TIMELINE

Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy

Pierre G. Coulie, Benoît J. Van den Eynde, Pierre van der Bruggen and Thierry Boon

Abstract | In this Timeline, we describe the characteristics of tumour antigens that are recognized by spontaneous T cell responses in cancer patients and the paths that led to their identification. We explain on what genetic basis most, but not all, of these antigens are tumour specific: that is, present on tumour cells but not on normal cells. We also discuss how strategies that target these tumour-specific antigens can lead either to tumour-specific or to crossreactive T cell responses, which is an issue that has important safety implications in immunotherapy. These safety issues are even more of a concern for strategies targeting antigens that are not known to induce spontaneous T cell responses in patients.

Cancer immunotherapy that involves the deliberate use of the adaptive immune system to reject tumours or to prevent their recurrence is gaining momentum. Interesting clinical results have been obtained using cancer vaccines, adoptive T cell therapies and antibodies that stimulate the activity of T lymphocytes. Moreover, increasing evidence suggests that adaptive immunity contributes to the long-term clinical benefits of anticancer treatments such as chemotherapy and radiotherapy. At the core of these clinical developments lies the fact that cancer patients can produce T lymphocytes that recognize tumour-specific antigens. The first human tumour-specific antigens that were recognized by T cells were discovered about 20 years ago (FIG. 1 (TIMELINE)). Considering the increasing number of clinical studies that rely on the presence of tumour-specific antigens that are recognized by T cells, it is worth summarizing the key steps that led to their identification, and it is worth describing the genetic processes that result in their presence on tumour cells. A proper understanding of the factors that affect the degree of specificity of the T lymphocyte response against tumour antigens is essential to aid the design of immunotherapy strategies that are not only efficient but also free of adverse side effects.

Identification of mouse antigens

Initial controversy about the existence of tumour rejection antigens. From 1940 to 1960, the study of mouse tumours that were induced with oncogenic viruses showed that the immune system could reject these tumours following the recognition of viral antigens[1]. The first evidence that mouse tumours that were not induced by viruses could also be recognized by the immune system was obtained by Gross and colleagues in 1943 (FIG. 1 (TIMELINE)). They induced tumours in mice through the use of chemical carcinogens and then resected these tumours. These mice were able to reject the same tumour cells on subsequent exposure[2]. Mice that were immunized with lethally irradiated tumour cells were similarly protected. These results were confirmed by other groups[3], and in the 1960s it became widely accepted that mouse tumour cells and therefore possibly human cancer cells could be recognized by the immune system.

In sharp contrast, in 1976, Hewitt[4] reported that a similar analysis carried out with spontaneous tumours that developed in mice failed to produce any evidence of immune control. He concluded that mouse tumour antigens were artefacts that were induced by the chemical treatment used to induce experimental tumours and were therefore unlikely to be present on human tumours.

In the 1970s, we treated a mouse teratocarcinoma cell line *in vitro* with a strong mutagen, and we showed that many cell clones that were derived from the mutated population were incapable of forming progressive tumours when injected into syngeneic mice[5,6]. These 'tum$^-$' variants were rejected by an immune response directed against new antigens that were different for every variant (tum$^-$ antigens). Remarkably, mice that had rejected tum$^-$ variants were also protected against a subsequent injection of the parental tumour cells[7], even though this teratocarcinoma was non-immunogenic, similar to the tumours that were described by Hewitt. We concluded that an efficacious response against the tum$^-$ antigens had an additional effect: it triggered a response against antigens that were present on the original tumour but that were apparently non-immunogenic on their own. In collaboration with Hewitt, we treated cells from spontaneous tumours with mutagens to obtain tum$^-$ variants, and we observed that these variants were also capable of inducing immune protection against the parental tumours[8]. This showed that spontaneous mouse tumours do express tumour antigens, albeit poorly immunogenic ones. We became convinced that human tumours might also be susceptible to immunological treatment and that we should first identify the nature of the rejection antigens that were observed on the mouse tum$^-$ variants and their parental tumour.

Molecular identification of antigens recognized by T lymphocytes on mouse tumours. After the discovery of T lymphocytes in the 1960s[9,10], their essential role in graft rejection and tumour rejection was soon realized[11,12]. In the tum$^-$ system, we observed that adoptive transfer of T cells, which were collected from mice following rejection of a tum$^-$ variant, protected irradiated mice against the growth of the same variant. This clearly indicated the involvement of T lymphocytes in the tum$^-$ phenomenon. Accordingly, for several years, we attempted to obtain specific cytolytic T cells that were directed against tum$^-$ variants; this was unsuccessful. We eventually turned to the P815 mastocytoma cell line, which proved to be remarkably easy to cultivate and to clone because it proliferated in suspension. Tum$^-$ variants were readily obtained for this cell line[13]. Moreover, excellent cytotoxic T lymphocyte (CTL) responses were obtained that showed clear specificity for each tum$^-$ variant[14].

We then benefited from a major advance in the CTL field: microcultures could be derived from a single CTL by repeated stimulations with irradiated target cells in the presence of a T cell growth factor that was later identified as interleukin-2 (IL-2)[15]. These clonal CTL cultures could be expanded to large numbers and could be maintained indefinitely. These stable CTL

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clones that were directed against a single antigen proved to be crucial for a rigorous analysis and dissection of the antigens recognized by T cells on several target cells.

With great help from Cerrotini and his group, who had had a prominent role in these developments, we obtained stable CTL clones that killed the stimulatory tum^{-} variant but not the other tum^{-} variants nor the parental tumour cells[16]. These CTL clones clearly recognized a tum^{-} antigen that was induced by the mutagen treatment. Other CTL clones killed both the tum^{-} and parental cells, evidently recognizing an antigen that was present on the original P815 tumour cells. That these antigens were genuine rejection antigens was shown by the *in vivo* observation that some tumours progressed, then nearly completely regressed, then progressed again. These 'escaping' tumours had invariably lost the antigen that was recognized by one of the CTL clones[17]. This was true not only for tum^{-} antigens but also for antigens that were present on the parental tumour[18]. In fact, these and other studies that were carried out in the early 1980s formally showed the reality of tumour immune surveillance and the occurrence of tumour escape after immune selection[19], which is a process that was recently renamed 'immunoediting' (REF. 20). Although immunoselected tumour variants were resistant to some CTL clones, they were still sensitive to others. A detailed analysis of a panel of such variants led to the conclusion that CTLs recognized several (typically less than ten) distinct antigens on a given tumour[17,18].

The next step was to define the molecular nature of these antigens. The only available tools were the stable CTL clones. The exact molecular nature of the antigens that were recognized by CTLs was unknown at that time. However, the notion that antigens are recognized by T lymphocytes in association with major histocompatibility complex (MHC; human leukocyte antigen (HLA) in humans) molecules had been known for a decade[21]. In 1986, Townsend showed that antiviral CTLs recognized small peptides of eight to ten amino acids, which were derived from a viral protein and presented at the surface of infected cells in association with MHC class I molecules[22]. Soon thereafter, an excellent crystallographic study showed that MHC class I molecules present small peptides in a groove that is located at the surface of the molecule[23]. We now know that these peptides are produced by partial digestion of the parental protein, mainly through the proteasome machinery (BOX 1). These peptides then become associated with the MHC class I molecule and are displayed at the cell surface following a process known as the 'antigen processing pathway' (FIG. 2).

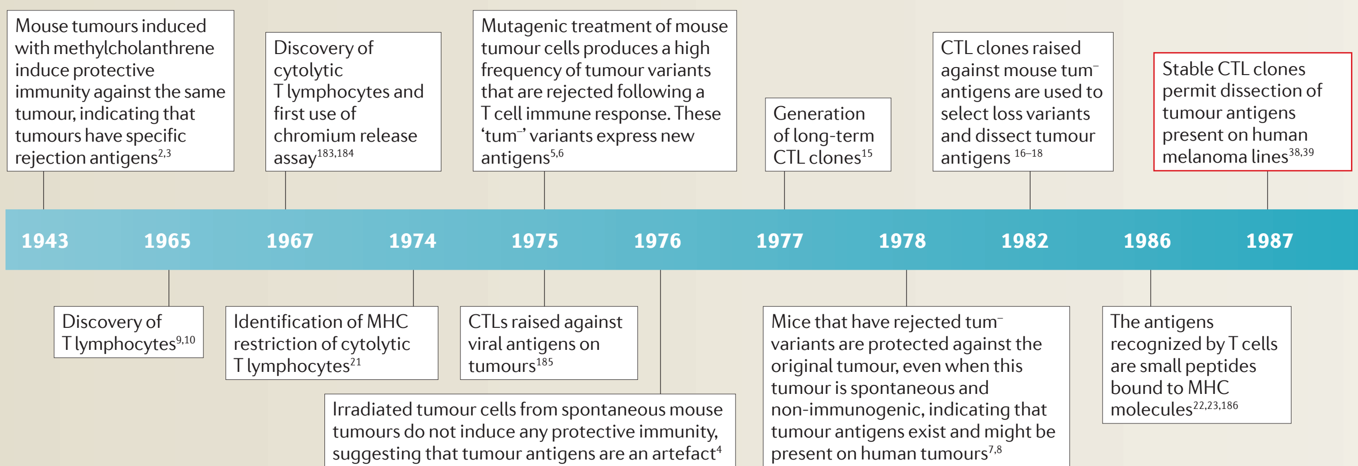
To identify our antigens, we used a genetic approach that aimed to clone the gene encoding the antigen. Once again, the P815 cells were invaluable, as we were able to select a highly transfectable variant named P1.HTR[24]. We transfected P1.HTR with a gene library derived from cells that expressed a given tum^{-} antigen and, using the relevant CTL clone, we identified a transfectant that expressed the antigen. The encoding gene was retrieved from the transfectant and sequenced.

The first gene that encoded a tum^{-} antigen was cloned in 1988 (REF. 25). It encoded a ubiquitous protein of unknown function. Crucially, the coding region contained a mutation that changed one amino acid in the protein. Small peptides that contained the mutated residue were shown to sensitize parental P815 cells to CTL-induced cell death, whereas corresponding wild-type peptides did not[26]. We concluded that the antigen was a complex between the mutated peptide and the presenting MHC class I molecule.

The identification of two other tum^{-} antigens[27,28] indicated that each of them also resulted from a point mutation in a ubiquitously expressed gene. Each mutation created a new antigenic peptide. In some cases, the mutation enabled the peptide to bind to the groove of the presenting MHC molecule. In other cases, the mutation created a new epitope in a peptide that was already bound to MHC, but the wild-type peptide was not recognized by T cells because of central tolerance (FIG. 3a). Even though tum^{-} antigens were artificially induced by mutagen treatment, their identification established the principle that rejection antigens can result from mutations in ubiquitously expressed genes. These results showed for the first time the occurrence of a process of immune surveillance of genome integrity.

We then set out to identify the tumour rejection antigen that was present on the parental mouse tumour P815. This time, the identified antigen, which was named P1A, did not result from a mutation. The antigenic peptide corresponded to the normal

Timeline | **Milestones in the discovery of tumour rejection antigens**



Black boxes refer to discoveries that are related to mouse tumours; red boxes refer to discoveries that are related to human tumours. Observations and discoveries that are related to viral antigens are not included. BAGE, B melanoma antigen; CTAG, cancer/testis antigen; CTL, cytotoxic T lymphocyte; GAGE, G antigen; *MAGEA1*, melanoma antigen family A, 1; MHC, major histocompatibility complex; WT1, Wilms' tumour protein.

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sequence of a gene of unknown function that was named *Trap1a*[29]. The antigen was recognized by T cells on the tumour because of the complete lack of expression of the gene in normal adult tissues, which prevented the establishment of immune tolerance. The only cell types in which the gene was expressed were spermatogonia and placental trophoblasts, which are two cell types that do not express MHC class I molecules on their surface and therefore cannot present the antigen to T cells (FIG. 3a). Tumour antigen P1A is therefore clearly tumour-specific, even though it is not mutated.

Trap1a was expressed in several mouse tumours of different histological types[30,31]. P1A was therefore the first example of a tumour-specific antigen that was shared among distinct tumours. Vaccination of mice against P1A induced protective responses that led to the rejection of P815 tumours, which further validated this type of antigen as a genuine tumour rejection antigen[32]. As expected, vaccination did not induce any deleterious immune response against normal organs, including testes. *Trap1a* shares its characteristic expression profile and location on the X chromosome with human 'cancer-germline' genes (discussed below).

Thus, the work that was carried out with mouse tumours provided a method of identification of tumour antigens, and it identified the two main genetic mechanisms that produce tumour-specific antigens recognized by T cells: namely, gene mutation and activation of genes that are silent in normal tissues.

Identification of human antigens

In the early 1980s, several groups began to stimulate *in vitro* blood[33–36] or tumour-infiltrating lymphocytes[37] that were isolated from cancer patients with autologous tumour cells killed by irradiation. This produced CTLs with higher lytic activity towards autologous tumour cells than control cells. However, such T cell populations always had some lytic activity against normal cells, which made their degree of tumour specificity difficult to establish.

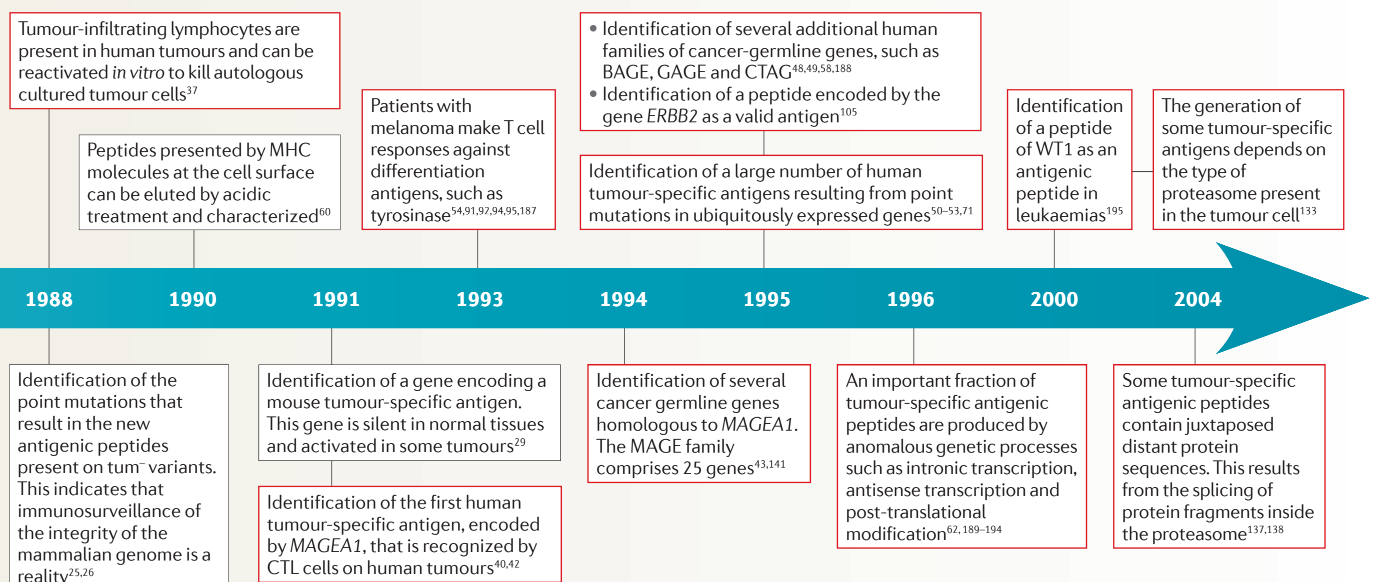
Once again, the production of stable CTL clones was crucial. In the late 1980s, using T cells from a patient with melanoma, we obtained stable anti-melanoma CTL clones that were completely inactive against a wide range of normal cells[38]. Such CTL clones were used in immunoselection experiments to dissect the various antigens that were present on the autologous tumour, and the results indicated the presence of at least six distinct antigens[39].

Genetic approach. To identify these antigens, we used a CTL clone and a DNA library that was derived from the autologous melanoma; this was the same strategy that we had used with the mouse tumour cells. In 1991, this led to the identification of the first human gene that coded for a tumour-specific antigen recognized by T cells[40]. This new gene, which was named melanoma antigen family A, 1 (*MAGEA1*), was expressed in many human tumours of different histological types. No expression was found in normal tissues, with the exception of male germline cells and trophoblastic cells. In humans, as in mice, these two cell types do not produce MHC molecules and therefore cannot present antigens to T cells (FIG. 3a)[41]. Thus, the expression profile of *MAGEA1* was similar to that of mouse *Trap1a*. The antigenic MAGEA1 peptide, which is presented to CTLs by HLA-A1 molecules, was identified by transfecting short DNA fragments, thereby narrowing down the peptide-encoding region until candidate peptides could be synthesized and tested for CTL recognition[42].

MAGEA1 proved to be a member of a large new gene family[43–47]. The MAGE family comprises 25 cancer-germline genes with a similar pattern of expression. Several other cancer-germline gene families were identified in the following years[48,49] — our procedure having been updated by the use of cDNA instead of genomic libraries.

Other antitumour CTLs were shown to recognize peptides that were encoded by mutated genes[50–53]. The first was caused by a mutation in a ubiquitously expressed gene that encoded a protein of unknown function[50]; the second was caused by a mutation in cyclin-dependent kinase 4 (*CDK4*)[51].

In addition, to our surprise, CTLs from patients with melanoma were found to recognize peptides derived from melanocyte-specific proteins. The first identified peptide was derived from tyrosinase, which is present in normal melanocytes and in most melanomas[54]. Interestingly, a peptide of tyrosinase was also the first tumour antigen that was found to be recognized by $CD4^+$ T cells[55].



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Box 1 | Proteasome-generated antigenic peptides

In the steady state, most cells contain the standard proteasome, which has three catalytic subunits called β1, β2 and β5. Under inflammatory conditions, these standard catalytic subunits are replaced by their interferon-inducible counterparts, β1i, β2i and β5i. The resulting 'immunoproteasome' has slightly different catalytic activities. In addition, some normal tissues and tumoural tissues contain intermediate proteasomes that comprise a mixed assortment of catalytic subunits (β1–β2–β5i or β1i–β2–β5i)[132]. Several tumour antigens are produced only by some types of proteasome. For example, the peptide Melan-$A_{26–35}$ is produced only by the standard proteasome[133,135], the peptide $MAGEA3_{114–122}$ is produced by the immunoproteasome and the intermediate proteasomes[134,136], and the peptides $MAGEA3_{271–279}$ and $MAGEC2_{336–344}$ are exclusively produced by one and both of the intermediate proteasomes, respectively[132,136]. Therefore, the expression of a given tumour antigen does not always parallel that of the parental protein and major histocompatibility complex (MHC) class I, but it also depends on the proteasome content of the cell.

Proteasomes can also splice peptide fragments that are located at distance from each other in the parental protein[137,138]. Peptide fragments can be spliced either in the same order or in the reverse order to that in which they occur in the parental protein[63]. Therefore, the sequence of an antigenic peptide cannot always be directly deduced from that of the encoding gene.

In 1995, another approach to identify genes that were preferentially expressed in tumours made use not of CTLs but of antibodies from cancer patients. Using a new methodology, which was developed by Pfreundschuh and colleagues[56] and named serological analysis of recombinant cDNA expression libraries (SEREX), libraries of tumour cDNA that was expressed in bacteria were screened using serum samples from cancer patients, with the expectation that the serum contained antibodies that would bind to surface or intracellular proteins that were specifically or preferentially present in tumours[56]. This led to the identification of several genes, a few of which turned out to be new cancer-germline genes, such as synovial sarcoma X breakpoint 1 (*SSX1*), *SSX2* (REF. 57) and cancer/testis antigen 1A (*CTAG1A*; also known as *LAGE2* and *NYESO1*)[58]. A repertoire of genes encoding proteins that elicit an antibody response in cancer patients can be found in the SEREX database[59].

Biochemical approach. Following pioneering work by the group of Rammensee[60] on the acid elution of antigenic peptides bound to MHC molecules, Hunt and colleagues[61] used this approach to identify a new melanoma antigen. The identified antigenic peptide was derived from the melanocytic differentiation protein GP100 (also known as PMEL and PMEL17)[61]. Relatively few tumour-specific antigens have been identified by this approach, which is technically very demanding. However, biochemical analysis of eluted peptides was essential to show that some peptides have undergone post-translational modifications[62,63]. For example, the deamidation of an asparagine into an aspartic acid residue was observed for a tyrosinase peptide, and this change was essential for the efficient T cell recognition of this particular peptide[62].

Reverse immunology. The numerous genes of the cancer-germline families are expected to be sources of a vast number of antigenic peptides that bind to a wide range of HLA molecules. Another possible source of tumour-specific antigens is genes that are mutated in many tumours, such as *KRAS*, *TP53* or the *BCR–ABL1* fusion gene. Thus, there is a need to identify new antigenic peptides on the basis of gene sequences and in the absence of a T cell that is directed against these antigens.

To achieve this, the first step is to identify candidate peptides that bind to a given HLA molecule. This is carried out by using computer-generated algorithms[64,65] that select peptides within a protein that are likely to bind to an HLA molecule. The resulting candidate peptides of about nine amino acids are synthesized, and their binding to HLA is tested *in vitro*. Cells pulsed with peptides that most efficiently bind to HLA are used to stimulate T lymphocytes in order to derive populations or, preferably, clones of T cells that recognize cells expressing the appropriate HLA pulsed with the peptide[66]. It is essential to verify that these CTLs also recognize unpulsed tumour cells that express the protein from which the peptide is derived, because many peptides against which T cells can be raised are not produced by the antigen-processing machinery (FIG. 2). In a related approach, T lymphocytes are stimulated with dendritic cells that are loaded with a recombinant protein or that are infected with a recombinant virus containing a tumour-specific DNA sequence[67,68].

Several antigenic peptides that are recognized by $CD4^+$ or $CD8^+$ T lymphocytes have been identified using these approaches[66,69–72].

The peptides that are expressed by tumour cells are listed in a database, which is regularly updated[73].

Human antigens: classes

Antigens of high tumoural specificity. Three types of tumour antigens have the potential to elicit immune responses that are strictly tumour specific: viral antigens, antigens that result from a mutation or a rearrangement of a gene-coding sequence and antigens that are encoded by cancer-germline genes (FIG. 3a).

Glossary

Adoptive transfer
In cancer immunotherapy, the infusion into patients of autologous antitumour T cells that have been amplified *in vitro*. The lymphocytes can also be transduced with retroviral expression vectors in order to express a given T cell receptor or other gene products.

Anergy
Hyporesponsiveness or unresponsiveness of T lymphocytes after recognition of their antigen.

Central tolerance
The deletion or inactivation of immature autoreactive B cells and T cells of the primary lymphoid organs: the bone marrow (B cells) and the thymus (T cells). The remaining mature autoreactive B cells and T cells are dealt with by the mechanisms of peripheral tolerance.

Deamidation
The removal of an amide group. In *N*-glycosylated proteins, deglycosylation of an asparagine by the peptide *N*-glycanase generates an aspartate by deamidation. This can result in an antigenic peptide.

Epitope
The molecular configuration of a peptide that is recognized by a T cell receptor or by an antibody.

Lymphoablation
The elimination of lymphocytes by a combination of lymphocyte-depleting chemotherapy (cyclophosphamide and fludarabine) and total body irradiation.

Serological analysis of recombinant cDNA expression libraries
(SEREX). A procedure whereby proteins from human tumours are screened for recognition by autologous serum.

Thymic epithelial cells
The thymus contains developing T lymphocytes and a stroma that consists of epithelial cells and dendritic cells. Epithelial cells of the thymic medulla, in which the transcription factor autoimmune regulator controls the expression of peripheral tissue antigens, contribute to the induction of central tolerance for T lymphocytes.

Uveitis
Inflammation of the uvea, which is the middle layer of the eye, between the retina and the sclera.

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A viral origin has now been shown for an important subset of human tumours, including cervical carcinoma, hepatocarcinoma, nasopharyngeal carcinoma and adult T cell leukaemia. Viral antigens that are useful for cancer prevention and treatment have been reviewed elsewhere[74–77] and will not be discussed further here.

Mutated genes greatly contribute to the immunogenicity of human tumours. Gene mutations produce new antigenic peptides by changing one amino acid, by altering the phase of the reading frame or by extending the coding sequence beyond the normal stop codon. In cancer patients, about one-half of the tumour-specific antigens that are recognized by spontaneous T cell responses are encoded by mutated genes — the other half being encoded by cancer-germline genes[73]. In some patients, the majority of the tumour-specific T cells recognize mutated antigens[78]. The contribution of these antigens to tumour immunogenicity is expected to vary according to the mutation rate: the contribution is expected to be higher in lung carcinomas that arise in tobacco smokers, in melanomas that are due to mutations induced by ultraviolet radiation and in the 15% of colorectal carcinomas that have hypermutated DNA owing to defects in the DNA mismatch repair pathway[79].

Most of the mutated antigens are caused by passenger mutations. However, several mutations that were discovered using tumour-specific T cells proved to be oncogenic. A mutation in *CDK4* results in the loss of binding of CDK4 to the inhibitor INK4A (also known as CDKN2A), and this disrupts the cell cycle regulation that is exerted by INK4A, which is a known tumour suppressor[51]. A mutation in the gene caspase 8 (*CASP8*) modifies the stop codon, and the resulting lengthened CASP8 protein has a reduced ability to trigger apoptosis[80]. Another oncogenic process involves chromosomal translocations. In this case, the breakpoints can code for chimeric peptides that can be processed in the tumour cells and presented on HLA molecules. Such peptides from BCR–ABL or ETV6–AML1 (also known as RUNX1) fusion proteins are recognized by T cells that respond to leukaemic cells that contain these fusion proteins[71,81–84].

To date, mutated antigens have not been used for therapeutic cancer vaccines because their diversity is such that no vaccine can be devised that is applicable to many patients. One interesting approach that might change this state of affairs involves extracting patients' dendritic cells, transfecting the cells with total mRNA from their tumour and then reinfusing the cells into the patient[85,86].

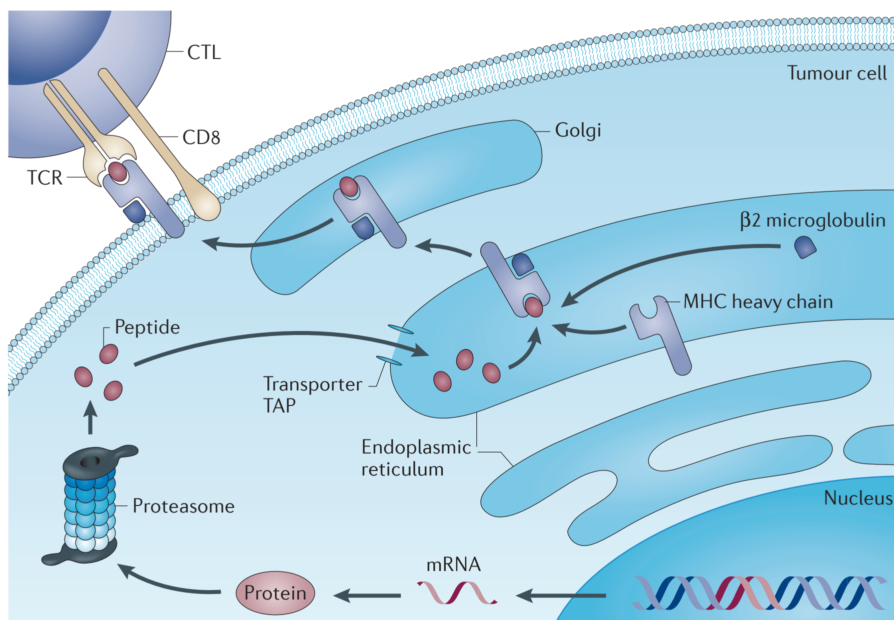

Figure 2 | **Processing of tumour antigens that are recognized by CD8⁺ T cells.** Most antigenic peptides that are presented to cytotoxic T lymphocytes (CTLs) by major histocompatibility complex (MHC) class I molecules are produced through the degradation of intracellular proteins by the proteasome, which is a large proteolytic complex that is mainly located in the cytosol. A dedicated transporter called transporter associated with antigen processing (TAP) transports the resulting peptides into the endoplasmic reticulum, where they associate with newly synthesized MHC class I molecules and migrate through the Golgi to the cell surface. A few antigenic peptides are produced by other proteases[139,140]. TCR, T cell receptor.

Cancer-germline genes are an important source of tumour-specific antigens. The MAGE gene family comprises 25 genes, which are located on the X chromosome in three gene clusters, *MAGEA*, *MAGEB* and *MAGEC*. Other cancer-germline gene families that are located on the X chromosome are G antigen (GAGE), CTAG and SSX. Altogether, more than 60 cancer-germline genes have been identified (TABLE 1). These genes show a very low degree of conservation among species. Despite recent progress, the functions of most of these genes remain unclear.

Cancer-germline genes are expressed in a substantial fraction of a large range of tumours (TABLE 2). For this reason, the antigens that they encode can be used as targets for immunotherapy that involves vaccination or adoptive T cell transfer. However, each candidate patient must be assessed for the expression of these genes in their tumour. There is a moderate positive correlation between the expressions of different cancer germline genes in the same tumours. The mechanism that leads to the activation of these genes in tumour cells involves the demethylation of their promoter, which is methylated in all normal cells except in germline cells[87–89] (FIG. 3a). This demethylation seems to be more frequent in advanced tumours, which is in line with the increasingly aberrant pattern of DNA methylation that occurs during tumour progression. Interestingly, 5′-aza-2′-deoxycytidine, which is an agent that promotes demethylation, could be tested in a clinical context to see whether it increases the expression of cancer-germline genes in tumours[87].

A thorough PCR analysis of the expression of cancer-germline genes in a wide array of normal tissues did not show any expression except in male-germline cells, hence their name. In female-germline cells MAGE protein was detected by immunohistology[90]. Besides germline cells, a low level of expression of MAGEA12 in brain cells has recently been reported[114]. Trophoblastic cells express many cancer-germline genes. As stated above, the expression of cancer-germline genes in trophoblastic cells and male germ cells is inconsequential to the treatment of cancer patients, because in the healthy state these cells are devoid of HLA class I molecules and therefore cannot present antigens to T cells. For this reason, we feel that it is inappropriate to refer to the antigens that are encoded by cancer-germline genes as 'cancer-testis antigens'. This name misleadingly suggests that testicular autoimmunity is a concern when immunizing against

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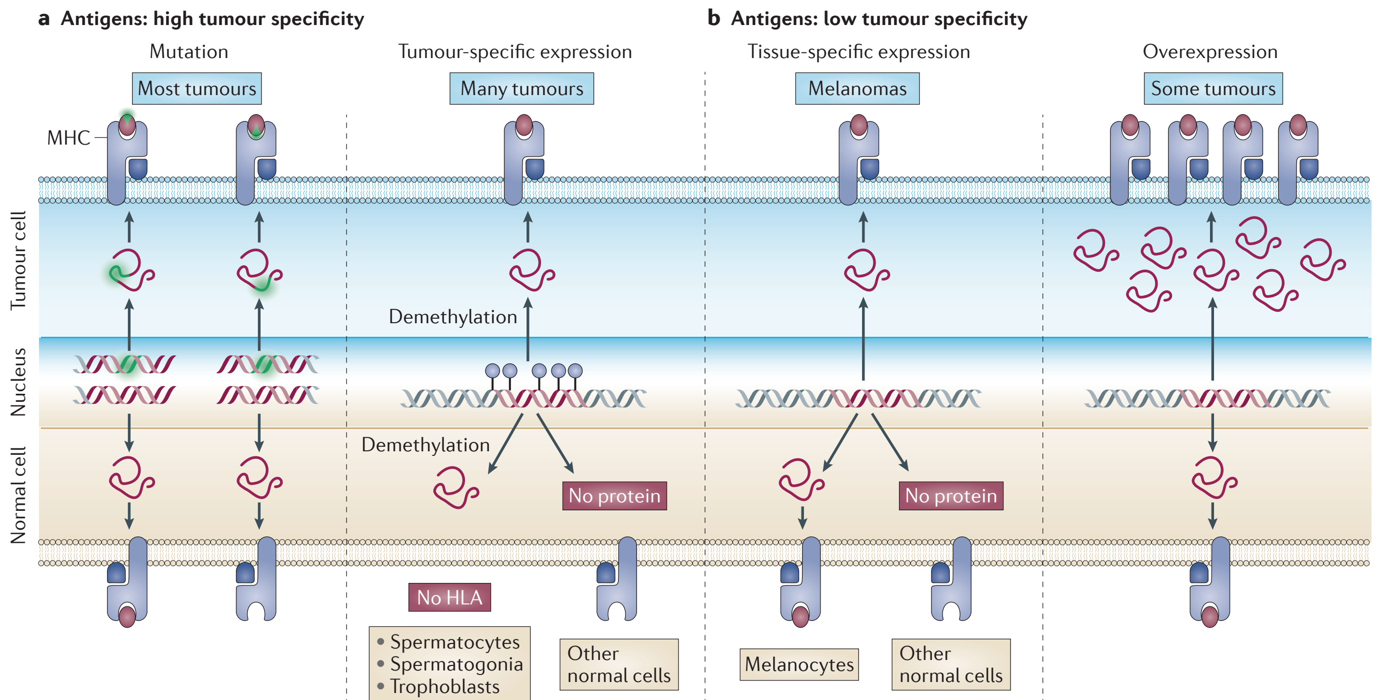

Figure 3 | **Classes of human tumour antigens that are recognized by T lymphocytes. a** | Tumour antigens with high tumour specificity and the mechanisms that lead to their generation are shown. Point mutations can modify a peptide that already binds to the major histocompatibility complex (MHC; known as the human leukocyte antigen (HLA) system in humans) or can enable a non-binding peptide to bind to the MHC. Cancer-germline genes are selectively expressed in tumours and germline cells because of DNA demethylation. Their antigens are not present on germline cells because of the lack of HLA molecules. **b** | Antigens of low tumour specificity and the mechanisms that produce them are shown. A melanocyte-specific gene is used as an example of tissue-specific gene expression. Overexpression of particular proteins, such as ERBB2, can also trigger an antitumour immune response. Only HLA class I molecules are represented, but the genetic processes shown can also lead to the presence on tumour cells of antigenic peptides that are presented by HLA class II molecules to $CD4^+$ T cells.

these antigens. We favour the terms 'cancer-germline' for the genes and 'MAGE-type' for the antigens.

Antigens of low tumoural specificity. This group includes differentiation antigens and antigens derived from proteins that are overexpressed in tumours (FIG. 3b). Differentiation antigens are expressed only in the tumour cells and in the normal tissue of origin.

Spontaneous T cell responses to differentiation antigens have been well documented only in patients with melanoma, with T cells recognizing tumour cells and normal melanocytes. The main antigenic peptides that are recognized by such CTLs are derived from tyrosinase[54,91], Melan-A (also known as MART1)[92–94] and GP100 (REFS 61,95,96). It is not known why tolerance is incomplete against these melanocytic antigens.

One melanocyte differentiation antigen — namely, a Melan-A peptide (amino acids 26–35; Melan-$A_{26–35}$) that is presented by HLA-A2 — deserves a comment. T cells against Melan-$A_{26–35}$ with a naive phenotype were found in non-cancerous HLA-A2-expressing individuals at remarkably high frequencies, about 1 per 10^3 of the blood $CD8^+$ T cells, which is 100-fold to 1,000-fold higher than frequencies of naive $CD8^+$ T cells against other antigenic peptides[97–100]. This frequency of naive T cells is due to an exceptionally high level of positive selection in the thymus by ligands that have not been identified[101]. Patients with melanoma spontaneously mount T cell responses against the Melan-$A_{26–35}$ peptide, and the resulting memory T cells can amount to 1–3% of the $CD8^+$ T cells[102].

The prostate expresses differentiation proteins, such as the prostate-specific antigen and prostatic acid phosphatase, which are absent from other tissues. Reverse immunology has been used to identify antigenic peptides within these proteins that could be used for therapeutic immunization of patients with prostate cancer[103,104].

Overexpression of proteins in tumours may provide an opportunity for a specific T cell response. This is because a threshold level of antigen is required for recognition by T cells. If tumour cells present an amount of peptide–HLA complexes that is above the threshold and if normal cells do not, a specific antitumoural T cell response could occur. However, such tumoural overexpression is difficult to rigorously show. Quantitative reverse transcription PCR of tumoural and normal tissues can provide a useful indication of appropriate overexpression. However, this approach provides average values for expression within tissues, and it is therefore difficult to rule out that a high expression occurs in a small subset of cells from normal tissues. Immunohistochemical analysis can offer complementary information but is not easily amenable to quantification.

The oncogene and growth factor receptor ERBB2 (also known as HER2 and NEU) is overexpressed in many epithelial tumours, including ovarian and breast carcinomas, owing to increased transcription and to gene amplification. Several antigenic peptides have been defined[73,105]. Vaccination with these peptides in a therapeutic and adjuvant setting does not seem to produce harmful side effects in patients with breast cancer. Treatment of patients with breast cancer with trastuzumab (Herceptin; Genentech), which is an antibody that blocks ERBB2, might also trigger immune responses that target the receptor.

The gene that encodes the transcription factor Wilms' tumour protein (WT1) is expressed at a 10-fold to 1,000-fold higher

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level in leukaemic cells than in normal cells[106,107]. After birth, it is mainly expressed in kidney podocytes and CD34+ haematopoietic stem cells. Patients with leukaemia received an allogenic haematopoietic cell transplant, followed by an infusion of donor-derived CTL clones that recognized peptide $WT1_{126-134}$ on HLA-A2. A decrease in the number of leukaemic cells was observed, without evidence of autoimmune toxicity[108].

An interesting case of protein overexpression on most adenocarcinomas is mucin 1 (MUC1), which also presents tumour-specific glycoforms that bear novel T cell and B cell epitopes[109].

Tumour specificity and potential side effects of immunotherapy. Everyone has an intuitive idea of the advantages of tumour-specific antigens as targets for cancer immunotherapy mediated by T lymphocytes. First, T cell responses that are elicited against such antigens in cancer patients ought to leave normal tissues completely unharmed. Second, our natural tolerance mechanisms should not prevent or repress these responses. However, we should bear in mind that some procedures that are used to generate responder T cells against tumour-specific antigens do generate T cells that crossreact with other antigens that are present on normal cells. This can result in harmful side effects, as described below.

The tumour antigens of high specificity occur because of a mutation in the encoding gene or because the gene has a tumour-specific pattern of expression. It is important to realize that, even when a remarkably tumour-specific pattern of expression is observed, it is never possible to completely exclude that a small subgroup of cells located in one organ will eventually show a substantial degree of expression — no matter how exhaustive the gene expression studies are.

Regarding natural tolerance, one should bear in mind that genes that are not expressed in any normal adult tissue nevertheless have some expression in thymic epithelial cells, which is promoted by transcription factors such as autoimmune regulator (AIRE)[110]. This might result in the elimination of the high-affinity T cell clones. Such expression has been observed for MAGE genes[111]. However, it is clear that this limited thymic expression does not prevent patients from mounting spontaneous MAGE-specific T cell responses and from responding to vaccines. A similar thymic expression has been observed for P1A-encoding gene *Trap1a*, but compared with the P1A-specific responses in normal mice, we found that the P1A-specific responses in mice in which the gene had been deleted showed only a small increase[112]. Accordingly, the often-encountered statement that MAGE-type antigens are 'self antigens' is rather deceptive. They are 'self' in the sense of being encoded by normal genes but not in the sense of being present in the normal organism. The belief that the term self antigens implies that the T cell response against these antigens is strongly reduced by tolerance seems to be erroneous.

The only completely reliable way to ascertain the safety of an antigen lies in clinical experimentation by repeatedly showing that a vaccine can cause the elimination of tumour masses without having noticeable adverse effects on normal tissues. A large number of patients have received peptide, protein or recombinant virus vaccines that contain MAGEA1, MAGEA3 and CTAG1A without any significant adverse effect, even in the minority of patients in which clinical responses and anti-vaccine CTL responses have been observed. This contrasts sharply with therapy against CTL-associated 4 (CTLA4), which is remarkably effective in a substantial fraction of cancer patients but which frequently causes severe autoimmunity[113].

In the absence of repeated clinical experiments, the best option is to use a tumour-specific antigenic peptide against which at least one spontaneous T cell response in a cancer patient has been documented in the absence of harmful side effects. We now believe that the usual procedures that involve the *in vitro* stimulation of T cells of cancer patients with the autologous tumour cell line do not activate naive cells but only activate memory T cells that result from a previous spontaneous response against the tumour. The target antigens that are identified by this approach are therefore relatively safe.

By contrast, the reverse immunology approach involves the selection of antigenic peptides that are good HLA binders and that can stimulate naive T cells *in vitro*.

Table 1 | **Cancer-germline genes that encode an antigenic peptide**

Gene family or single gene (number of genes*)	Gene locus (number of genes*)	Chromosomal location	Refs: Proposed functions	Refs: First identified antigenic peptide
MAGE‡	*MAGEA*[43,141] (12)	Xq28	142–149	42,150–152
	MAGEB[44–46] (9)	Xp21.3		
	MAGEC[46,47] (4)	Xq27.2	142	153
BAGE[154,155] (5)		13 and 21		48
GAGE	*GAGE*[156] (9)	Xp11.23	157	49
	XAGE[158] (10)	Xp11.22		159
CTAG‡ (REF. 161) (2)		Xq28		160
SSX[162] (9)		Xq28	162–164	165
Cyclin A[166] (2)		13q13.3 and 4q27	167	168
KKLC1 (REF. 169) (1)		Xq23		169
KMHN1 (also known as *CCDC110*)[171] (1)		4q35.1	170	171
SAGE[172] (1)		Xq26.3		173
SPA17 (REF. 174) (1)		11q24.2	175	176

BAGE, B melanoma antigen; CTAG, cancer/testis antigen; GAGE, G antigen; *KKLC1*, Kita-kyushu lung cancer antigen 1; MAGE, melanoma antigen; *SAGE*, sarcoma antigen; *SPA17*, sperm autoantigenic protein 17; SSX, synovial sarcoma X; *XAGE*, X antigen. *Pseudogenes are not included. ‡MAGE[177,178] and CTAG[179] gene families share homology with genes that are expressed in many, if not all, normal tissues but do not encode any of the known antigenic peptides that are recognized by tumour-specific T cells.

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Table 2 | **Tumour expression profile of cancer-germline genes***

Genes	Metastatic melanoma	Lung carcinoma	Colorectal carcinoma	Breast carcinoma	Prostate carcinoma	Refs
MAGEA1	46	46	0	19	18	69
MAGEA3	74	47	17	13	18	69
MAGEA4	25	51	11	6	0	69
MAGEA12	62	30	11	13	5	69
MAGEC2	43	11	0	15	1 of 10‡	46
BAGE1	31	10	0	12	0	69
GAGE1	41	38	0	10	15	69
XAGE1B	43	2 of 3‡	4 of 12‡			180
CTAG2	33	41	0	23	27	69
CTAG1	35	27	0	23	27	69
SSX2	50	0	26	19	25	181,182

BAGE1, B melanoma antigen 1; *CTAG*, cancer/testis antigen (*CTAG2* is also known as *LAGE1*; *CTAG1* is also known as *NYESO1*); *GAGE1*, G antigen 1; *MAGEA*, melanoma antigen family A; *SSX2*, synovial sarcoma X breakpoint 2; *XAGE1B*, X antigen family member 1B. *Percentage of tumours that express the gene. ‡The numbers of tested tumours are low, and the real numbers are shown.

This approach is effective, but the use of these antigens requires more caution because it is not clear whether a response against these antigens can occur safely in humans.

A recent report provides strong evidence not only that caution must be exerted but also that, by distancing oneself from the principles described above, one incurs a substantial risk of adverse side effects[114]. Following adoptive transfer with T cells transfected with a receptor directed against a MAGEA3 peptide presented by HLA-A2, which was identified by reverse immunology, severe brain toxicity was induced. These side effects were thought to have occurred because *MAGEA12,* which encodes a peptide that differs only by one amino acid from MAGEA3, is expressed at low levels in the white matter of the brain. Why was a major problem encountered in this case, whereas none was observed after MAGEA3 protein vaccination in humans? We suspect that three factors may have been of importance. First and foremost, the T cell receptor that was transfected into the T cells of patients was obtained in an HLA-A2 transgenic mouse that was immunized against MAGEA3. The mouse homologues are considerably different from the human MAGE genes. Accordingly, the subtle natural tolerance mechanisms that could have prevented the development of anti-MAGEA3 T cells crossreacting with MAGEA12 in humans could not operate in mice. Second, the T cell receptor was modified *in vitro* to increase its affinity for the peptide, thereby further bypassing any natural tolerance mechanism. Third, these patients had undergone lymphoablation and received, together with large doses of IL-2, an enormous number of T cells that were transduced with the receptor, and this number exceeded the total number of $CD8^+$ T cells that were normally present in the body. One month after the transfer, the number of T cells that bound to MAGEA3 constituted about one-half of all peripheral blood mononuclear cells (PBMCs). By comparison, the highest number of T cells that recognized MAGEA3 that we ever observed in a vaccinated patient was 1 in 2,000 T cells[98].

Another recent report describes striking cardiac toxicity after the use of a modified anti-MAGEA3 T cell receptor that cross-reacts with a cardiac protein that is totally unrelated to MAGE proteins[115].

The probability of attack of normal tissues by the antitumoural T cells clearly increases for antigens of low tumoural specificity, such as differentiation antigens or overexpressed antigens, as experienced with carcinoembryonic antigen[116] and carbonic anhydrase IX[117]. Nevertheless, most patients with melanoma make spontaneous T cell responses against melanocyte differentiation antigens without signs of autoimmunity. Some patients have vitiligo (white skin patches that are due to the disappearance of melanocytes), but this is not a serious side effect and it has a favourable prognostic value[118].

Adoptive transfer of expanded populations of tumour-infiltrating lymphocytes from patients with melanoma has shown clinical efficacy in a significant proportion of the patients, both in a therapeutic setting and in an adjuvant setting[37,119]. In several trials in which the patients received $5–50 \times 10^9$ T cells, no sign of harmful effects on normal tissues was observed. However, in a patient who was lymphodepleted before the transfer of 140×10^9 T cells, uveitis was observed, which was presumably related to the presence of melanocytes in the choroid layer of the retina[120].

Perspectives for immunotherapy

The presence of several tumour-specific antigens on every tumour provides a rationale for three approaches to cancer immunotherapy that are currently under development: namely, the use of T cell stimulatory antibodies, adoptive transfer of antitumoural T cells and vaccination.

Antibodies against CTLA4, programmed cell death protein 1 (PD1) and programmed cell death ligand 1 (PDL1), which boost overall T cell activity, have shown clinical efficacy in cancer patients[121–123]. However, harmful autoimmune side effects have been observed in many patients. The damage to normal tissues is likely to be due to a release of the peripheral tolerance relative to antigens expressed by these tissues. The relevant T cells, which are yet to be identified, are probably completely unrelated to those that target the tumour antigens.

As stated above, the adoptive transfer of autologous antitumoural T cells that have been expanded *in vitro* has shown some efficacy in the absence of deleterious side effects[119,124,125]. We suggest that the use of expanded autologous CTLs directed against MAGE-type antigens, or the use of their unmodified T cell receptors, could make it possible to inject large numbers of T cells without harmful side effects.

To date, the therapeutic vaccination procedures that have been applied to tumour-bearing patients have shown little success: clinical responses occur in only 5–10% of the patients, whereas 80% of the patients do not show any sign of tumour regression. Our initial view of the process occurring in the responding patients was that the CTLs that were activated by the vaccine would have to move in large numbers to the tumour sites and directly destroy the tumour cells. We now believe that this view is erroneous. First, large numbers of T cells directed against various tumour-specific antigens are already present in the blood and the tumour before vaccination[126,127]. Second, in most responding patients, very low numbers of anti-vaccine T cells are observed in the blood and in the tumours. The frequency of these T cells in regressing tumours can be as low as 1 per 10^6 tumour cells[127]. Third, in the regressing tumour, we observe a considerable expansion of pre-existing and new T cells against various

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antigens expressed by the tumour, so that the frequency of these antitumoural T cells can reach 1 per 100 tumour cells[127,128].

On the basis of these and other findings, it is now clear that most patients with melanoma produce a spontaneous T cell response against their tumour[126,129]. In many patients, this attempt at eliminating the tumour evidently fails and a large number of antitumour T cells remain in an inactive state. Inside the tumours, this anergy seems to be reinforced by an immunosuppressive environment. For the vaccinated patients who show tumour regression, we propose the following sequence of events: a small number of active anti-vaccine T cells penetrate a tumour and attack some tumour cells. As a result of this interaction, these CTLs are re-stimulated and produce cytokines that focally reverse the local immunosuppressive environment around them to create an immunostimulatory environment. This reawakens many of the inactive antitumour T cells that are already present in the tumours, and it stimulates new naive antitumour T cells. The mobilization of all these T cells that are directed against tumour antigens other than the vaccine antigen provides the numbers that are required to reject the tumour. We think that these active T cells can then migrate to other tumour sites and trigger a response there. To summarize, vaccination produces a spark that reactivates the anergic memory T cells that recognize tumour antigens[130].

This revised view has several consequences. First, even if only a fraction of the tumour cells expresses the vaccine antigen, this should not prevent a rejection response because the sparking effect results in the activation of CTLs that are directed against other antigens. Second, as the response is directed against multiple antigens, antigen-loss variants for one antigen should not escape the T cell response that is triggered by the vaccination. This does not apply to variants that have lost all HLA expression, but natural killer cells should destroy these tumour cells.

A third consequence is the possibility that the immunosuppressive nature of the tumour microenvironment is a key limitation to cancer therapeutic vaccination. This could prevent the sparking effects of anti-vaccine T lymphocytes[131]. The minority of patients who respond to anticancer vaccination might not be those who made a higher T cell response to the vaccine antigen, but rather they might be those who have a low degree of immunosuppression at their tumour site. Research efforts are now devoted to understanding these immunosuppressive processes. Local and systemic treatments with non-toxic inhibitors of immunosuppression are being developed and could complement vaccination or adoptive transfer.

Pierre G. Coulie, Benoît J. Van den Eynde, Pierre van der Bruggen and Thierry Boon are at the de Duve Institute and the Université catholique de Louvain, B-1200 Brussels, Belgium.

Benoît J. Van den Eynde, Pierre van der Bruggen and Thierry Boon are also at the Ludwig Institute for Cancer Research, B-1200 Brussels, Belgium.

Pierre G. Coulie, Benoît J. Van den Eynde and Pierre van der Bruggen are also at WELBIO (Walloon Excellence in Lifesciences and Biotechnology), B-1200 Brussels, Belgium.

Correspondence to P.G.C.
e-mail: pierre.coulie@uclouvain.be

doi:10.1038/nrc3670

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Acknowledgements
The authors thank S. Depelchin and J. Klein for editorial work, and D. Godelaine and A. Van Pel for reviewing the manuscript.

Competing interests statement
The authors declare competing interests: see Web version for details.

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