antigen-presenting cells for processing and presentation through MHC class I molecules to CD8⁺ T cells. In certain cases, the acquired antigen can be presented by MHC class I molecules through a transporter associated with antigen processing (TAP)-independent mechanism that involves delivery of the antigen to MHC class I-containing phagolysosomes.

DCs, promote T cell immunity in mice[33]. Furthermore, vaccination with extracellular vesicles that have been released by *Toxoplasma gondii*-pulsed DCs confers protection against parasite infection in mice, which suggests that the extracellular vesicles transport toxoplasma antigens[54]. Certain viral antigens are also targeted into the exosome pathway. T cells segregate the HIV Gag protein into plasma membrane-derived extracellular vesicles[5], and CMV-infected endothelial cells release extracellular vesicles that contain CMV glycoprotein B, which stimulate memory CD4⁺ T cells in the presence of APCs[55]. EBV-infected cells release extracellular vesicles that carry

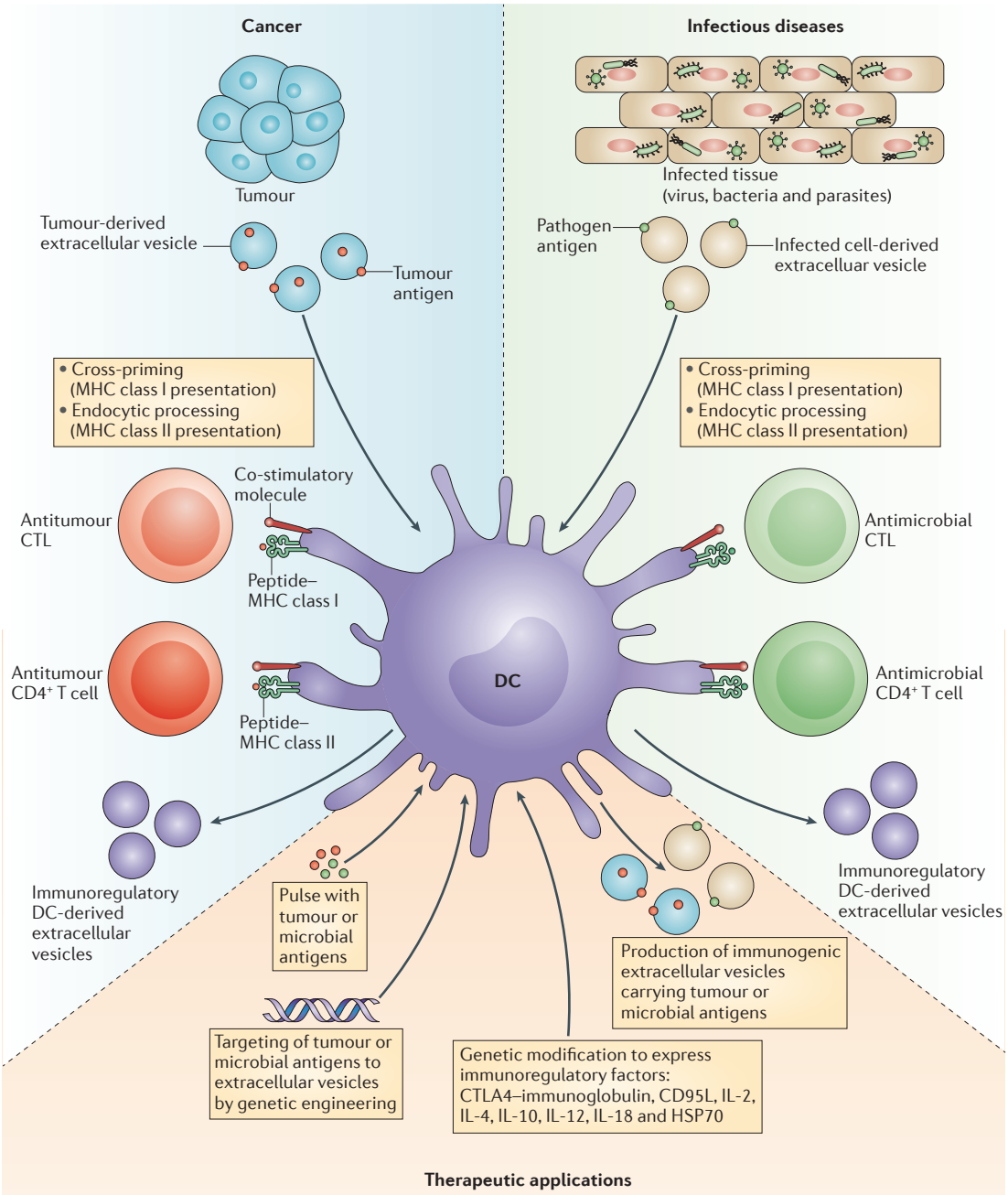

Figure 3 | **Role of extracellular vesicles in regulating tumour and microorganism immunity that can be modified for therapeutic applications.** Professional antigen-presenting cells (APCs) (that is, dendritic cells (DCs)) process tumour-derived or microorganism-derived antigens for presentation to cytotoxic T lymphocytes (CTLs) or CD4⁺ T cells. A similar approach can be used *in vitro* to generate immunogenic extracellular vesicles for therapeutic applications, by pulsing the APCs with tumour-derived or pathogen-derived antigens, or by genetically engineering the APCs to target the desired antigens to the exosome membrane. Similarly, the APC can be modified to express immunosuppressive or immunostimulatory cytokines or ligands, rendering the extracellular vesicles that are released from the APCs able to suppress or to stimulate antigen-specific immune responses. CTLA4, cytotoxic T lymphocyte antigen 4; IL, interleukin; HSP70, heat shock protein 70.

the oncogenic viral antigen latent membrane protein 1 (LMP1)[56]. Influenza virus haemagglutinin, because of its localization in raft domains, is sorted into the exosome membrane with its extracellular domain facing outwards[57]. This incorporation of haemagglutinin increases the ability of the extracellular vesicles to bind to acceptor cells through terminal sialic acid residues[57].

As exosomes originate in MVBs that are positioned along the endocytic route, they can transport antigens that are internalized by the parent cells, which could be important for their therapeutic applications[58] (FIG. 3; see Supplementary information S1 (table)). Alternatively, by genetically engineering the parent cells, antigens that are not naturally present on extracellular vesicles could be delivered into the vesicles[59–61]. Given their high immunogenic potential, antigen-associated extracellular vesicles that are released by antigen-pulsed or genetically-engineered parent cells constitute a promising cell-free system for vaccination, particularly for peptides that are present at low frequency or that are weakly immunogenic[59].

BCR antigens. B cells recognize conformational epitopes on native antigens, which are presented as immune complexes on follicular DCs (FDCs) within B cell follicles[62]. The immune complexes activate the complement system, leading to the generation of iC3b, C3dg and C3d fragments, which bind to complement receptor type 1 (CR1; also known as CD35) and CR2 (also known as CD21) on FDCs. Multiple copies of native antigens arranged at regular intervals, together with iC3b, C3dg and C3d, are presented to B cells on FDC-derived iccosomes, which promote B cell receptor (BCR) crosslinking and CR2-mediated B cell activation, respectively. Increasing evidence suggests that extracellular vesicles that are attached to FDCs could use similar mechanisms to promote B cell immunity.

FDCs do not synthesize MHC class II molecules, but they can acquire peptide–MHC class II complexes by capturing MHC class II$^+$ extracellular vesicles that are probably released by follicular B cells[63]. Since FDCs are non-phagocytic, the captured extracellular vesicles might remain on the cell surface for a long time. Interestingly, FDCs synthesize MFGE8 and express ICAM1, CR1 and CR2, which could participate in the docking or the binding of extracellular vesicles through ligation to phosphatidylserine, CD11a and/or C3-derived fragments[62,64]. Indeed, B cells release extracellular vesicles bearing C3-derived fragments as a mechanism of elimination of C3 fragments that are deposited on the cell membrane in physiological conditions[65]. Exosomes are protected from complement lysis because they have the complement regulatory proteins CD55 and CD59 on their surface, which inhibit C3b deposition and the assembly of the membrane attack complex, respectively[66].

After the immunization of mice with exosomes from DCs that were loaded with ovalbumin (OVA), the generation of T cell immunity was shown to require antigen-specific B cell assistance[29]. As B cells recognize conformational epitopes, this finding suggests that, to be effective immunogens, extracellular vesicles must carry B cell epitopes as native or partially-degraded antigens[29]. Resting B cells release extracellular vesicles after stimulation with IgM-specific antibodies and CD40 signalling[19], which are conditions that mimic the activation of B cells by FDCs *in vivo*[62]. Extracellular vesicles released by B cells undergoing activation express MHC molecules that are loaded with peptides derived from the internalized native antigens. Therefore, it is likely that at the FDC surface extracellular vesicles bearing copies of native antigens stimulate antigen-specific B cells and extracellular vesicles carrying peptide–MHC class II complexes (which are released by follicular B cells undergoing activation) attract and/or stimulate follicular helper T cells that are specific for that particular peptide–MHC complex. This scenario would lead to an antigen-specific B cell–T cell interaction and to the development of B cell immunity. B cells also augment the release of exosomes following cognate interactions with CD4$^+$ T cells[19,67]. At later stages of B cell differentiation, *ex vivo* experiments suggest that, following stimulation, allergen-specific B cells release exosomes that activate T helper 2 cells[25].

Extracellular vesicles in the transfer of RNAs

As well as being carriers of protein antigens, extracellular vesicles constitute a mechanism for the intercellular passage of genetic information in the form of RNAs, which suggests that tissue-targeted extracellular vesicles could be potential delivery platforms for gene therapy (see below)[2,60,68]. Extracellular vesicles that are secreted by normal or cancer cells have been found to contain functional mRNAs and small ncRNAs, including miRNAs[69–76], but generally not DNA (with the exception of amplified *MYC* extra-chromosomal DNA that is found in certain tumour-derived extracellular vesicles[6]) or ribosomal RNAs[70]. The RNAs that are transported by extracellular vesicles are protected from degradation by RNases[70]. Some mRNAs and miRNAs are detected in both extracellular vesicles and parent cells, whereas others are identified in either extracellular vesicles or in parent cells, which suggests a preferential sorting of certain RNA sequences into extracellular vesicles[70,77].

The passage of RNAs from the lumen of the extracellular vesicles into the target cells requires the release of the vesicle contents into acceptor cells (FIG. 4). This involves the docking of extracellular vesicles on target cells (predominantly at cholesterol-rich microdomains) and the fusion of their membranes, as shown by incubating DCs with extracellular vesicles that have been labelled with a lipophilic probe[78,79]. The delivery of exosome contents into the cytosol of target cells has been shown by content-mixing assays, in which DCs that express transgenic luciferase in the cytoplasm were incubated with luciferin-loaded extracellular vesicles[78].

The finding that the miRNAs miR-148a and miR-451 that are delivered by DC-derived exosomes are functional in miRNA reporter assays in acceptor DCs suggests that this phenomenon is a means of horizontal propagation of post-transcriptional regulation among

Iccosomes
Membrane vesicles (0.25–0.38 μm in diameter) that are pinched off from the delicate processes of follicular dendritic cells. Iccosomes carry immune complexes with native antigens and C3-derived complement fragments on the surface, which activate B cells. During B cell activation, iccosomes are recognized and internalized by follicular B cells in an antigen-specific manner.

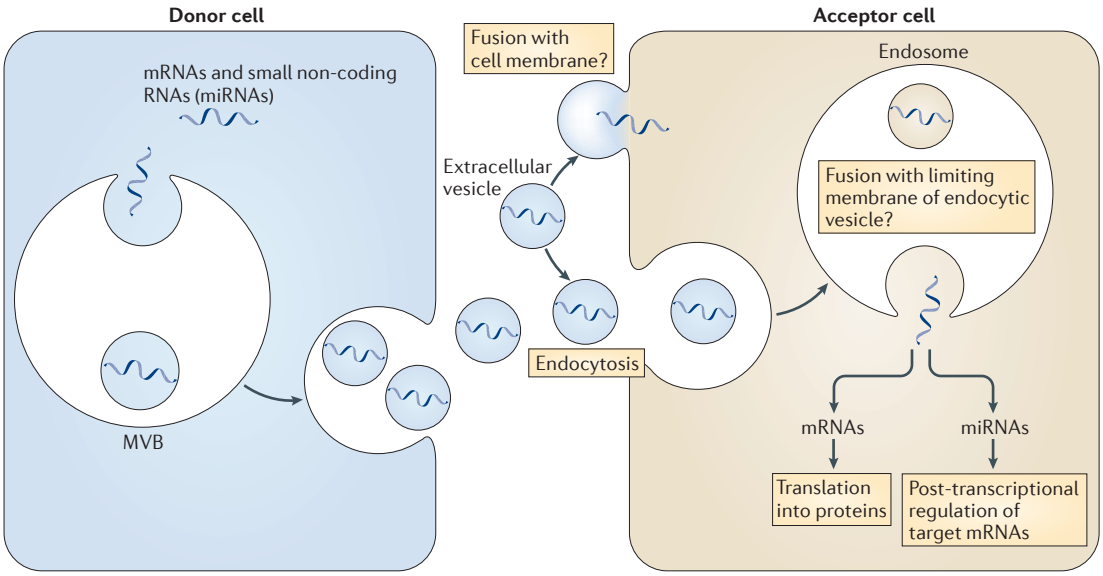

Figure 4 | **Mechanism of transfer of exosomal shuttle RNAs between cells.** mRNAs and small non-coding RNAs, including microRNAs (miRNAs), are transported inside the lumen of secreted extracellular vesicles. Once released, the extracellular vesicles are trapped by acceptor cells. Release of the vesicular RNAs into the cytosol of the acceptor cell requires fusion of the vesicle membrane with the plasma membrane or probably with the limiting membrane of endocytic vesicles, after the extracellular vesicles have been internalized. MVB, multivesicular body.

APCs[78], or between APCs and other cell types (FIG. 4). Indeed, antigen-induced assembly of the immunological synapse triggers the polarization of T cell MVBs to the point of APC–T cell contact, and promotes the transfer of exosomal miR-335, which is functional in miRNA reporter assays in acceptor APCs[77]. EBV-infected B cells transfer exosome-derived viral miRNAs to DCs, which silences mRNA transcripts that encode immune-stimulatory molecules[80].

Activation of immunity by extracellular vesicles

Increasing evidence suggests that extracellular vesicles transfer not only antigens to APCs but also signals that may promote the activation of the acceptor cells into immunogenic APCs. Mast cell-derived extracellular vesicles, which contain a relatively high content of heat shock protein 60 (HSP60) and HSPA8 (also known as HSC70), promote DC maturation in mice[58]. Macrophages that have been infected with *Mycobacterium tuberculosis*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium or *T. gondii* release extracellular vesicles that carry microbial antigens, as well as pathogen-associated molecular patterns that promote a Toll-like receptor-dependent inflammatory response by macrophages[81]. This adjuvant-intrinsic effect, together with the vesicular nature of extracellular vesicles, may explain why extracellular vesicles are more efficient than soluble peptides at transferring antigens between APCs.

Interleukin-1β (IL-1β), which is a cytokine that lacks the leader sequence needed for secretion by the classical pathway, is released inside the extracellular vesicles secreted by DCs and macrophages[82–84]. How IL-1β is then released from the vesicle lumen remains unknown. Unlike IL-1β, other cytokines are transported on the extracellular vesicle surface. Tumour necrosis factor (TNF) superfamily members, including CD95 ligand (CD95L; also known as FASL), TNF-related apoptosis-inducing ligand (TRAIL; also known as TNFSF10) and CD40 ligand (CD40L; also known as CD154), are sorted into the exosome membrane. CTLs, natural killer (NK) cells and DCs kill target cells through the polarized release of CD95L-carrying extracellular vesicles[85–88]. Tumour cells and parenchymal cells of immune-privileged tissues also secrete CD95L via extracellular vesicles as a mechanism of immune escape[89,90]. In addition, mast cells release CD40L-bearing extracellular vesicles[91] and platelets secrete vesicles that deliver CD40L–CD40 signalling[92]. The release of TNF superfamily molecules through extracellular vesicles decreases the degradation of these molecules by surface proteases, augments their local concentration in the extracellular milieu and favours their aggregation into trimers, thereby increasing their biological activity[87]. DC-derived and macrophage-derived exosomes carry enzymes that synthesize leukotriene B4 (LTB4) and LTC4 (REF. 93). At sites of inflammation, unstable LTA4 that is released by neutrophils could be converted into pro-inflammatory LTB4 and LTC4 by APC-derived extracellular vesicles.

In addition, extracellular vesicles seem to have a role in mediating inflammatory and autoimmune diseases; for example, extracellular vesicles in the serum and that are derived from the synovial fibroblasts of patients with rheumatoid arthritis have higher levels of a membrane-bound form of TNF — which is a key target in the treatment of rheumatoid arthritis — than extracellular vesicles from healthy control individuals[94]. Interestingly, these TNF-positive extracellular vesicles

render activated T cells resistant to apoptosis, which could contribute to the T cell-mediated pathogenesis of rheumatoid arthritis. In addition, citrullinated proteins and the nuclear protein DEK, which are known to be autoantigens in rheumatoid arthritis, were detected in extracellular vesicles that were purified from the synovial fluid of patients with rheumatoid arthritis and juvenile arthritis, respectively[95]. In addition, extracellular vesicles that are found in the articular cartilage of patients with osteoarthritis mediate mineral formation and the destruction of articular cartilage[96].

Immunosuppression by extracellular vesicles

Tolerosomes. Besides their obvious roles in promoting immune responses, endogenous extracellular vesicles have also been shown to have immunosuppressive effects following antigen immunization through certain routes and in tumour models. Oral administration of OVA resulted in the generation of MHC class II$^+$ vesicles in the serum that could suppress OVA-specific immune responses and were therefore termed tolerosomes[97]. These MHC class II$^+$ circulating vesicles seem to be derived from intestinal epithelial cells that acquire antigens after oral administration[97]. Similarly, following intradermal immunization with OVA, extracellular vesicles that suppress an OVA-specific delayed-type hypersensitivity response (DTH response) in the footpad could be isolated from the plasma[98]. The OVA-specific, circulating suppressive extracellular vesicles were MHC class II$^+$ and required both MHC class I and CD95L for their suppressive effects. Interestingly, the number and/or the activity of the OVA-specific immunosuppressive extracellular vesicles in the blood peaked 14 days post immunization. Thus, it has been hypothesized that the functional relevance of blood-borne extracellular vesicles is to reduce an active immune response to an antigen that has been encountered through certain immunization routes[98] and that they may suppress responses to peripheral self antigens and to commonly encountered foreign antigens to inhibit chronic inflammation and autoimmunity. Exosomes isolated from the bronchoalveolar fluid of mice that were previously tolerized to the pollen allergen Ole e 1 by repetitive intranasal inoculation prevented allergic reactions against Ole e 1 in a mouse model, which suggests that bodily fluids other than the blood also contain antigen-specific immunosuppressive extracellular vesicles[99].

Tumour-derived extracellular vesicles. Tumour-derived extracellular vesicles have been reported both to stimulate and to suppress tumour-specific and nonspecific immune responses. Given that the protein composition of extracellular vesicles is similar to the parental cell type, tumour-derived extracellular vesicles contain tumour-specific antigens. In fact, certain tumour-derived extracellular vesicles are enriched for tumour antigens such as melan A, carcinoembryonic antigen (CEA) and mesothelin compared with the parental tumour cells[52,53,100]. Therefore, tumour-derived extracellular vesicles have been used as a source of tumour antigens to stimulate an antitumour response (see below). However, despite this, there is substantial evidence that suggests that tumour-derived extracellular vesicles primarily suppress antigen-specific and nonspecific antitumour responses; for example, tumour-derived extracellular vesicles are enriched for CD95L, TRAIL or galectin 9, which can promote T cell apoptosis[89,101,102]. Moreover, tumour-derived extracellular vesicles suppress CD3 ζ-chain expression by T cells and block NK group 2 member D (NKG2D)-dependent cytotoxicity of NK cells and CD8$^+$ T cells[103].

In addition, tumour-derived extracellular vesicles have numerous effects on APC function; for example, monocyte differentiation can be altered to favour the generation of myeloid-derived suppressor cells (MDSCs), through the presence of prostaglandin E2 (PGE2), transforming growth factor-β (TGFβ), HSP72 and miRNAs in tumour-derived vesicles[104–107]. MDSCs in turn induce or support the function of regulatory T (T_{Reg}) cells, which inhibit antitumour responses. In addition, tumour-derived extracellular vesicles can directly affect T_{Reg} cells, both by enhancing their function and by inhibiting their apoptosis. Tumour-derived extracellular vesicles can also block the maturation of DCs and macrophages *in vivo* and *in vitro* through a TGFβ1-dependent mechanism[108].

An example of antigen-specific immune suppression by tumour-derived extracellular vesicles has been shown using OVA as a model tumour antigen. Extracellular vesicles that were derived from an OVA-expressing melanoma and that contained the full-length OVA protein were shown to effectively suppress an OVA-specific immune response[109]. In addition, in tumour-bearing mice, blood-borne extracellular vesicles that were positive for CD11b were shown to suppress tumour antigen-specific responses through an MHC class II-dependent mechanism[110]. These observations suggest that tumour-derived extracellular vesicles initially modulate the function of CD11b$^+$ APCs in the tumour microenvironment, which in turn release immunosuppressive MHC class II$^+$CD11b$^+$ vesicles into the circulation. How these endogenous vesicles suppress tumour antigen-specific responses is unclear, but they probably have an important role in tumour immune escape.

Although tumour-derived extracellular vesicles can suppress tumour-specific immune responses, vesicles also have a role in facilitating tumour invasion and metastasis. Tumour-derived extracellular vesicles have been shown to help to establish the pre-metastatic niche through the generation of a suitable microenvironment in distant metastatic sites[14,111–114]. In addition, melanoma-derived extracellular vesicles that have been injected in peripheral tissues preferentially home to sentinel lymph nodes to prepare lymph nodes to become remote niches for metastatic tumour growth[14]. Taken together, it seems that tumour-derived extracellular vesicles have important roles at multiple stages of tumour pathogenesis, from suppressing the antitumour responses to facilitating the formation of the metastatic niche.

Delayed-type hypersensitivity response
(DTH response). A CD4$^+$ T cell-driven, antigen-specific immune response that is induced by intradermal antigen immunization followed by injection of the antigen in the skin of the ear or the footpad. The immune response is measured by swelling and redness.

Myeloid-derived suppressor cells
(MDSCs). A heterogeneous population of leukocytes of myeloid lineage that increase in number during cancer and inflammation and that suppress T cell responses. They include myeloid progenitor cells, immature macrophages, granulocytes and dendritic cells.

Immunosuppression by stem cell-derived extracellular vesicles. Adult stem cells, including mesenchymal stem cells (MSCs), from different sources confer regenerative effects in animal models of disease and tissue injury and are in Phase I and II trials for disorders including limb ischaemia, congestive heart failure and acute myocardial infarction[115]. In addition, MSCs confer immunosuppressive effects and have shown promise in clinical trials for Crohn's disease and graft-versus-host disease. However, in many of the cases where therapeutic effects have been observed using MSCs, the transplanted stem cells have not been documented to persist following injection or to contribute to tissue regeneration[116,117]. Therefore, it is likely that the main immunosuppressive effects of adult stem cells result from paracrine mechanisms mediated by secreted factors, including extracellular vesicles. Indeed, in certain cases, injection of conditioned media from MSC cultures has been confirmed to suppress inflammation as efficiently as injection of the stem cells themselves[118–123]. MSCs express several T cell-attracting chemokines, as well as immunosuppressive factors, such as indoleamine 2,3-dioxygenase (IDO), TGFβ1, PGE2 and IL-10 (REF. 118). Interestingly, analysis in animal models of inflammation suggests that MSC-derived vesicles are also immunosuppressive, probably through the transfer of RNAs and proteins carried by the extracellular vesicles[123,124]. Thus, extracellular vesicles that are released from MSCs could be easily and safely administered for the treatment of autoimmune and inflammatory diseases, and possibly for tissue regeneration. Indeed, clinical studies have been initiated to treat graft-versus-host disease with MSC-derived extracellular vesicles.

Therapeutic uses of extracellular vesicles

APC-derived extracellular vesicles. The ability of extracellular vesicles, especially those derived from APCs, to regulate the immune response can be enhanced through both pharmacological and biological treatments, including gene transfer of APCs. APCs, particularly DCs, can both positively and negatively regulate antigen-specific and nonspecific immune responses (FIG. 3; see Supplementary information S1 (table)). This differential immune regulation partly depends on the level of expression of MHC molecules and co-stimulatory molecules (such as CD80 and CD86) and co-regulatory molecules (such as programmed cell death 1 ligand 1 (PDL1) and PDL2)[125]. Immature DCs express a low ratio of co-stimulatory molecules to co-regulatory molecules on their surface and are therefore immunosuppressive. Treatment of *in vitro*-generated DCs with immunosuppressive drugs or cytokines or genetically modifying them to express immunosuppressive agents renders the DCs immunosuppressive; for example, treatment of bone marrow-derived DCs with IL-10 or IL-4 resulted in DCs that could suppress established collagen-induced arthritis (CIA) and that could prevent the onset of hyperglycaemia in non-obese diabetic mice[126–129]. Interestingly, treatment with extracellular vesicles that are derived from these *in vitro*-generated immunosuppressive DCs also reversed early onset CIA as effectively as, or more effectively than, the parental cells[129–132]. Although the mechanisms of immunosuppression have not been completely elucidated, the suppressive DC-derived extracellular vesicles were internalized *in vivo* by DCs and macrophages of the spleen, liver and draining lymph nodes, and might then modify the behaviour of these cells to confer a systemic suppressive effect. The fact that injected extracellular vesicles are rapidly cleared by phagocytic leukocytes probably increases the therapeutic efficacy of this approach. Of note, the suppressive activity of the extracellular vesicles was MHC class II dependent, as extracellular vesicles from MHC class II-deficient DCs had no suppressive activity, whereas MHC class I-deficient extracellular vesicles retained their function[131]. Interestingly, the suppressive effect was also dependent on the presence of CD95 in the host mice and CD95L in the extracellular vesicles[131]. However, no change in the level of T cell apoptosis was observed following the injection of extracellular vesicles, which suggests a different mechanism of action from that of the CD95–CD95L pathway.

Immunosuppressive exosomes have also been used in transplantation. Donor-specific allograft immunosuppression was induced by co-treatment with donor DC-derived exosomes and a drug that blocks the maturation of recipient DCs[133]. Similarly, treatment of recipient mice with exosomes released by immature donor DCs, combined with a suboptimal dose of rapamycin before and after the transplant, markedly prolonged the survival of cardiac allografts in a mouse model[134].

The therapeutic effects of APCs and particularly APC-derived extracellular vesicles can also be efficiently enhanced by gene transfer. In this situation, APCs can be modified by nonviral methods, including liposomes, and virus-mediated gene transfer, including lentiviral and adenoviral vectors. The transfer of genes encoding immunoregulatory cytokines such as IL-4, IL-10 or TGFβ1 was shown to result in the release of APC-derived extracellular vesicles that are as immunosuppressive as the parent APCs. It is possible that the genetic modification results in the generation of APCs and extracellular vesicles that are more immunosuppressive. Alternatively, it is possible that a low level of cytokines contained within the vesicles contributes to the suppressive effects. Similarly, gene transfer of *IDO* to APCs resulted in the release of immunosuppressive extracellular vesicles[132]. Although IDO expression could be detected in the APC-derived extracellular vesicles, treatment of the genetically modified APCs with an IDO inhibitor reduced the suppressive effects of the extracellular vesicles[132]. This suggests that it is the activity of IDO in the APCs that renders the extracellular vesicles immunosuppressive, but the mechanism is unclear. In addition, gene transfer of CD95L to APCs results in the generation of highly immunosuppressive vesicles. In this context, the presence of exogenously expressed CD95L on the surface of the vesicles is required for immunosuppression[131]. Similar approaches could be used to genetically modify MSCs to render the MSC-derived extracellular vesicles more immunosuppressive.

Mesenchymal stem cells (MSCs). Multipotent stromal cells that can differentiate into a variety of cell types that are derived from the mesoderm including osteoblasts, chondrocytes and adipocytes, but not into haematopoietic cells.

Naturally-occurring immunosuppressive extracellular vesicles. In addition to extracellular vesicles that are derived from immunosuppressive APCs, there are naturally occurring immunosuppressive extracellular vesicles that could be therapeutically used. Autologous extracellular vesicles that have been isolated from plasma shortly after oral administration of antigen or following antigen inoculation could be used to induce antigen-specific immunosuppression[97,98]. In addition, extracellular vesicles that have been isolated from the bronchoalveolar lavage fluid following exposure to specific antigens could be used to prevent antigen-specific allergic reactions[99,135]. Similarly, extracellular vesicles that are present in human breast milk and colostrum and that can increase the number of T_{Reg} cells could be used to suppress immune responses[136,137]. Finally, pregnancy has been shown to alleviate the severity of rheumatoid arthritis and multiple sclerosis; thus, trophoblast cell-derived extracellular vesicles present in the maternal peripheral circulation could be used to inhibit T cell signalling[138]. The isolation and enrichment of these different types of autologous or allogeneic vesicle have clinical applications for the control of autoimmunity, alloimmunity and inflammatory diseases. Indeed, blood-based therapeutics such as platelet rich plasma (PRP) are already routinely used. In an unblinded clinical study, extracellular vesicles from a blood-based therapeutic treatment similar to using PRP showed evidence of efficacy in patients with severe rheumatoid arthritis, which shows the feasibility of therapeutically using autologous extracellular vesicles.

Enhancing immunostimulation by tumour-derived extracellular vesicles. Although tumour-derived extracellular vesicles seem to be predominantly immunosuppressive, they clearly are a source of tumour antigens. APCs that have been pulsed with extracellular vesicles derived from tumour cells cross-present the antigens to antigen-specific CTLs. The ability of APCs that have been treated with tumour-derived extracellular vesicles to stimulate T cell responses has resulted in the initiation of several clinical trials and the generation of some evidence of immunostimulation[139,140]. In a clinical trial for malignant glioma, tumour exosome-loaded DCs were shown to stimulate a tumour-specific CTL response against autologous tumour cells[141]. However, generally the antitumour efficacy observed in these clinical studies has been limited, probably because of the need to render the tumour-derived extracellular vesicles or the DCs that are treated with tumour-derived extracellular vesicles more immunostimulatory. Enhancing immunostimulatory capacity could be achieved through different approaches including the treatment of the DCs with IFNγ[142] and the transfer of genes for cytokines, such as IL-12 and IL-2 (REFS 143,144), membrane-bound tumour antigens or HSPs[59,145–147]. In addition, heat shocking tumour cells resulted in the release of extracellular vesicles that contain DC- and T cell-attracting chemokines[146] — a process that could be further enhanced by genetically modifying the tumour cells. Similar approaches could also be used to elicit immune responses to viral and bacterial antigens.

Several miRNAs and other ncRNAs that are present in extracellular vesicles probably contribute to the biological effects of these vesicles[2]. The ability to alter the RNA composition of the vesicles either by transfering a specific gene or ncRNA to the parent cell or by directly delivering miRNAs or small interfering RNAs (siRNAs) to the vesicle enables vesicles to be used as carriers of therapeutic RNAs. The feasibility of this approach has been shown using tumour-derived extracellular vesicles to deliver a therapeutic siRNA, but, in theory, this approach could be applied to a variety of extracellular vesicles[68].

Concluding remarks

Most, if not all, cells release extracellular vesicles containing mRNAs, miRNAs, ncRNAs, proteins, lipids, carbohydrates and even metabolites. Thus, extracellular vesicles, which are similar in several aspects to enveloped viral particles, concentrate, transport and deliver molecules that function in important biological processes. Extracellular vesicles from both immune and non-immune cells positively and negatively modulate the immune response. In addition to the role of extracellular vesicles in the regulation of the immune response, it is likely that extracellular vesicles participate in age-related tissue degeneration, in the modulation of microbial infection, in autoimmune and inflammatory disease pathology, and in tumour initiation and progression. However, it is important to note that, although extracellular vesicles have been shown to exert effects on leukocytes *in vitro* or on innate and adaptive immune cells following the injection of purified extracellular vesicles that have been released by cells in culture, very little is known about the immunoregulatory role of these vesicles *in vivo*. There are only a few examples in which the effect of inhibiting the secretion of extracellular vesicles from a certain cell type has been examined *in vivo*[148].

Understanding how the extracellular vesicles from different immune cell types function to regulate immunity should result in the development of new therapeutic approaches; for example, inhibiting tumour-derived exosome function should result in reduced metastasis and a better antitumour immune response. Alternatively, it could be possible to deplete extracellular vesicles from the blood of cancer patients by apheresis. By contrast, modifying APC or MSC function *in vivo* or *in vitro* to facilitate the release of extracellular vesicles that are more immunosuppressive could be used to treat autoimmune disease, inflammatory disorders and graft-versus-host disease, as well as to facilitate transplantation. The feasibility and the safety of clinically using extracellular vesicles has been shown in several clinical studies using autologous, blood-derived or MSC-derived extracellular vesicles. Clearly, extracellular vesicles are important mediators and regulators of immunity for which there are and will be widespread therapeutic applications.

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The good, the bad and the ugly — T_{FH} cells in human health and disease

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Abstract | Antibody production is an important feature of the vertebrate immune system. Antibodies neutralize and clear pathogens, thereby protecting against infectious diseases. Such humoral immunity has great longevity, often persisting for the host's lifetime. Long-lived humoral immunity depends on help provided by $CD4^+$ T cells, namely T follicular helper (T_{FH}) cells, which support the differentiation of antigen-specific B cells into memory and plasma cells. T_{FH} cells are stringently regulated, as aberrant T_{FH} cell activity is involved in immunopathologies such as autoimmunity, immunodeficiencies and lymphomas. The elucidation of the mechanisms that regulate T_{FH} cell differentiation, function and fate should highlight targets for novel therapeutics.

Germinal centres
The structures that are formed by the expansion of antigen-activated B cell blasts that have migrated into the follicles of lymph nodes. The B cells in these structures proliferate and the immunoglobulin genes undergo somatic hypermutation before the cells leave as plasma cells or memory cells.

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Following infection or vaccination, the induction of protective immunity against invading pathogens depends on the generation of an appropriate type of immune response. This relies on the flexibility of naive $CD4^+$ T cells, which differentiate into diverse subsets with specialized effector functions to protect against infection by distinct pathogens[1] (TABLE 1). The importance of having distinct subsets of $CD4^+$ T cells is evident in disease states that arise from the perturbed differentiation or function of specific effector T cell populations[2] (TABLE 1).

The generation of these effector T cell subsets depends on the stimulatory cytokines that are present in the microenvironment during activation; these cytokines induce transcription factors that prime naive precursor cells for differentiation. Interleukin-12 (IL-12) induces the T-box transcription factor T-bet (also known as TBX21) in the case of T helper 1 (T_H1) cells, IL-4 induces GATA-binding protein 3 (GATA3) in the case of T_H2 cells and IL-6 or IL-23 induce retinoic acid receptor-related orphan receptor-γt (RORγt) in the case of T_H17 cells[1] (TABLE 1). However, the concept of 'master regulators' for lymphocyte differentiation is an overly simplified idea, as numerous transcription factors are required for the commitment of $CD4^+$ T cells to specific effector lineages (TABLE 1). Thus, the ultimate outcome of T_H cell differentiation depends on the coordinated functions of several important molecular regulators that operate to control gene expression and effector function.

T_H cell subsets have been identified and characterized for immunity against specific pathogenic threats (TABLE 1), but the production of neutralizing antibodies is required for the development of protective immunity to most infectious diseases. In this case, the differentiation of B cells to antibody-secreting cells is dependent on instructive signals that arise from T follicular helper (T_{FH}) cells. The importance of antibody production by antigen-specific B cells is exemplified not only by our ability to establish serological memory that provides long-lasting protection against pathogen infection but also by the success of most vaccines, which rely on antibody responses for their efficacy. The fundamental role of T cells in B cell differentiation was first reported nearly 50 years ago[3]. However, it is only in the past decade that we have gained a clear understanding of the biology of T_{FH} cells.

T_{FH} cells were first described in humans as $CD4^+$ T cells in secondary lymphoid tissues that expressed the B cell zone-homing chemokine receptor CXC-chemokine receptor 5 (CXCR5) and therefore localized to B cell follicles, including germinal centres (GCs). $CD4^+CXCR5^+$ T cells were more efficient than $CD4^+CXCR5^-$ T cells at inducing class switching and antibody secretion in B cells[4–7]. Further studies showed remarkable similarities between human and murine T_{FH} cells[8–10], which allowed elucidation of the mechanisms by which T_{FH} cells drive affinity maturation as well as antibody and autoantibody production in GCs (reviewed in REFS 11–13) (BOXES 1,2).

The most accurate definition of T_{FH} cells relates to their function as cells that migrate to follicles and interact with antigen-specific B cells to support B cell differentiation. However, using this empirical definition to isolate and study T_{FH} cells makes detailed analysis of this subset of cells challenging owing to the inherent difficulty in isolating cells from anatomically discrete regions of lymphoid tissues. For this reason, T_{FH} cells are more commonly defined

Table 1 | **Effector subsets of CD4⁺ T cells: ontogenic and functional requirements, and roles in disease**

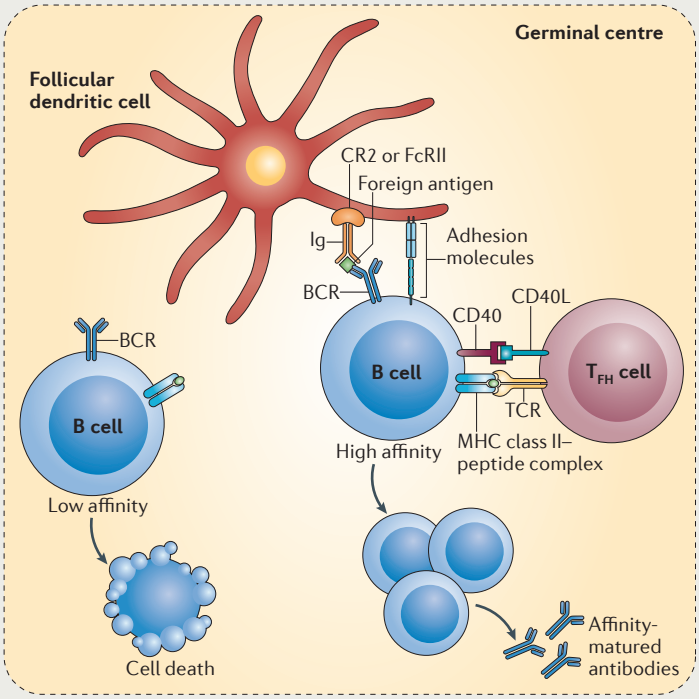
CD4⁺ T cell subset	Inducing cytokines	Activated STATs	Transcription factors	Suppressing cytokines	Canonical cytokines produced	Roles in host protection	Associated pathologies	Refs
T_H1 cells	• IL-12 • IFNγ	• STAT4 • STAT1	T-bet	IL-4 and IL-10	IFNγ	• Antiviral and antimicrobial immunity • Cell-mediated immunity	• Mendelian susceptibility to mycobacterial disease (decrease in T_H1 cells) • Multiple sclerosis (increase in T_H1 cells)	1,121, 122
T_H2 cells	• IL-4	• STAT6	GATA3 and MAF	IFNγ	IL-4, IL-5 and IL-13	• Immunity to extracellular parasites	• Allergy, asthma or eczema (increase in T_H2 cells)	1,123
T_H17 cells	• IL-23 and IL-1β • IL-6 and IL-1β • TGFβ	• STAT3	RORγt and RORα	• IL-4, IFNγ, IL-27 and IL-2 • TGFβ (suppresses IL-22 expression)	IL-17A, IL-17F, IL-21, IL-22 and IL-26*	• Protection at mucocutaneous sites • Antimicrobial immunity (for example, against *Candida* spp. and *Staphylococcus* spp.) • Inflammatory bowel disease	• Inflammatory bowel disease (increase in T_H17 cells) • Susceptibility to fungal infections (decrease in T_H17 cells)	1,121, 122,124
T_H9 cells	• TGFβ • IL-4	• STAT6	PU-1 and IRF4	IFNγ and IL-27‡	IL-9	• Protection against helminth infections	• Allergy (atopic dermatitis) and asthma (increase in T_H9 cells)	125
T_H22 cells	• TNF • IL-6	• STAT1 • STAT3 • STAT5	RORγt and AHR	High doses of TGFβ	IL-22	• Barrier immunity (skin, intestines and airways) • Enhancement of innate immunity • Tissue regeneration	• Allergy (atopic dermatitis) (increase in T_H22 cells) • Inflammation at joints and barriers (increase in T_H22 cells in mice)	126
T_{Reg} cells	• TGFβ and IL-2	• STAT5	FOXP3	IL-6	TGFβ and IL-10	• Immune suppression	• IPEX syndrome (decrease in T_{Reg} cells)	75
T_{FH} cells	• IL-6, IL-21 and/or IL-27 • IL-12	• STAT3 • STAT4 • STAT1	BCL-6, IRF4, MAF and BATF	IL-2 and IL-10	IL-21, IL-4 and IL-10	• Help for B cell activation or differentiation • Generation of long-lived antibody responses	• Humoral immunodeficiency (decrease in T_{FH} cells) • Autoimmunity (increase in T_{FH} cells) • T cell lymphoma (increase in T_{FH} cells) (see TABLE 2)	23–25, 45

AHR, aryl hydrocarbon receptor; BATF, basic leukine zipper transcriptional factor ATF-like; BCL-6, B cell lymphoma 6; FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; IFNγ, interferon-γ; IL, interleukin; IPEX, immunodysregulation, polyendocrinopathy and enteropathy X-linked; IRF4, interferon-regulatory factor 4; ROR, retinoid-related orphan receptor; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor-β; T_{FH}, T follicular helper; T_H, T helper; TNF, tumour necrosis factor; T_{Reg}, T regulatory. *Human-specific cytokine. ‡Reported in mice.

on the basis of their surface phenotype. Consequently, in both humans and mice[8–10,14–17], T_{FH} cells are considered to be CD4⁺ T cells that express the highest levels of CXCR5, together with the surface receptors inducible T cell co-stimulator (ICOS) and programmed cell death protein 1 (PD1; also known as PDCD1), the transcriptional repressor B cell lymphoma 6 (BCL-6) and the cytokine IL-21 (BOX 3), and that have downregulated the T cell zone-homing receptor CC-chemokine receptor 7 (CCR7) and IL-7 receptor-α (IL-7Rα) (TABLE 1).

This phenotypic approach to defining T_{FH} cells has facilitated their molecular and cellular characterization. However, it needs to be appreciated that, just as B cells undergo important differentiation events at the T cell–B cell border (such as the formation of extrafollicular plasmablasts) and in GCs (such as the formation of memory and plasma cells), CD4⁺ T cells can differentiate into T_{FH} cell subsets that are strategically located at these regions to facilitate distinct phases of a T cell-dependent B cell response[6,7,18,19]. These T_{FH} cell subsets probably provide early B cell help at the T cell–B cell border, and/or they represent precursor cells that differentiate into GC T_{FH} cells following receipt of appropriate signals from inside the active B cell follicle and that guide the differentiation of GC B cells into memory or plasma cells. In this Review, we discuss recent developments in the investigation of the mechanisms that underlie T_{FH} cell development and function, the discovery of specialized subsets of T_{FH} cells and how perturbations to T_{FH} cells potentially contribute to numerous human diseases.

Box 1 | **T_{FH} cells and affinity maturation of GC B cells**



Affinity maturation of the antibody response is based on the selective perpetuation (known as 'positive selection') of germinal centre (GC) B cells that have acquired increased affinity for foreign antigens through somatic hypermutation (SHM) of their immunoglobulin variable-region genes. Whereas SHM itself takes place in B cells that occupy the GC dark zone, high-affinity GC B cells only undergo positive selection after returning to the light zone where they gain preferential access to foreign antigens that are expressed on the surface of follicular dendritic cells (see the figure). However, the precise mechanisms by which preferential access to antigens translates into positive selection of high-affinity GC B cells have not been clearly established.

The specific provision of T follicular helper (T_{FH}) cell-derived helper signals to high-affinity GC B cells is proposed to be one of, if not the only, major drivers of antibody affinity maturation[12,13]. In theory, the greater propensity of high-affinity GC B cells to access foreign antigens should augment their ability to internalize, process and present foreign peptides to the T_{FH} cells that reside in the light zone of GCs (see the figure). However, although it is clear that T_{FH} cells are required to support the GC response, it has been difficult to establish their precise role in driving affinity maturation. In a recent study, a GC response was manipulated in mice such that antigen presentation by GC B cells to T_{FH} cells was decoupled from B cell receptor (BCR)-mediated antigen recognition. This resulted in the delivery of T_{FH} cell help to all GC B cells and the proliferative expansion of the population of T_{FH} cells regardless of their affinity for antigen[12]. This result does not prove that preferential delivery of T_{FH} cell help is the fundamental driver of affinity maturation but it clearly demonstrates that T_{FH} cells have the potential to perform this role if high-affinity GC B cells are indeed superior at presenting antigen to T_{FH} cells.

CD40L, CD40 ligand; CR2, complement receptor 2; FcRII, low affinity Fc receptor for immunoglobulin; Ig, immunoglobulin; TCR, T cell receptor.

Requirements for T_{FH} cell formation

T_{FH} cell differentiation requires input from several surface receptors (including CD28, ICOS, CD40 ligand (CD40L) and signalling lymphocytic activation molecule (SLAM) family members), as well as from cytokines and their associated signalling pathways (for example, signal transducer and activator of transcription 3 (STAT3) or SLAM-associated protein (SAP; also known as SH2D1A)), which all culminate in the induction of BCL-6, an important regulator of the T_{FH} cell lineage[20–22]. However, additional transcription factors (including interferon-regulatory factor 4 (IRF4), basic leucine zipper transcriptional factor ATF-like (BATF) and MAF) and microRNAs also have important regulatory functions during T_{FH} cell development[22–25] (FIG. 1; TABLE 1; Supplementary information S1 (table)). The requirements for T_{FH} cell formation have been extensively reviewed in the literature[23–26] and are summarized in Supplementary information S1 (table). In this Review, we discuss the most recent studies that have identified pathways that positively and negatively influence T_{FH} cell generation and function, and that clarify previous inconsistencies.

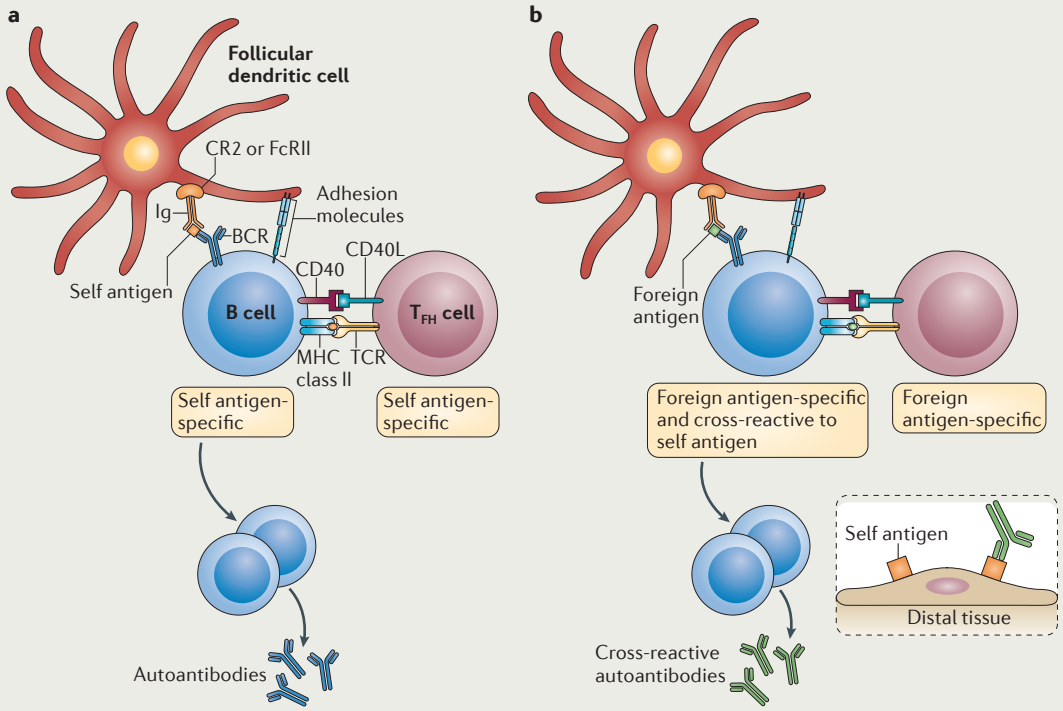
B cell lymphoma 6 (BCL-6). A transcriptional repressor identified as being crucial for the formation of T follicular helper (T_{FH}) cells. Several mechanisms have been proposed for the role of BCL-6 in T_{FH} cell commitment, including suppression of the expression of transcription factors that are required for the generation of alternative T_H fates, suppression of microRNAs and cooperation with other transcriptional regulators to induce the expression of important T_{FH} cell-related genes.

The TNF receptor superfamily. B cell-activating factor (BAFF; also known as TNFSF13B) and a proliferation-inducing ligand (APRIL; also known as TNFSF13) are ligands of the tumour necrosis factor (TNF) superfamily that regulate B cell survival and differentiation[11]. Both ligands bind to the common receptors transmembrane activator and CAML interactor (TACI; also known as TNFRSF13B) and B cell maturation antigen (BCMA; also known as TNFRSF17), and BAFF also binds to the BAFF receptor (BAFFR; also known as TNFRSF13C); receptors which are predominantly expressed on B cells[11]. NF-κB-inducing kinase (NIK; also known as MAP3K14) is a component of the BAFFR signalling pathway[11]. Expression of NIK and BAFFR by B cells was found to be required for their constitutive expression of ICOS ligand (ICOSL). The significance of this is that NIK deficiency in B cells compromises T_{FH} cell induction[27]. Thus, sustained BAFF–BAFFR–NIK signalling in B cells maintains ICOSL expression, thereby facilitating cognate ICOS–ICOSL interactions between activated $CD4^+$ T cells and B cells, which result in optimal T_{FH} cell differentiation.

Box 2 | T_{FH} cells and autoantibody production at the germinal centre

Although the generation of high-affinity antibodies directed against foreign pathogens is the '*raison d'être*' of the germinal centre (GC), the random nature of the somatic hypermutation (SHM) process can lead to the generation of antibodies that recognize self antigens with high affinity. Given the fact that many pathogenic autoantibodies are both somatically hypermutated and of high affinity[13], it has long been presumed that T follicular helper (T_{FH}) cell-driven selection of self-reactive GC B cells can make an important contribution to autoimmune disease. Indeed, it has recently been demonstrated that in some circumstances T_{FH} cells are required to drive systemic autoimmunity[34].

A failure of T cell self-tolerance in the T_{FH} cell repertoire is no doubt an important driver of autoimmunity (see the figure, panel **a**), but recent data indicate that T_{FH} cell responses that are directed against foreign antigens can also support an autoantibody response under certain circumstances[70]. Thus, if a somatically mutating GC B cell acquires self-reactivity but also maintains its cross-reactivity with foreign antigens, then foreign antigen-specific T_{FH} cells can support B cell maturation and drive autoantibody production (see the figure, panel **b**). This mechanism selectively operates against peripheral tissue-specific self antigen targets, as cross-reactivity with self antigens that are present in the GC microenvironment results in the deletion of the self-reactive GC B cells[70]. Thus, these data indicate that pathogen-specific T_{FH} cells might drive the production of peripheral tissue-specific cross-reactive autoantibodies that are found in post-infectious autoimmune diseases such as rheumatic fever and Guillain–Barre syndrome[13].



BCR, B cell receptor; CD40L, CD40 ligand; CR2, complement receptor 2; FcRII, low affinity Fc receptor for immunoglobulin; Ig, immunoglobulin; TCR, T cell receptor.

In contrast to BAFFR, TACI negatively regulates B cell function[11]. In keeping with this, *Taci*$^{-/-}$ mice have expanded populations of T_{FH} cells and GC B cells[28]. In the absence of TACI, greater ICOSL expression on B cells results in an increase in the number of T_{FH} cells[28]. These observations demonstrate that an important extrinsic determinant of T_{FH} cell formation is the balance between positive and negative signalling that is provided by BAFF and APRIL through BAFFR and TACI, respectively. These interactions cooperatively regulate ICOSL expression on antigen-presenting B cells (FIG. 1). These studies highlight the crucial role for ICOS–ICOSL signalling in T_{FH} cell formation, particularly as the phenotype of NIK-deficient mice recapitulates that of mice that are globally deficient in ICOS[29] or mice with B cells that lack expression of ICOSL[30].

The SLAM family of surface receptors. The SLAM family of receptors includes SLAM (also known as SLAMF1), CD84 (also known as SLAMF5), natural killer cell receptor 2B4 (also known as CD244), T lymphocyte surface antigen LY9 and NTBA (natural killer, T and B cell antigen; also known as SLAMF6 and LY108 in mice). These receptors recruit SAP, thereby allowing $CD4^+$ T cells to activate signalling intermediates, including protein kinase Cθ (PKCθ), BCL-10, nuclear factor-κB (NF-κB) and FYN, that are important for receptor function[31]. Naive SAP-deficient $CD4^+$ T cells fail to form stable conjugates with cognate B cells[32,33], and are therefore unable to differentiate into T_{FH} cells and to help T cell-dependent B cell responses[17,18,23,34]. This explains why individuals with X-linked lymphoproliferative disease (XLP) caused by mutations in *SH2D1A* (the gene encoding

X-linked lymphoproliferative disease
A rare, often fatal, primary immunodeficiency disease that is characterized by an inability to mount an effective immune response against Epstein–Barr virus, as well as a susceptibility to developing lymphoma and/or hypogammaglobulinaemia.

Box 3 | **IL-21 is a potent differentiation factor for human B cells**

Interleukin-21 (IL-21) was discovered in 2000 as a pleiotropic cytokine that is capable of activating most lymphocyte populations[116]. The initial description reported that IL-21 strongly induced the proliferation of CD40-stimulated human B cells, but that it inhibited IL-4-induced B cell proliferation[116]. Since then, many studies have established the potency of IL-21 as a growth and differentiation factor for human B cells. When human B cells are primed with T cell help in the form of a CD40-specific monoclonal antibody or CD40 ligand (CD40L), IL-21 induces robust B cell proliferation as well as the expression of activation-induced cytidine deaminase (*AICDA*; required for immunoglobulin class switching), B lymphocyte-induced maturation protein 1 (*BLIMP1*; also known as *PRDM1*) and X box-binding protein 1 (*XBP1*)[83,117,118], all of which mediate the differentiation of B cells into plasma cells. Consequently, at least *in vitro*, IL-21 efficiently induces subsets of activated B cells to undergo class switching either to become IgG- or IgA-expressing cells, or to become plasmablasts secreting IgM, IgG, IgA or IgE[83,117–120]. IL-21 predominantly induces switching to IgG3, IgG1 and IgA1 subclasses[117,119,120], whereas IL-21-stimulated naive, GC or memory B cells produce large quantities of immunoglobulins[83,117,119]. IL-21 can also induce the expression of B cell lymphoma 6 (*BCL6*) in human naive B cells[117], which is consistent with IL-21 having a role in establishing GCs. Before the discovery of IL-21, it was well recognized that class switching by human B cells was regulated by IL-4 (which promotes class switching to IgG4 and IgE), IL-10 (which promotes class switching to IgG1 and IgG3), IL-13 (which promotes class switching to IgG4 and IgE) and transforming growth factor-β (TGFβ; which promotes class switching to IgA), and IL-10 was also considered to be a strong inducer of immunoglobulin secretion. Furthermore, B cell survival was shown to be positively regulated by IL-4 or IL-10 (reviewed in REF. 82). Studies over the past decade have highlighted the importance of IL-21 in humoral immunity in humans by demonstrating that it has the remarkable ability to exert all of these functions on human B cells.

SAP) have poor humoral immunity[31]. A role for CD84 in SAP-dependent T_{FH} cell generation following immunization with protein antigen was recently demonstrated[33]. However, the T_{FH} cell deficiency in *Cd84*$^{-/-}$ mice was less severe than in SAP-deficient mice[33], and T_{FH} cell formation following viral infection was unaffected by the absence of CD84 (REF. 35).

Analysis of gene-targeted mice has failed to show a requirement for SLAM family receptors other than CD84 in T_{FH} cell formation (reviewed in REF. 23), although SLAM has been shown to be required for IL-4 expression by GC T_{FH} cells[18]. One interpretation of these observations is that the severe effect of SAP deficiency in T_{FH} cells reflects a requirement for numerous SLAM receptors during the differentiation of T_{FH} cells. Alternatively, as SLAM receptors can also recruit inhibitors of signalling (such as lipid and tyrosine phosphatases), SAP deficiency might exacerbate negative signals that are delivered through one or more of the SLAM receptors. Consistent with this idea, loss of LY108 reversed the inability of SAP-deficient $CD4^+$ T cells to form T_{FH} cells and to support B cell responses[35]. This was due to a reduced recruitment of SH2 domain-containing protein tyrosine phosphatase 1 (SHP1; also known as PTPN6) to the immune synapse by LY108 (REF. 35). Thus, LY108 functions as a rheostat that is capable of delivering positive SAP-dependent and negative SHP1-dependent signals that dynamically regulate T_{FH} cells (FIG. 1).

The role of PD1. An important phenotypic determinant of T_{FH} cells is their high expression of PD1 (REF. 14). PD1 has an inhibitory role in T_{FH} cell differentiation, as mice with impaired PD1 function have more T_{FH} cells ($CCR7^{low}ICOS^{hi}$ cells in *Pd1*$^{-/-}$ mice) as a result of increased proliferation and reduced apoptosis[36–39]. Expression of PD1 ligand 1 (PDL1; also known as CD274), rather than PDL2, on B cells constrains T_{FH} cell formation via the PD1 pathway[38] (FIG. 1). Although these studies showed that ablating PD1 signalling increased T_{FH} cell numbers, they yielded conflicting results about how this affected the outcome of the GC response[36–39]. For instance, some groups reported increased antigen-specific antibody responses in PDL1-deficient mice[38] or in *Plasmodium*-infected mice that had been treated with a PDL1-specific mAb[39]. These findings in mice are consistent with data demonstrating that ligation of PD1 suppresses the proliferation, activation and function of human T_{FH} cells *in vitro*[40]. However, other groups have reported impaired plasma cell and GC responses in the absence of PD1 signalling[36,37]. These differences might reflect nuances in the experimental systems that have been used, but they might also result from distinct functions of PD1 in the development and function of not only T_{FH} cells but also follicular T regulatory cells (follicular T_{Reg} cells), which express higher levels of PD1 than T_{FH} cells[41].

Cytokines. Numerous cytokines have been shown to be important for T_{FH} cell generation *in vivo* and *in vitro*. The initial cytokines that were identified to induce T_{FH} cell-like features in cultured $CD4^+$ T cells were IL-6 and IL-21 (TABLE 1). However, subsequent analyses of IL-6- and IL-21- or IL-21R-deficient mice yielded conflicting results regarding the necessity of these cytokines in regulating T_{FH} cell formation *in vivo* (reviewed in REFS 23,24). Furthermore, recent studies have provided greater insights into the roles of these cytokines in T_{FH} cell commitment. Using several mouse models of viral infection, investigators found varying — and transient — degrees of impairment in T_{FH} cell numbers in the absence of IL-6, but they observed consistently reduced levels of virus-specific IgG[42–45]. A more severe decrease in T_{FH} cell formation and protective IgG production occurred in the absence of both IL-6 and IL-21 (REFS 42,44) (FIG. 1). This requirement for IL-6 and IL-21 is consistent with reductions in the number of T_{FH} cells in the absence of functional STAT3 (REFS 30,46), which acts downstream of both cytokines (TABLE 1). Interestingly, the early reduction in the number of mouse T_{FH} cells in the absence of STAT3 was

SH2 domain-containing protein tyrosine phosphatase 1
(SHP1). A protein tyrosine phosphatase that is involved in suppressing intracellular signals delivered via numerous activating receptors, including T cell and B cell antigen receptors, as well as members of the signalling lymphocytic activation molecule (SLAM) family of surface receptors. One proposed mechanism of action is the direct or indirect dephosphorylation of components of the T cell receptor signalling pathway, such as CD3ζ, LCK, ζ-chain-associated protein kinase of 70 kDa (ZAP70) and phosphoinositide 3-kinase.

Follicular T regulatory cells
A subset of T regulatory (T_{Reg}) cells that co-opts the transcriptional machinery of T follicular helper (T_{FH}) cells to facilitate their migration to germinal centres, where they can appropriately restrain humoral immune responses, thereby potentially preventing overzealous antibody responses. Follicular T_{Reg} cells can be identified by the expression of typical T_{FH} cell surface markers (CXC-chemokine receptor 5 (CXCR5), inducible T cell co-stimulator (ICOS) and programmed cell death protein 1 (PD1)) along with the T_{Reg} transcription factor forkhead box P3. Their mechanism of action remains to be completely elucidated.

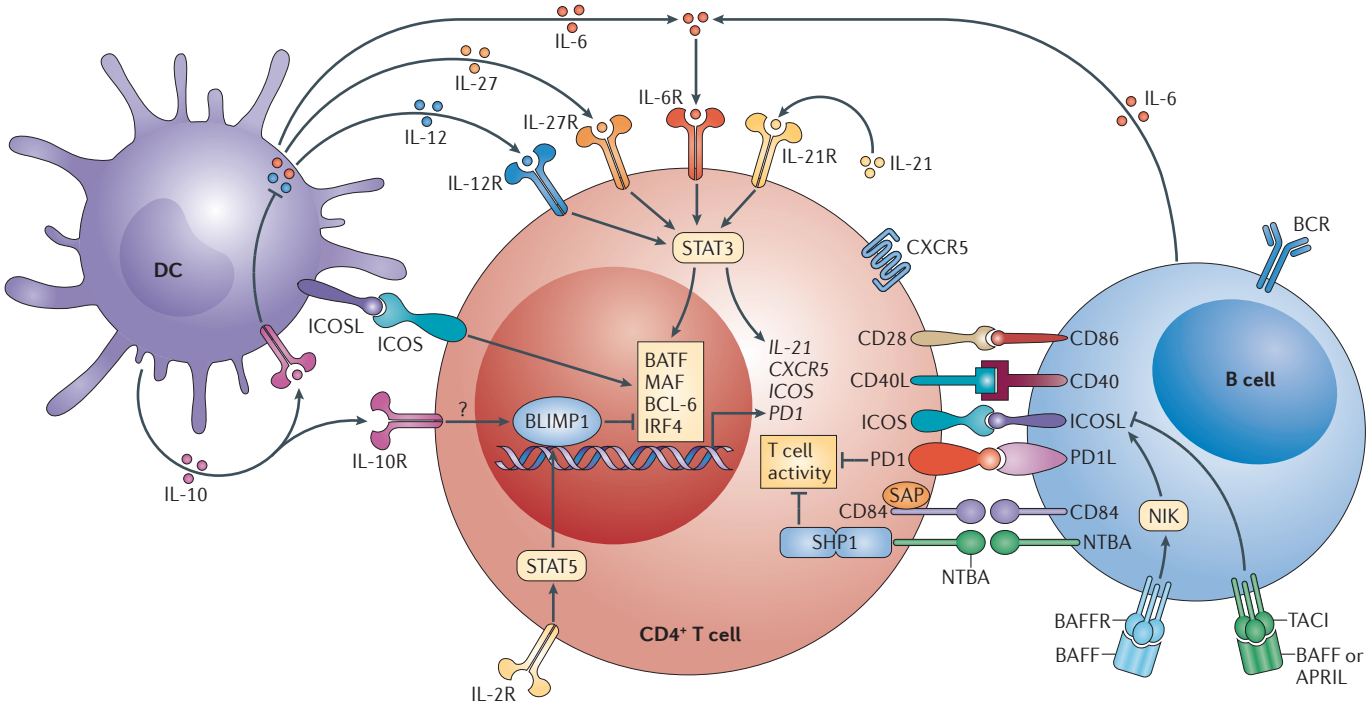


Figure 1 | **Cellular and molecular regulation of T_{FH} cell formation.** Naive CD4$^+$ T cells interact with antigen-presenting dendritic cells (DCs) in the interfollicular or T cell zones; DC-primed CD4$^+$ T cells acquire expression of CXC-chemokine receptor 5 (CXCR5) and B cell lymphoma 6 (BCL-6) to become early T follicular helper (T_{FH}) cells. These cells then migrate to the T cell–B cell border and — following interactions with cognate B cells — differentiate into germinal centre T_{FH} cells. This differentiation process is governed by signals provided by signal transducer and activator of transcription 3 (STAT3)-activating cytokines, including interleukin-6 (IL-6), IL-12, IL-21 and IL-27. These cytokines are secreted by DCs (which produce IL-6, IL-12 and IL-27), B cells (which produce IL-6 and possibly IL-27) and CD4$^+$ T cells (which produce IL-21). Cytokine-mediated activation of STAT1 might also contribute to this process (not shown). These cytokines operate individually or collectively to induce or enhance expression of the transcription factors BCL-6, MAF, basic leucine zipper transcriptional factor ATF-like (BATF) and interferon-regulatory factor 4 (IRF4), which then imprint the T_{FH} cell fate on a T cell by inducing the transcription of signature genes, including *CXCR5*, inducible T cell co-stimulator (*ICOS*), *IL21* and programmed cell death protein 1 (*PD1*). Cell–cell interactions among activated CD4$^+$ T cells, antigen-presenting DCs and B cells also promote T_{FH} cell formation. CD28–CD86, CD40 ligand (CD40L)–CD40 and ICOS–ICOS ligand (ICOSL) interactions are central to this process. Notably, ICOSL expression on B cells is controlled through the opposing effects of B cell-activating factor (BAFF) signalling via the distinct receptors BAFF receptor (BAFFR) and transmembrane activator and CAML interactor (TACI). In addition, signalling lymphocytic activation molecule (SLAM) family receptors have a dual role in T_{FH} cell generation: the recruitment of SLAM-associated protein (SAP) to these receptors facilitates and maintains conjugate formation between T and B cells, whereas recruitment of inhibitory phosphatases (such as SH2 domain-containing protein tyrosine phosphatase 1 (SHP1)) suppresses these interactions, thereby influencing the ability of CD4$^+$ T cells to form T_{FH} cells and to support B cell responses. T_{FH} cell generation is restricted by B lymphocyte-induced maturation protein 1 (BLIMP1), which is induced by IL-2 in a STAT5-dependent manner; BLIMP1 functions by repressing the expression of BCL-6. IL-10 also suppresses T_{FH} cell formation, but it is unknown whether this is also mediated via BLIMP1. APRIL, a proliferation-inducing ligand; BCR, B cell receptor; IL-10R, IL-10 receptor; NIK, NF-κB-inducing kinase; NTBA, natural killer, T and B cell antigen (also known as LY108 in mice); PDL1, PD1 ligand 1.

also transient and not as severe as that observed in IL-6-deficient mice, which indicates that there might be a requirement for an alternative signalling pathway downstream of IL-6R[45]. Indeed, the T_{FH} cell deficit in IL-6-deficient mice was recapitulated when STAT1 and STAT3 were deleted from CD4$^+$ T cells, which indicates that IL-6-mediated activation of both STATs is required for full T_{FH} cell development[45]. These results suggest that significant functional redundancy exists between IL-6 and IL-21, and that the relative importance of either cytokine is related to the level of its production at a given time and is inversely proportional to the levels of other compensatory cytokines.

Whereas IL-21 is likely to be produced by CD4$^+$ T cells themselves, IL-6 could be derived from activated B cells[44], from dendritic cells (DCs)[47] or from follicular dendritic cells[43]. IL-6 expression by B cells or DCs has been shown to promote IL-21 production by CD4$^+$ T cells that have been activated *in vitro*[44,47], and B cell-derived IL-6 restored T_{FH} cell numbers following transfer into mice that were deficient in both IL-6 and IL-21 (REF. 44). Thus, although interactions between DCs and naive CD4$^+$ T cells are important in initiating the T_{FH} cell programme[23], subsequent cognate interactions between these early T_{FH} cells and antigen-specific B cells are required to ensure their progression to a T_{FH} cell fate (FIG. 1).

Although it is known that numerous cytokines can promote T_{FH} cell formation (reviewed in REFS 23,24) (Supplementary information S1 (table)), much less is known about the factors that restrain this process. An important attribute of all T_H cell lineages is their ability to suppress the generation of cells with alternative effector fates[1] (TABLE 1). Thus, it is not surprising that T_{FH} cell formation can also be suppressed by several immunoregulatory cytokines. Impaired IL-10 signalling was shown to promote T_{FH} cell formation through at least two separate mechanisms. First, IL-10R deficiency enhanced T_{FH} cell function, as was shown by the increased antibody responses of cognate B cells following interactions with *Il10rb*$^{-/-}$ T_{FH} cells. Second, the inability of DCs to respond to IL-10 resulted in greater production of IL-6, IL-23 and IL-12; this, in turn, contributed to an increased frequency of T_{FH} cells in *Il10rb*$^{-/-}$ mice (FIG. 1). Furthermore, *Il10rb*$^{-/-}$ T_{FH} cells expressed higher levels of IL-17 and IL-21 than wild-type T_{FH} cells, which indicates that IL-10 regulates T_{FH} cells both quantitatively and qualitatively[48].

Importantly, the increased production of both IL-17 and IL-21 substantially shaped humoral immune responses in *Il10rb*$^{-/-}$ mice, as blockade of these cytokines not only impeded T_{FH} cell formation but also reduced the augmented antigen-specific antibody response[48]. Thus, consistent with its ability to suppress T_H1 and T_H17 cells[1] (TABLE 1), IL-10 also negatively regulates T_{FH} cells. However, the molecular mechanism through which IL-10 exerts its repressive effect is unknown. T_{FH} cell formation can also be limited by IL-2–STAT5 signalling, which induces B lymphocyte-induced maturation protein 1 (BLIMP1; also known as PRDM1) to suppress BCL-6 function[49] (FIG. 1); therefore, IL-10-mediated BLIMP1-induction might also contribute to the inhibitory effect of IL-10 on T_{FH} cells.

Overall, positive and negative signals from numerous receptors and cytokines fine-tune T_{FH} cell formation, homeostasis and function. Consequently, perturbations in this balance might deregulate T_{FH} cell differentiation, thereby accelerating disease development in genetically susceptible hosts. The exact role of specific cytokines in the regulation of human T_{FH} cells remains unknown, but the identification of individuals with inactivating mutations in important cytokines and cytokine receptors will shed light on these requirements.

T_{FH} cell memory

Although T_{FH} cells are unquestionably important in establishing B cell memory, the fate of T_{FH} cells that are generated during GC reactions is unclear. Several recent studies in mice have investigated whether T_{FH} cells are short-lived effector cells or whether they differentiate into long-lived memory-type cells that can resume a T_{FH} cell state following re-exposure to the same initiating antigen. Although both CXCR5$^+$PD1^{low} T_{FH} cells and CXCR5^{hi}PD1^{hi} GC T_{FH} cells are generated following vaccination or infection, the number of GC T_{FH} cells decreases more rapidly than that of CXCR5$^+$PD1^{low} T_{FH} cells[50,51]. The decrease in GC T_{FH} cells is partly due to their loss of the T_{FH} phenotype rather than as a result of apoptosis. Thus, these 'former' T_{FH} cells most probably differentiate into memory cells that downregulate CXCR5, PD1 and BCL-6, that re-express CCR7, IL-7Rα and CD62L (also known as L-selectin), and that persist for a long time.

To add further support to the idea that T_{FH} cells can generate memory cells, it was recently shown that gene expression profiles of early T_{FH} cells share many similarities with precursor memory CD8$^+$ T cells[52]. Upon subsequent antigenic challenge, these memory-like cells form T_{FH} cells more quickly and promote GC formation and antibody production more effectively than naive or memory cells that originated from non-T_{FH} cells[50,51,53–55]. Interestingly, it was shown that not all of these cells adopted a T_{FH} cell phenotype, which indicates that cellular plasticity might allow them to differentiate into other T_H cell subsets. A caveat to these studies is the reliance on identifying T_{FH} cells by phenotype alone. In the future it would be useful to more precisely track T_{FH} cells in GCs and to determine their fate. However, taken together, these data demonstrate that, at least in mice, some T_{FH} cells can enter the memory pool and can provide long-term protection following reinfection.

Antigen-specific T cells have also been identified within the population of T_{FH}-like cells that is detected in the peripheral blood of humans. These cells seem to be more readily detected following recent antigen exposure, which indicates that they exist as T_{FH}-like cells or that their precursors adopt a T_{FH} cell phenotype in response to antigenic stimulation[24,56,57], as discussed below.

The expanding universe of T_{FH}-like cells

T_{FH} cells that provide B cell help during responses to 'conventional' T cell-dependent antigens have been well characterized, but 'unconventional' subsets of T_{FH} cells have also been identified, and these are discussed below.

Extrafollicular T_H cells. Humoral immune responses are initiated in extrafollicular areas of lymphoid tissues where B cells differentiate into short-lived plasmablasts following interactions with T_H cells[11,13,26]. Similar to T_{FH} cells, the formation of extrafollicular T_H cells requires BCL-6, and their function is mediated by CD40L, ICOS and IL-21 (REF. 26). However, extrafollicular T_H cells do not express high levels of CXCR5; rather, they are attracted to these sites by the CXCR4– CXCL12 (CXC-chemokine ligand 12) axis[26]. Extrafollicular T_H cells might also give rise to GC T_{FH} cells following interactions with cognate B cells[19,23,24].

NKT$_{FH}$ cells. Natural killer T (NKT) cells are innate-like T cells expressing a semi-invariant T cell receptor (TCR) that recognizes lipid antigens presented by the non-polymorphic MHC molecule CD1d. The ability of the NKT cell population to rapidly expand and to produce effector cytokines following encounter with an antigen — typically α-galactosyl ceramide (αGalCer) — links the innate and adaptive immune responses. NKT cells can initiate or enhance antibody responses to lipid and protein antigens via cognate[58–60] and non-cognate[61] mechanisms, respectively.

Follicular dendritic cells
Specialized non-haematopoietic stromal cells that reside in lymphoid follicles and germinal centres. These cells possess long dendrites and carry intact antigen on their surface. They are crucial for the optimal selection of B cells that produce antigen-binding antibodies.

Recent studies have reassessed the nature of NKT cells that provide B cell help. It was shown that following immunization with αGalCer–antigen conjugates, a small proportion of mouse NKT cells acquired a T_{FH}-like phenotype[62,63]. These NKT follicular helper (NKT_{FH}) cells were detected in human tonsils[62] and their development depended on the same factors that are important for conventional T_{FH} cells[62]. By producing IL-21, NKT_{FH} cells supported the rapid formation of GCs, yielding detectable levels of antigen-specific IgG, with some evidence of affinity maturation[62–64] (FIG. 2).

Although NKT_{FH} cells resembled T_{FH} cells, the most striking difference was their inability to invoke long-lived memory responses to lipid antigens (FIG. 2). Furthermore, the magnitude of the NKT_{FH} cell-induced antibody responses to lipid antigens was inferior to those driven by conventional T_{FH} cells[62–64]. Despite this, administering αGalCer as an adjuvant or as a conjugated component of immunizing antigens increased the production of antigen-specific antibodies. This probably reflects the direct actions of NKT_{FH} cells on antigen-specific $CD1d^+$ B cells, as well as the indirect actions of NKT_{FH} cells, such as cytokine production, on other cell types[58–61]. It might also indicate that there is synergy between NKT_{FH} and T_{FH} cells. Taken together, these findings support the inclusion of αGalCer in vaccine adjuvants.

γδ T_{FH} cells. The finding that mice and humans lacking the conventional αβ TCR have T_H cells that elicit humoral responses against T cell-dependent antigens led to the realization that γδ T cells can provide help to B cells to generate GCs[65]. Similar to conventional T_{FH} cells, some human γδ T_{FH} cells express CXCR5 and localize to follicles and GCs[66–68]. γδ T cells express a semi-invariant TCR repertoire and recognize non-peptidic phosphoantigens that are derived from microbial metabolites[65]. These antigens rapidly activate γδ T cells, which can subsequently acquire T_{FH} cell features[66,68,69]; the differentiation of γδ T cells to T_{FH}-like cells is increased by exogenous IL-21 (REFS 68,69) (FIG. 2). As γδ T cells do not produce IL-21 (REFS 68,69), they are dependent on extrinsic sources of this cytokine to differentiate into T_{FH}-like cells.

As conventional $CD4^+$ T cells, γδ T cells and NKT cells recognize different repertoires of microbial antigens, their ability to differentiate into T_{FH}-like effector cells provides a mechanism whereby humoral immunity can be generated against a broad range of pathogen-associated antigens (FIG. 2). This expands the number of molecular targets that initiate protective immunity, but it might also contribute to post-infection autoimmunity by generating cross-reactive autoantibodies[13,70] (BOX 2).

Follicular T_{Reg} cells. Recent studies have proposed that T_{FH} cells are controlled by follicular T_{Reg} cells — a specialized subset of T_{Reg} cells that colocalize within B cell follicles. Follicular T_{Reg}-like cells were first described in human tonsils in 2004 (REF. 71), but it took another 7–8 years for them to be examined in greater detail.

Follicular T_{Reg} cells comprise approximately 10–15% of the T_{FH} cell population in human and murine lymphoid tissues[72–74]. They show characteristics of both T_{FH} cells and T_{Reg} cells, but they lack expression of CD40L, IL-4 and IL-21 (REF. 73). Abrogating either follicular T_{Reg} cell development or their follicular localization was shown to enhance GC responses and subsequent antibody production[72,74]. Although follicular T_{Reg} cells show similar requirements to conventional T_{FH} cells for their development[73], they actually originate from thymus-derived T_{Reg} cells, rather than from peripherally derived T_{Reg} or T_{FH} cells. Similar to other specialized T_{Reg} cell subsets that have evolved to selectively control T_H1, T_H2 and T_H17 cells[75], follicular T_{Reg} cells seem to have co-opted the transcriptional machinery of T_{FH} cells to migrate into GCs to exert their regulatory effects[72–74].

PD1 is more highly expressed on follicular T_{Reg} cells than on T_{FH} cells. Interestingly, PD1 or PDL1 deficiency was shown to favour the development of follicular T_{Reg} cells but not T_{FH} cells[41]. Furthermore, PD1-deficient follicular T_{Reg} cells were more effective than normal follicular T_{Reg} cells at impeding T_{FH} cell-mediated B cell differentiation[41]. Thus, PD1 seems to negatively regulate not only the development and maintenance of follicular T_{Reg} cells but also their suppressive function. The increase in the number of follicular T_{Reg} cells compared with T_{FH} cells, together with the increased suppressive function of PD1-deficient follicular T_{Reg} cells[41], might explain some of the discrepancies in the findings regarding the consequences of PD1 deficiency on T_{FH} cell-dependent B cell responses[36–39].

The mechanisms by which follicular T_{Reg} cells attenuate humoral immunity remain unknown. Follicular T_{Reg} cells express *IL10* mRNA[73] (TABLE 1), and IL-10 can attenuate T_{FH} cell formation in normal[48] and autoimmune[76] settings. Thus, follicular T_{Reg} cell-derived IL-10 might be one means by which T_{FH} cell-mediated B cell responses are regulated. However, as follicular T_{Reg} and T_{FH} cells express similar levels of IL-10, it is difficult to predict how follicular T_{Reg}-derived IL-10 would affect T_{FH} cells in a different manner to their own endogenous IL-10. Although it is known that follicular T_{Reg} cells are likely to limit GC reactions by impeding both T_{FH} cells and GC B cells, therapeutically exploiting the immunoregulatory function of follicular T_{Reg} cells will require further delineation of their mechanism of action.

Human circulating T_{FH}-like cells

As access to human lymphoid tissues is limited, studies of human T_{FH} cells have exploited the fact that $CD4^+CXCR5^+$ T cells comprise a small subset of circulating lymphocytes[4,5]. However, there are clear differences between $CD4^+CXCR5^+$ T cells in the blood and those in the tonsils. For instance, $CD4^+CXCR5^+$ T cells in the blood do not express BCL-6 and their expression of ICOS and PD1 is substantially lower than that of T_{FH} cells[4,5,56,77,78]. Despite this, *in vitro*-cultured blood-derived $CD4^+CXCR5^+$ T cells produce more IL-21, IL-10 and CXCL13 — which are all features of T_{FH} cells[8,9] — and are more efficient at inducing B cell differentiation

γδ T cells
T cells that express the γδ T cell receptor. These T cells are present in the skin, vagina and intestinal epithelium as intraepithelial lymphocytes. Although the exact function of γδ T cells is unknown, it has been suggested that mucosal γδ T cells are involved in innate immune responses.

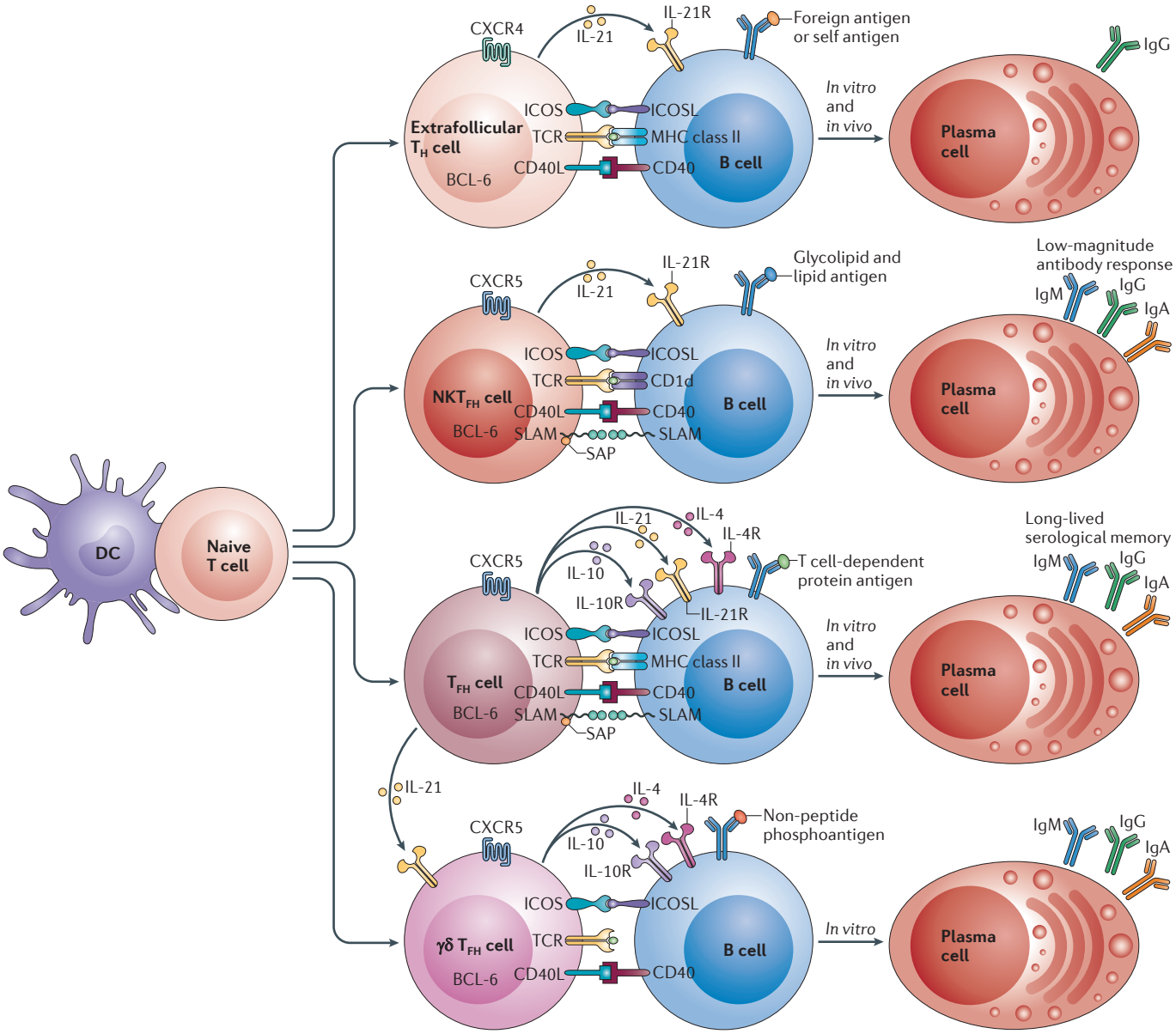


Figure 2 | **T_{FH} cell subsets with specialized effector functions.** Naive CD4$^+$ T cells, natural killer T (NKT) cells and γδ T cells can all differentiate into T follicular helper (T_{FH}) cells following instructive signals that are derived from antigen-presenting cells. These cells share many features, including their requirements for differentiation, their expression of transcription factors and their surface phenotype, which underlies their ability to induce B cell differentiation. Extrafollicular T helper (T_H) cells, NKT follicular helper (NKT_{FH}) cells and T_{FH} cells require inducible T cell co-stimulator (ICOS), B cell lymphoma 6 (BCL-6), SLAM-associated protein (SAP) and CD28 signalling for their differentiation, which occurs *in vitro* and *in vivo* in the presence of B cells. γδ T_{FH} cells also express ICOS, but the molecular requirements for their generation remain unknown. Extrafollicular T_H cells provide help to B cells outside the follicles; unlike other T_{FH}-type cell populations, these cells do not express CXC-chemokine receptor 5 (CXCR5). Rather, their localization to extrafollicular areas seems to be mediated by CXCR4. NKT_{FH} cells and T_{FH} cells support the rapid formation of GCs and the generation of antigen-specific antibodies in an interleukin-21 (IL-21)-dependent manner. T_{FH} and γδ T_{FH} cells might also support GC responses by secreting IL-4 and IL-10. γδ T_{FH} cells do not express IL-21 but might rely on exogenous IL-21 expression for their T_H cell function. T_{FH} cells respond to protein antigens and induce long-lived serological memory. Thus, they have a central role in pathogen-specific responses as well as in several immunopathologies. NKT_{FH} cells respond to glycolipid antigens and induce antibody responses of a lower magnitude than those induced by T_{FH} cells. NKT_{FH} cells might have a role in antibody responses against pathogens that express glycolipid antigens such as *Borrelia hermsii*, *Streptococcus pneumonia* and *Plasmodium falciparum*[62–64]. As γδ T cells recognize microbial metabolites, γδ T_{FH} cells might have an adjuvant role in responses to most infectious pathogens. Extrafollicular T_H cells have been most effectively characterized for their role in disease pathogenesis in murine models of human autoimmunity[26]. These cells might also represent precursors of T_{FH} cells[19]. CD40L, CD40 ligand; DC, dendritic cell; ICOSL, ICOS ligand; IL-21R, IL-21 receptor; SLAM, signalling lymphocytic activation molecule; TCR, T cell receptor.

than $CD4^+CXCR5^-$ T cells[56,78]. Thus, human circulating $CD4^+CXCR5^+$ T cells have some features of T_{FH} cells, which indicates that precursors of circulating $CD4^+CXCR5^+$ T cells might have experienced some aspects of a 'T_{FH} cell differentiation programme' *in vivo*.

Interestingly, subsets of circulating $CD4^+CXCR5^+$ T cells that have distinct effector functions also exist. By assessing the expression of CXCR3 and CCR6 — chemokine receptors that are associated with T_H1 and T_H17 cells, respectively — $CD4^+CXCR5^+$ T cells could be classified into T_H1-like, T_H17-like and T_H2-like ($CXCR3^-CCR6^-$) T_{FH} cell subsets[56]. Interestingly, the T_H2- and T_H17-like T_{FH} cell subsets were shown to produce higher levels of IL-21 and to induce naive B cell differentiation more efficiently than the T_H1-like T_{FH} cell subset. This showed that there is substantial heterogeneity among blood-derived $CD4^+CXCR5^+$ T cells and potentially clarified previous findings indicating that circulating $CD4^+CXCR5^+$ T cells might be inefficient 'helpers' for B cell differentiation[5].

Despite the improved characterization of circulating $CD4^+CXCR5^+$ T cells, their exact relationship with GC T_{FH} cells remains incompletely defined. It is also unclear what functional role BCL-6 has in blood-derived $CD4^+CXCR5^+$ T cells, as it does not seem to be required for their persistence, expression of CXCR5, production of IL-21 or for their ability to induce B cell differentiation. Kinetic analysis of T_H cell activation in mice showed that although BCL-6 is expressed by developing T_{FH} cells, it is downregulated at later time points[79]. Apart from BCL-6 expression, there seemed to be few differences between $BCL\text{-}6^+$ and $BCL\text{-}6^-$ T_{FH} cells with respect to their expression of important T_{FH} cell-related genes[79]. This indicates that although BCL-6 is required for T_{FH} cell formation, it might be dispensable for their maintenance following antigen clearance and the resolution of an immune response. Furthermore, $BCL\text{-}6^-$ T_{FH} cells upregulated *Il7r* and *Ccr7* and downregulated the cell cycle machinery[79].

Thus, T_{FH} cells probably yield a population of cells that exit the GC and lymphoid tissues, and that return to the circulation as a population of quiescent memory-type $CD4^+CXCR5^+$ T cells.

Human T_{FH} cells in immunity and disease

Dysregulated behaviour of T_{FH} cells and extrafollicular T_H cells has been found to contribute to autoimmune or immune-deficient states in several mouse models of human disease[10,24,26]. Consequently, there is great interest in determining the role of T_{FH} cells in human disease. Although the exact nature of circulating $CD4^+CXCR5^+$ T cells in humans remains unclear, investigation of this population of cells in various contexts has nonetheless provided important insights into their potential functions during normal immune responses and in immunopathologies (TABLE 2).

Circulating $CD4^+CXCR5^+$ T cells as biomarkers of effective humoral immunity. Long-lived T cell-dependent antibody responses underlie the success of most vaccines that are currently in use. For this reason, there is growing interest in identifying appropriate biomarkers of successful vaccination. Several publications reported the outcomes of immunization against influenza during the 2009 H1N1 epidemic. These studies included healthy individuals and a cohort of HIV-infected individuals. Whereas all healthy individuals generated protective levels of H1N1-specific IgG 4 weeks after vaccination, such a response was observed in only 50% of HIV-positive individuals. Successful induction of H1N1-specific IgG coincided with significant increases in both serum levels of IL-21 and frequencies of circulating $CD4^+CXCR5^+$ T cells and memory B cells[80,81]; this correlated with titres of serum H1N1-specific antibodies[81]. Thus, the ability of the H1N1 vaccine to induce protective antibody responses correlated with the induction of detectable features of T_{FH} cell-mediated immunity[80,81].

Another study found that the emergence of $CD4^+CXCR5^+CXCR3^+ICOS^+$ T cells in influenza-vaccinated individuals correlated with increased levels of neutralizing antibodies and — in adults — with the appearance of circulating plasmablasts[57]. This was unexpected, as it was previously found that circulating $CXCR3^+$ T_{FH}-like cells were poor inducers of naive B cell differentiation and that they did not produce IL-21 (REF. 56). However, $CD4^+CXCR5^+CXCR3^+ICOS^+$ T cells did promote the differentiation of memory, but not naive, B cells into plasmablasts *in vitro* through the production of IL-10 and IL-21 (REF. 57). This suggests that increased numbers of $CD4^+CXCR5^+CXCR3^+ICOS^+$ T cells could be used as an indicator of the development of protective antibody responses from pre-existing memory B cells, rather than of primary responses of naive B cells[57]. Despite this conclusion, it remains unknown which signals induce IL-21 expression in $CD4^+CXCR5^+CXCR3^+$ $ICOS^+$ T cells and why these cells fail to activate naive B cells even though they express CD40L, IL-10 and IL-21, which, in combination, can strongly induce the differentiation of naive B cells to plasmablasts[82,83].

Taken together, these studies show that quantifying the frequencies of circulating $CD4^+CXCR5^+$ T cells, or of their subsets, is a reliable predictor of vaccine success and of the magnitude of the induced response[57,80,81]. It also allowed for the separation of vaccine responders from non-responders[80,81]. Understanding why some individuals failed to elicit a substantial vaccine-specific T_{FH} cell-dependent antibody response might facilitate the development of improved vaccination strategies, particularly in individuals with suboptimal humoral immunity.

Autoimmune diseases. The detection of $CD4^+CXCR5^+$ T cells and CXCL13 in organs that are affected by autoimmune disorders, such as the salivary glands in Sjögren's syndrome, suggested that aberrant T_{FH} cell development can drive autoimmunity[84]. As a result of this finding, several studies have assessed the possibility of enumerating circulating $CD4^+CXCR5^+$ T cells as a potential biomarker of autoimmune diseases, especially given the difficulty of accessing and analysing

Sjögren's syndrome
A systemic autoimmune disease in which autoantibodies target and destroy exocrine glands such as the tear ducts and the salivary glands.

Table 2 | **Human diseases associated with aberrant T_{FH} cell function**

Human disease	T_{FH} cell-related phenotype	Correlated pathology	Refs
Autoimmunity			
Systemic lupus erythematosus	• Increased frequencies of circulating $CD4^+CXCR5^+PD1^{hi}$ cells, $CXCR5^+ICOS^{hi}$ cells or $ICOS^{hi}$ T cells • Increased serum levels of IL-21 and CXCL13	• Increased severity of end-organ damage • Higher serum levels of dsDNA-specific autoantibodies	77,85,94
Sjögren's syndrome	• Increased frequencies of circulating $CD4^+PD1^{hi}$ T cells, $CD4^+CXCR5^+ICOS^{hi}$ T cells or $CD4^+CXCR5^+CCR6^+$ (T_H17-type) T cells	• Higher serum levels of autoantibodies	77,93
Rheumatoid arthritis	• Increased frequencies of circulating $CD4^+CXCR5^+PD1^{hi}$ cells or $CD4^+CXCR5^+ICOS^{hi}$ T cells • Increased serum levels of IL-21	• Higher serum levels of CCP-specific autoantibodies • Higher disease score and serum levels of IL-21- and CCP-specific autoantibodies	90,92
Juvenile dermatomyositis	• Increased T_H2– and T_H17-like subsets among circulating $CD4^+CD45RO^+CXCR5^+$ T cells	• Higher disease score • Increased number of circulating plasmablasts	56
Autoimmune thyroid disease (Graves' disease or Hashimoto's thyroiditis)	• Increased frequencies of circulating $CD4^+CXCR5^+PD1^{hi}$ T cells or $CD4^+CXCR5^+ICOS^{hi}$ T cells	• Increased titres of serum autoantibodies against thyroid stimulating hormone receptor and thyroglobulin	91
Myasthenia gravis	• Increased frequencies of circulating $CD4^+CXCR5^+$ T cells or $CXCR5^+CD57^+$ T cells	• Increased disease severity	86,87
Immunodeficiency			
X-linked lymphoproliferative disease	• Normal frequency of circulating $CD4^+CXCR5^+$ T cells but impaired helper function *in vitro*	• Impaired cytokine production, ICOS expression and B cell help *in vitro*	17,96
CVID (ICOS deficiency) and hyper-IgM syndrome (CD40 or CD40L deficiency)	• Reduced frequency of circulating $CD4^+CD45RO^+CXCR5^+$ T cells	• Impaired development of T_{FH} cells owing to an inabilty to form GCs	29
AD-HIES (STAT3 deficiency)	• Reduced frequency of circulating $CD4^+CD45RO^+CXCR5^+$ T cells • Impaired generation of T_{FH}-like cells from naive precursors *in vitro*	• Inability of naive $CD4^+$ T cells to express IL-21 and to provide help to B cells in response to T_{FH} cell-inducing cytokines (for example, IL-12)	46
HIV	• Increased frequency of T_{FH} cells in lymph nodes of HIV-infected individuals • Enrichment of HIV-specific $CD4^+$ T cells in the T_{FH} cell compartment	• Positive correlations with plasma viraemia, numbers of GC B cells and plasma cells, hypergammaglobulinaemia and virus-specific antibodies	99, 101
Lymphoma			
AITL and FTCL	• Increased number of $CXCR5^+PD1^{hi}ICOS^{hi}OX40^+SAP^{hi}BCL\text{-}6^+MAF^+$ malignant cells • Detectable expression of CXCL13 and IL-21	• Might be correlated with aberrant humoral features such as follicular hyperplasia, hypergammaglobulinaemia and autoantibody production in these PTCLs	104–111

AD-HIES, autosomal dominant hyper-IgE syndrome; AITL, angioimmunoblastic T cell lymphoma; BCL-6, B cell lymphoma 6; CCP, cyclic citrullinated peptide; CCR6, CC-chemokine receptor 6; CD40L, CD40 ligand; CVID, common variable immunodeficiency syndrome; CXCL13, CXC-chemokine ligand 13; CXCR5, CXC-chemokine receptor 5; dsDNA, double-stranded DNA; FTCL, follicular T cell lymphoma; GC, germinal centre; ICOS, inducible T cell co-stimulator; IL, interleukin; PD1, programmed cell death protein 1; PTCL: peripheral T cell lymphoma; SAP, SLAM-associated protein; STAT3, signal transducer and activator of transcription 3; T_{FH}, T follicular helper; T_H; T helper.

secondary lymphoid organs in affected individuals. Early studies showed increases in the frequency of $CD4^+ICOS^+$ T cells in the peripheral blood of patients with systemic lupus erythematosus (SLE)[85] and in the frequency of $CD4^+CXCR5^+$ T cells in the peripheral blood of patients with myasthenia gravis. Furthermore, in myasthenia gravis, the frequency of $CD4^+CXCR5^+$ T cells in the circulation positively correlated with clinical disease scores[86,87]. Subsequent studies reported increases in circulating $CD4^+CXCR5^+ICOS^{hi}PD1^{hi}$ T cells in some patients with SLE or Sjögren's syndrome, and these were shown to correlate with autoantibody titres, frequencies of circulating GC B cells, plasma cells and disease severity[77,88,89]. Increased frequencies of circulating $CXCR5^+ICOS^{hi}$ or $CD4^+CXCR5^+PD1^+$T cells have also been observed in patients with rheumatoid arthritis and autoimmune thyroid diseases (Graves' disease and Hashimoto's thyroiditis), and have been shown to be accompanied by elevated levels of serum autoantibodies[90–92] (TABLE 2).

Furthermore, it was found that the T_H2- and T_H17 (but not T_H1)-like $CD4^+CXCR5^+$ T cell subsets that provide B cell help are increased in juvenile dermatomyositis[56]. In light of this observation, circulating $CD4^+CXCR5^+$ T cells from patients with Sjögren's syndrome were re-examined with respect to their T_H-like phenotypes[93]. A positive correlation was found between the levels of serum autoantibodies and the numbers of circulating $CD4^+CXCR5^+$ T cells, particularly with regard to those that also expressed CCR6 (TABLE 2).

Systemic lupus erythematosus
(SLE). An autoimmune disease in which autoantibodies that are specific for DNA, RNA or proteins associated with nucleic acids form immune complexes. These complexes damage small blood vessels, especially in the kidneys. Patients with SLE generally have abnormal B and T cell function.

As these $CD4^+CXCR5^+CCR6^+$ T cells did not produce IL-17 (REF. 93), this study highlighted the possibility that $CD4^+CXCR5^+CCR6^+$ T cells are not strictly T_H17-like T_{FH} cells, but that they acquire CCR6 expression to facilitate migration to inflamed tissues.

Although some studies did not detect increases in T_{FH}-like cells in SLE[94] and rheumatoid arthritis[95], the concept has emerged that levels of circulating $CD4^+CXCR5^+$ T cells are generally increased in humoral autoimmune conditions. As this increase is usually correlated with clinical disease, the frequency of circulating $CD4^+CXCR5^+$ T cells or the serum levels of IL-21, which are both indicative of T_{FH} cell activity, seem to be useful biomarkers for predicting disease outcome. Furthermore, in patients with SLE, both the increased frequency of circulating $CD4^+CXCR5^+$ T cells and the clinical manifestations that are associated with this disease were reduced following corticosteroid treatment[88]. This indicates that T_{FH} cells are likely to contribute to disease pathogenesis, and it highlights the possibility of targeting T_{FH} cells to effectively treat antibody-mediated autoimmune conditions. However, it remains to be determined whether the association between autoimmunity and the expansion of the population of T_{FH} cells might reflect the activation of self-reactive T_{FH} cells or whether might reflect the increased stimulation of GC B cells producing cross-reactive autoantibodies as a result of increased T_{FH} cell help[13,70] (BOX 2).

Primary immunodeficiencies. Several primary immunodeficiencies have been associated with genetic defects that might affect T_{FH} cell differentiation and function. Mutations in the gene encoding SAP cause XLP[31], whereas mutations that affect the genes encoding CD40L and ICOS cause hyper-IgM syndrome and common variable immunodeficiency, respectively[2]. Important features of these immunodeficiencies include impaired humoral immune responses and a paucity of well-formed GCs in secondary lymphoid tissues[2]. The numbers of circulating $CD4^+CXCR5^+$ T cells are considerably decreased in CD40L- or ICOS-deficient individuals[29]. Although patients with XLP have normal numbers of $CD4^+CXCR5^+$ T cells, possibly as a result of persistent activation with viral antigens[17], their $CD4^+$ T cells fail to acquire features of T_{FH} cells *in vitro*[17,96] (TABLE 2).

Patients with autosomal dominant hyper-IgE syndrome — a primary immunodeficiency that is caused by mutations in *STAT3* — also show impaired functional antibody responses[2]. These patients have a partial deficiency in numbers of circulating $CD4^+CXCR5^+$ T cells, and their naive $CD4^+$ T cells fail to differentiate into T_{FH}-like cells *in vitro*[46]. STAT3 is likely to function downstream of several cytokines — including IL-6, IL-12, IL-21 and IL-27 — which indicates that signal integration among multiple cytokine receptors is required for T_{FH} cell formation[46] (TABLE 1). Patients with mutations in *IL12RB1*, which encodes IL-12Rβ1, also have fewer circulating $CD4^+CXCR5^+$ T cells[97]. This correlates with reduced numbers of memory B cells, abnormal GCs and low-avidity antibody responses to tetanus. Interestingly, antibody responses following natural infections or vaccination have been shown to be unaffected — or even enhanced — by IL-12Rβ1 deficiency, and the defect in circulating $CD4^+CXCR5^+$ T cells improved with age[97]. Thus, IL-12 signalling may only be required for T_{FH} cell formation and function early in life and for responses to only some antigens (for example, antigens that are derived from non-replicating pathogens)[97].

Taken together, these analyses of monogenic immunodeficiencies showed that *CD40L*, *ICOS*, *SH2D1A*, *STAT3* and possibly *IL12RB1* are required for T_{FH} cell formation, function and/or maintenance. These studies also provided further correlative evidence that circulating $CD4^+CXCR5^+$ T cells are related to bona fide T_{FH} cells, as a deficit in $CD4^+CXCR5^+$ T cells mimicked a deficiency of GCs and presumably lymphoid organ-resident T_{FH} cells. Importantly, the impaired formation and/or function of circulating $CD4^+CXCR5^+$ T cells is associated with compromised humoral immune responses, which highlights the contribution of T_{FH} cells to successful T cell-dependent antibody responses. As some immunodeficiencies are associated with mutations in *TACI* and *BAFFR*[2], and as studies in mice indicate that these receptors modulate T_{FH} cell formation[27,28], it will be interesting to assess the formation and function of circulating T_{FH}-type cells in individuals with these genetic lesions.

Acquired immunodeficiencies. $CD4^+$ T cells are targets of HIV infection and are therefore depleted in infected individuals. Paradoxically, several aspects of humoral immunity are augmented following HIV infection, including polyclonal B cell activation, the appearance of peripheral blood plasmablasts and hypergammaglobulinaemia[98]. To investigate the basis for these contrasting phenomena, T_{FH} cells have recently been assessed in individuals with HIV and in simian immunodeficiency virus (SIV)-infected macaques. A marked accumulation of T_{FH} cells was observed in the lymph nodes both of individuals with chronic HIV infection and of macaques that were chronically infected with SIV[40,99–102]. Interestingly, a substantially higher proportion of T_{FH} cells were specific for HIV compared with effector and memory $CD4^+$ T cell subsets[99,101] (TABLE 2). T_{FH} cells were found to be infected with SIV or HIV[100–102], and human T_{FH} cells were shown to contain substantially more copies of HIV DNA than naive, memory or effector T cell populations[101]. Strikingly, human T_{FH} cells were also better at supporting viral replication and infection of susceptible host cells[101]. Furthermore, T_{FH} cells that were present during chronic infection seemed to be persistently activated[99–101], unlike those in uninfected donors. Thus, ongoing antigenic stimulation of activated $CD4^+$ T cells in HIV-infected individuals might cooperatively drive these cells to a T_{FH} cell fate. Sustained signalling through IL-6R — which is highly expressed on T_{FH} cells derived from both normal donors and those with

Myasthenia gravis
A chronic autoimmune disease that involves the generation of T cell-dependent autoantibodies that are specific for the acetylcholine receptor. These antibodies interfere with the transmission of signals at neuromuscular junctions.

Rheumatoid arthritis
An immunological disorder that is characterized by symmetrical polyarthritis, often progressing to crippling deformation after years of synovitis. It is associated with systemic immune activation and the presence of acute-phase reactants in the peripheral blood, as well as rheumatoid factor (immunoglobulins that are specific for IgG), which forms immune complexes that are deposited in many tissues.

Graves' disease
A type of autoimmune disease, and the most common form of hyperthyroidism in humans. It results from activating antibodies that are specific for the thyroid stimulating hormone receptor (TSHR). In mouse models of Graves' thyroiditis, the disease is induced by immunization with the TSHR.

Hashimoto's thyroiditis
An autoimmune disease in which self-reactive B cells and T cells target the thyroid, resulting in hypothyroidism.

Juvenile dermatomyositis
A chronic, multisystem autoimmune and inflammatory disease involving muscle, skin, blood vessels, the gastrointestinal tract and other organs. Autoantibodies are often detected in these patients, but their specificities have not yet been completely defined.

chronic HIV or SIV infection[16,100,102] — in response to IL-6, which is abundantly produced during HIV infection[98], might also cooperatively drive these cells to a T_{FH} cell fate[24]. As IL-2 can suppress T_{FH} cell differentiation[49], it is possible that the loss of IL-2-producing T cells following HIV infection[102] also contributes to the increased numbers of T_{FH} cells in HIV-infected individuals.

The increase in T_{FH} cell numbers has functional consequences for humoral immunity during HIV infection. Thus, the frequency of T_{FH} cells positively correlates with plasma viraemia, the numbers of GCs and plasma cells, the levels of virus-specific IgG and the onset of hypergammaglogulinaemia[40,99–101] (TABLE 2). Furthermore, antiretroviral therapy has been shown to reduce the frequencies of T_{FH} cells, GC B cells and plasma cells, as well as the numbers of HIV-specific and HIV-infected T_{FH} cells[101]. These findings strongly indicate that the expansion of the T_{FH} cell population and the subsequent dysregulated antibody response might be driven by chronic viral infection.

It is possible that increases in HIV-specific T_{FH} cells would result in protective HIV-specific antibody responses; however, these are usually not broadly neutralizing[98]. It was recently found that although T_{FH} cells from uninfected and HIV-infected individuals show similar phenotypes and express comparable levels of cytokines, the T_{FH} cells from HIV-infected individuals were unable to promote B cell differentiation *in vitro*[40] as a result of heightened PDL1 expression on GC B cells, which inhibits T_{FH} cell function via PD1 (REF. 40). This extrinsically mediated functional impairment of T_{FH} cells might contribute to the inability of HIV-infected individuals to generate neutralizing HIV-specific antibodies. Elucidating mechanisms to promote the effector function of HIV-specific T_{FH} cells — for example, through PD1–PDL1 blockade — might facilitate the development of improved HIV vaccines.

Malignancies. Peripheral T cell lymphomas (PTCLs) are rare haematological malignancies constituting approximately 5–10% of all non-Hodgkin's lymphomas. PTCLs include angioimmunoblastic T cell lymphoma (AITL), follicular T cell lymphoma (FTCL) and PTCL-not otherwise specified (PTCL-NOS)[103,104].

Morphological, phenotypic and molecular characterization of malignant cells in AITL has shown that these cells have many of the features of T_{FH} cells[105–109] (TABLE 2). In a substantial proportion of cases of FTCL, which was previously considered to be a follicular variant of PTCL-NOS, the malignant cells were also found to show T_{FH} cell traits[104,108–111]. Importantly, the assessment of a set of T_{FH} cell-related genes in different malignancies led to the reclassification of several cases of PTCL-NOS as AITL[109], which demonstrates the robustness of molecular diagnoses of PTCLs. These studies established that numerous PTCLs probably arise from normal T_{FH} cells. Some of the cardinal features of AITL and FTCL include B cell activation, follicular hyperplasia, hypergammaglobulinaemia and autoantibody production[103,104,111]. These humoral immunity-associated features of AITL and FTCL probably reflect the dysregulated activity of malignant T_{FH} cells, which parallels the aberrant function of T_{FH} cells in autoimmunity. Thus, it might be possible to treat these features of AITL and FTCL by targeting T_{FH} cell-related molecules — such as PD1, CXCL13 and IL-21 — that are abundantly expressed in these diseases.

It is unknown why T_{FH} cells give rise to several types of PTCLs. Interestingly, T_{FH} cells undergo prompt apoptosis, at least *in vitro*[9], which indicates that the mechanisms that control their survival might be stringently regulated. Thus, the molecular lesions that override T_{FH} cell apoptosis may contribute to the malignant transformation of these cells.

Cytogenetic abnormalities occur infrequently in PTCLs[103,104]. However, mutations in isocitrate dehydrogenase 2 (*IDH2*) and *TET2* were recently detected in approximately 25% and 45% of cases of AITL, respectively, and *TET2* was also shown to be mutated in approximately 30% of cases of PTCL-NOS[112–114]. In AITL, there was no difference in clinical outcome between patients with *IDH2* mutations and those without *IDH2* mutations[113]. However, patients with *TET2* mutations were associated with more advanced disease and poorer clinical outcome than patients without *TET2* mutations[114]. How *TET2* mutations lead to the transformation of T_{FH} cells remains to be determined. Interestingly, a novel mouse model of AITL was recently reported in which 50% of mice that were heterozygous for the *Roquin* (also known as *Rc3h1*) allele developed AITL-like disease[115]. This model might provide important insights into the pathophysiology of AITL in humans and might facilitate preclinical testing of potential therapeutics. It also raises the possibility that heterozygous mutations in *ROQUIN* underlie AITL in some individuals.

Conclusions

Nearly half a century has passed since Jacques Miller first described the fundamental requirement for thymus-derived cells to support antigen-specific antibody production[3]. It has taken a substantial period of time to identify the T cell subset that is responsible for mediating B cell responses but in the past decade there have been important advances in our knowledge and understanding of the molecular and cellular biology as well as the function and regulation of T_{FH} cells. Indeed, we now have an appreciation of how these cells operate during normal immune responses and, perhaps more importantly, how they might underlie immunological diseases as diverse as autoimmunity, immunodeficiency and lymphomas. These rapid discoveries in T_{FH} cell biology should allow us to exploit these cells (as well as their associated receptors, cytokines, chemokines and biochemical pathways) for the development of novel therapeutics to treat these conditions, as well as for the development of next-generation vaccines to induce sustained protection against infection. Hopefully it will not take another 50 years before T_{FH} cell-targeted therapies are available to improve human health.

Isocitrate dehydrogenase 2 (*IDH2*). An enzyme that catalyses the oxidative decarboxylation of isocitrate to 2-oxoglutarate and is a component of the tricarboxylic acid cycle. Mutations in *IDH1* and *IDH3* have been detected in glioma, glioblastomas and acute myeloid leukaemia.

TET2
The *TET2* gene encodes an oxygenase that catalyses the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine to alter the epigenetic status of DNA. It is frequently mutated in human lymphomas.

Roquin
A RING-type ubiquitin ligase that represses the expression of inducible T cell co-stimulator (ICOS), thereby restraining the development and function of T follicular helper cells. A mutation in the *Roquin* (also known as *Rc3h1*) gene results in lupus-like disease in mice.

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