SPINNING MOLECULAR IMMUNOLOGY INTO SUCCESSFUL IMMUNOTHERAPY

Drew M. Pardoll

Until recently, immunotherapies have been of limited success, particularly against cancer. However, recent insights into the cells, molecules and signalling pathways that regulate immune responsiveness are providing new approaches for immunotherapy. In this article, I review some of the most promising molecular and cellular targets for immunotherapy and discuss approaches that use these targets to amplify immune responses and potentially break antigen-specific tolerance. These strategies provide a blueprint for the development of successful immunotherapy over the next decade.

VACCINES

CYTOKINE
A protein released by one cell that affects the physiology of other cells in the vicinity in a particular fashion through binding to a specific receptor.

PROVIRUS
The latent form of a virus that exists within a cell without harming the cell or producing new virions.

Sidney Kimmel Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, USA.
e-mail: dmpardol@jhmi.edu
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One of the most successful and widely used types of medical intervention in human history is immunoprophylaxis. Preventive vaccines have virtually eliminated some of the worst diseases, such as polio and smallpox. By contrast, immunotherapy of established chronic infections, as well as cancer, has yet to be of widespread clinical use. The striking differences in success between immunoprophylaxis and immunotherapy are due to the fact that, to become established successfully within the host, pathogens and cancer cells have developed mechanisms to avoid recognition and elimination by the immune system. These mechanisms of immune evasion are understood best for tumours and viruses, which often use common strategies. The down-modulation of components of the system of antigen processing and presentation to T cells is one of the most well-defined mechanisms for both viruses and tumours[1–5]. The production of CYTOKINES that inhibit or divert productive effector responses and the induction of antigen-specific tolerance through normal pathways of self-tolerance generation are additional mechanisms by which tumours and some viruses can avoid recognition by the immune system[6–10].

Although they present significant challenges to successful immunotherapy, immune-evasion mechanisms and the induction of tolerance are relative, rather than absolute, barriers. Essentially, all tumours express unique antigens (the result of genetic alterations), tissue-specific antigens and/or upregulated self-antigens (the result of epigenetic effects) that can be recognized by T cells (BOX 1). Whereas prophylactic vaccines work by the induction of long-lived neutralizing antibody responses, the successful immunotherapy of established cancer or pathogenic infections will probably depend on diverse effector pathways that are regulated by both $CD4^+$ and $CD8^+$ T cells[11,12].

The principles and strategies for the development of immunotherapy discussed in this review can be applied to both cancer and chronic viral infections. Indeed, the immunobiology of these two types of disease intersects for several reasons. Chronic viral infections are maintained in the form of integrated or episomal PROVIRUSES within viable cells. The recognition and elimination of these infected cells seems to involve similar mechanisms to the recognition and elimination of tumour cells. In the case of viral infections, viral antigens expressed during the chronic carrier state are ideal targets for antigen-specific immunotherapy. The most significant link between cancer and viral immunotherapy comes from the fact that several common human cancers in immunocompetent individuals are induced by chronic viral infection, such as with human papillomavirus

Box 1 | **What makes a good tumour antigen?**

Antigen-based immunotherapy of cancer is crucially dependent on the identification of tumour-rejection antigens. The largest number of immunologically defined tumour antigens has been discovered by the serological screening of phage-display libraries from tumours using the serum of cancer patients[131,132]. Although this approach identifies hundreds of antigens recognized by antibodies circulating in cancer patients, it does not provide insights into which of these antigens could generate effective antitumour immunity. The screening of tumour libraries using tumour-reactive T cells from cancer patients is considered generally to be a superior approach to identify tumour-rejection antigens, but this approach requires established T-cell lines and clones[77,78]. Selective expression by the tumour relative to normal tissues is often considered to be an important feature of a tumour antigen; however, tissue-specific differentiation antigens are also potential targets in tumours of dispensable tissues, such as melanoma and prostate cancer. So, the quality of any candidate tumour antigen as an immunotherapeutic target depends on several interrelated factors, including its tissue distribution, the T-cell repertoire and any pre-existing tolerance. Tumour antigens can be divided into four categories.

Unique tumour-specific antigens that are the products of mutation
Many of these mutations are physiologically relevant to the cancer (for example, inactivating mutations in cyclin-dependent kinase 4 (CDK4) or activating mutations in Ras)[133–135]. Mutated tumour antigens might generate the most potent antitumour immunity of all but they are patient specific. Nonetheless, clinical trials using individually constructed idiotypic vaccines for B-cell lymphoma have shown pronounced antitumour responses in a significant number of patients[136].

Viral antigens in virus-associated cancers
Viral antigens, such as human papilloma virus 16 (HPV-16) E6 and E7, are highly promising antigens that are shared by a large proportion of the tumours that express these viruses[13–16]. Other important viruses, such as hepatitis B and C viruses, are probably better targets for the treatment of premalignant disease[17–20].

Tissue-specific differentiation antigens
The most commonly identified melanoma antigens are not tumour-specific but rather, melanocyte specific. Examples include tyrosinase, tyrosinase-related protein 1 (TRP1), TRP2, gp100 and MART1/melan-A[77,78]. The observed correlation between the induction of vitiligo (skin depigmentation owing to immune attack on melanocytes) and antimelanoma responses in both mouse and human immunotherapy trials indicates that, under some circumstances, tolerance to tissue-specific differentiation antigens might be broken with therapeutically beneficial outcomes[117,137,138].

Tumour-selective antigens
Many genes expressed at very low (or undetectable) levels in normal tissues are upregulated in tumours owing to epigenetic effects. The differential expression of antigens in tumours provides potential therapeutic windows, even when the expression is not completely tumour-specific. A large number of tumour antigens in this category have been identified as targets of T-cell responses in patients with melanoma and other types of tumour[77,139].

(HPV), Epstein–Barr virus and hepatitis B and C viruses[13–23]. Tumours that result from infection with these viruses contain proviral elements. Each of these viruses and the antigens they encode are excellent targets for specific immunotherapy. Furthermore, the ability to diagnose chronic viral carrier states before the advent of a full-blown neoplastic process provides opportunities for cancer prevention.

The explosion of knowledge about the molecular and cellular bases of immune regulation, particularly at the level of T-cell responses, provides a new arsenal of approaches to enhance antigen-specific responses to both viruses and cancer. In the case of cancer, immune responses to tumour antigens can be induced through the engineering of the tumour cells themselves or by engineering responses that are specific for particular tumour antigens. In this review, I discuss the ways in which molecular and cell-biological insights into immune regulation are being applied to design strategies of both quantitatively and qualitatively improved immunotherapy for cancer.

CO-STIMULATORY SIGNAL
A signal to a T cell (in the form of a soluble or membrane-bound molecule) that has little or no effect on its own, but either enhances or modifies the physiological effect of the primary signal mediated by engagement of the T-cell receptor.

Enhanced function of antigen-presenting cells

Growth and differentiation programmes of APCs. The most common target of active immunotherapy strategies is the enhancement or modulation of the function of antigen-presenting cells (APCs). This strategy is based on the concept that the quantitative and qualitative characteristics of a T-cell response to antigen depend on the signals that the T cell receives from an APC. Of the main subtypes of bone-marrow-derived APCs (B cells, macrophages and dendritic cells (DCs)), the DC is the most potent type of APC, and is responsible for initiating immune responses[24,25].

As virtually all stages of DC differentiation and function can be modulated by engineered vaccines, it is important to understand the molecular signals that regulate the role of DCs in the activation of T-cell-dependent immunity (FIG. 1). At sites of infection and inflammation, bone-marrow-derived progenitor cells respond to signals that induce proliferation and differentiation. Granulocyte–macrophage colony-stimulating factor (GM-CSF) and other cytokines, such as FLT3 ligand and interleukin-4 (IL-4), are mitogenic or co-mitogenic factors that induce an intermediate stage of DC differentiation that is characterized by the efficient uptake and processing of antigen[26–30]. Once they have taken up antigens at inflammatory tissue sites, immature DCs differentiate in response to several distinct maturation signals. Although many diverse molecules can induce

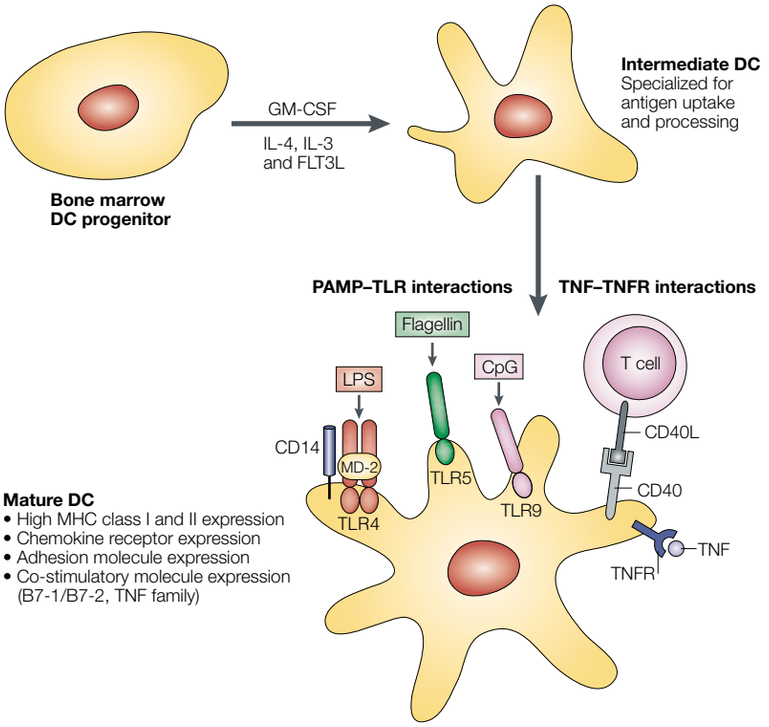


Figure 1 | **Multi-step pathway for dendritic-cell differentiation and activation.** Dendritic-cell (DC) differentiation is a complex multi-step genetic programme that is regulated by distinct signals. The first defined differentiation stage has been termed the immature or intermediate DC stage. Intermediate DCs differentiate from bone-marrow-derived progenitors in response to certain cytokines, of which granulocyte–macrophage colony-stimulating factor (GM-CSF) seems to be the most important. Other cytokines, such as FLT3 ligand (FLT3L), interleukin-3 (IL-3) and/or IL-4 can augment or modify this process. Intermediate DCs that develop and reside in peripheral tissues are specialized for antigen uptake and processing. Intermediate DCs express significant amounts of MHC class II, which is located predominantly in specialized antigen-processing vesicular compartments. The maturation or activation of DCs occurs in response to a broad array of signals, which can generally be divided into two categories — pathogen-associated molecular pattern molecules (PAMPs) or endogenously produced signals of the tumour-necrosis factor (TNF) family. These two types of signal activate DC maturation through Toll-like receptors (TLRs) or TNF receptor (TNFR) family members, respectively. DC maturation starts with the expression of homing and chemokine receptors (such as CC-chemokine receptor 7 (CCR7)) that mediate traffic out of the tissue space and into draining lymph nodes through afferent lymphatics. As they arrive in the paracortical regions of the draining lymph nodes, DCs upregulate their expression of co-stimulatory molecules, such as CD80 (B7-1) and CD86 (B7-2), and peptide-loaded MHC class II molecules transport from the intravesicular processing compartment to the cell surface. Mature DCs in the lymph node also secrete chemokines, such as thymus and activation-regulated chemokine (TARC; CC-chemokine17), which attract naive T cells. As described in the text, each of these DC maturation and activation steps is potentially amenable to regulation by engineered immunotherapy approaches. LPS, lipopolysaccharide; CD40L, CD40 ligand.

IDIOTYPE
The portion of either a T-cell receptor or immunoglobulin, defined by the hypervariable regions and involved in antigen recognition, that is completely unique.

T HELPER 1 (T_H1)/T_H2
Different phenotypes of helper T cells that are characterized by distinct patterns of cytokine release on activation.

DC maturation, most seem to signal to DCs by binding to two classes of receptor — the Toll-like receptor (TLR) and tumour-necrosis factor (TNF) receptor (TNFR) families. TLRs are pattern-recognition receptors, which bind common chemical moieties that are expressed by pathogens and known as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and unmethylated CpG DNA sequences[31]. The two best-characterized endogenous DC maturation factors of the TNF family are TNF-α[32] and CD40 ligand (CD40L)[33,34].

The maturation of DCs, which occurs as they migrate to draining lymph nodes, is characterized by the transport of peptide–MHC complexes to the cell surface[35,36]. In addition to the presentation of high concentrations of peptide–MHC complexes for T-cell stimulation (known as signal 1), DCs regulate T-cell activation and differentiation through the provision of CO-STIMULATORY SIGNALS, in the form of cytokines — such as IL-12 — and membrane-bound ligands of the B7 and TNF family (collectively known as signal 2). The ever-expanding collection of co-stimulatory signals used by DCs to instruct T cells as to their pathway of differentiation and effector function reflects the high degree of complexity of the communication between APCs and T cells. Each of the molecular events that are involved in proliferation, antigen presentation and co-stimulation is a potential target that can be exploited in the design of immunotherapeutic approaches.

Building DC growth or activation factors into vaccines. The elucidation of specific molecules that induce DC proliferation and maturation has provided an important tool kit for the engineering of vaccines with enhanced therapeutic potency. The prototypical example is the incorporation of GM-CSF into both cell-based and antigen-based vaccines. A detailed comparison of the potency of tumour-cell vaccines that are transduced with different cytokine-encoding and immunoregulatory genes has shown that GM-CSF-transduced cell vaccines induce the most potent systemic immunity against challenge with wild-type tumour[37,38]. Biopsy analysis of the local sites of vaccination of GM-CSF-transduced tumour vaccines shows a strong infiltrate of mononuclear cells that express markers characteristic of the DC lineage. A few days after vaccination, increased numbers of mature DCs could be detected in the draining lymph nodes, together with active T-cell proliferation in the paracortical regions of the lymph node. Using a combination of proliferative and maturation stimuli for DCs, Colombo and colleagues found that vaccination with tumour cells that were co-transduced with the genes that encode GM-CSF and CD40L generated a marked increase in the number of activated DCs at the site of vaccination, as well as enhanced vaccine potency[39].

The incorporation of GM-CSF or its encoding gene into recombinant protein, DNA or viral vaccines has also been shown to significantly enhance immunization. For protein vaccines, both preclinical studies and clinical trials of vaccination for B-cell lymphomas have been carried out; the vaccine consisted of the lymphoma immunoglobulin IDIOTYPE as the tumour antigen, either mixed with GM-CSF protein or covalently linked to GM-CSF as a recombinant chimeric protein[40,41]. Interestingly, the immunological effects of paracrine GM-CSF are characterized by an enhancement of diverse effector functions, involving both T HELPER 1 (T_H1) and T_H2 components. In addition to the generation of cytotoxic T lymphocytes (CTLs), documented T_H1 effector pathways that are induced by paracrine GM-CSF vaccines include the activation of macrophages, which results in the production of both superoxides and nitric oxide as tumoricidal effectors[11,42].

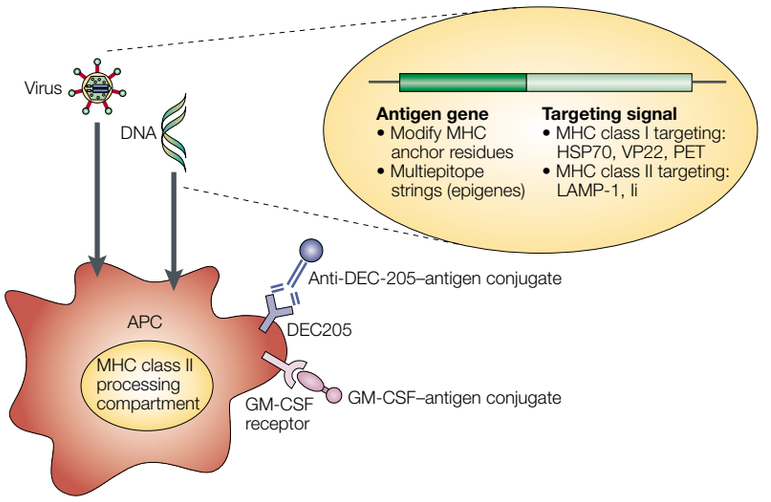


Figure 2 | **Approaches to enhance antigen targeting to antigen-presenting cells for improved immunization.** Several approaches are being actively explored to enhance the processing and presentation of antigens by crucial antigen-presenting cells (APCs), such as dendritic cells. Recombinant viruses that directly infect APCs can be engineered to express antigen together with various MHC-targeting signals that will enhance antigen transport into the MHC class I and/or II processing pathways. Targeting to the MHC class I processing pathway can be enhanced by linking antigen-encoding genes to genes that encode heat-shock proteins (HSPs), VP22 (an important coat protein of Marek's disease virus) or PET (*Pseudomonas* exotoxin translocation subunit). Targeting to the MHC class II pathway can be enhanced by linking the antigen to the lysosome-associated membrane protein 1 (LAMP1) or invariant chain (Ii; a molecule that is involved in MHC class II formation) targeting signals. Additionally, genes that encode co-stimulatory molecules can be engineered into recombinant vectors, thereby endowing infected cells with super-physiological levels of co-stimulatory ligands. Similar modifications have been engineered into DNA vaccines. Engineered protein vaccines are being constructed that link antigens to ligands for receptors on the surface of APCs (such as DEC-205 and the granulocyte–macrophage colony-stimulating factor (GM-CSF) receptor), which can effectively mediate endocytosis and targeting into MHC processing pathways. DEC-205 is also known as lymphocyte antigen 75 (LY75).

T_H2 effector pathways involve the activation of eosinophils at the site of tumour metastases. These mixed effector responses have been documented in clinical trials of GM-CSF-transduced vaccines for renal-cell cancer, melanoma and pancreatic cancer[43–45].

Ex vivo antigen-loaded DC vaccines. The ability to culture DCs *ex vivo* has led to numerous studies using *ex vivo* antigen-loaded DCs as tumour vaccines. Initially, it was shown that the loading of *ex-vivo*-cultured DCs with MHC class-I-restricted peptides, whole proteins or tumour lysates, followed by the re-administration of the DCs, led to the generation of immune responses against the loaded antigen, as well as antitumour responses[46–52]. More recently, the discovery of more-efficient gene-transfer vectors has led to approaches in which *ex-vivo*-cultured DCs are transduced with genes that encode relevant viral or tumour antigens[53–55]. Several different recombinant, replication-defective viruses have been used to transduce DCs. In addition, Gilboa and colleagues have shown that purified RNA can be used to transduce DCs effectively, leading to the presentation of encoded antigens[56]. This strategy offers the interesting possibility that DCs could be transduced with the entire amplified TRANSCRIPTOME of a tumour cell, even when only tiny amounts of tumour tissue are available. At present, the paucity of direct comparative studies leaves open the question of which method of loading DCs *ex vivo* is the most effective. Another important issue regarding *ex vivo* antigen-loaded DC vaccines is the degree of maturation that is induced *in vitro* and its relevance to the homing and function of loaded DCs after re-injection. At present, the maturation protocols used for DC vaccination are quite variable and range from the use of monocyte-conditioned medium to various defined agents, such as TNF-α, IL-1, soluble CD40L and prostaglandins[57,58]. Concern has been raised that the full-blown maturation and/or activation of DCs *ex vivo*, to a stage normally achieved only once they are within the paracortical regions of the lymph node, will impair the ability of DCs to home to lymph nodes after re-injection. This has led to the suggestion that DCs should be loaded and re-injected in an immature state and allowed to mature *in vivo*. But, such an approach has potential negative consequences, as Steinman, Bhardwaj and colleagues have shown — the immunization of patients with antigen-loaded immature DCs can result in tolerance or the suppression of antigen-specific responses[52].

The elucidation of proliferative and maturation signals for DCs has led recently to approaches in which DCs are not only loaded with antigen, but also transduced with genes that encode proliferation and maturation signals. This would result in autocrine DC stimulation *in vivo* after re-injection. In one study, DCs loaded with antigen were transduced with the genes that encode GM-CSF and CD40L. These genetically modified DCs were much more potent stimulators of antitumour immunity than DCs that were loaded with antigen alone[59].

Another approach aimed at providing DCs with a full complement of tumour antigens is the generation of DC–tumour fusion vaccines[60]. The concept behind this approach is to fuse autologous tumour cells with DCs, thereby allowing for the co-expression of all relevant tumour antigens and DC molecules within the same cell. One of the main limitations to the clinical use of an approach of this type is the efficiency with which fusion can be achieved between DCs and tumour cells in the absence of selection. Ultimately, preclinical and clinical DC vaccine studies must identify the crucial parameters of DC growth and maturation, as well as antigen loading, that result in therapeutically relevant levels of T-cell activation *in vivo*.

Antigen targeting to dendritic cells. The discovery of specific receptors on DCs that are responsible for receptor-mediated endocytosis has enabled the modification of antigens so that they can be more efficiently bound to these DC uptake receptors (FIG. 2). Indeed, antigen–GM-CSF fusion proteins are an example of potential DC targeting[40], because GM-CSF not only stimulates DC proliferation, but might act also to target the antigen into endosomal compartments by binding to the GM-CSF receptor. More-direct antigen-targeting approaches have used fusions of antigen and immunoglobulin Fc regions to enhance Fc receptor (FcR)-mediated antigen uptake

TRANSCRIPTOME
The full complement of mRNA that is transcribed within a particular cell type.

by APCs[61,62]. Such an approach is likely to be more effective in targeting antigens to macrophages than to DCs, because macrophages have higher levels of FcR. Approaches that modify antigens so that they can be selectively targeted to DC receptors — such as CD36 and the C-type lectin DEC-205 (also known as lymphocyte antigen 75 (LY75)) — might ultimately provide more-effective priming. A recent study has shown that the conjugation of antigens to antibodies that are specific for DEC-205 markedly enhances the targeting of antigen to DEC-205$^+$ DCs. Importantly, however, this approach failed to produce sustained antigen-specific immunity because of the failure to activate DCs *in vivo*. The addition of an activating CD40-specific antibody to the anti-DEC-205–antigen conjugate did result in sustained immunity, showing the importance of combining MHC-targeting and APC-activation strategies[63].

Another interesting group of proteins that might target antigen effectively to DCs and, furthermore, into MHC processing pathways is the heat-shock protein (HSP) family[64]. It is now well established that complexing peptide antigens to certain HSPs, such as glycoprotein 96 (gp96), HSP70, calreticulin and HSP110, significantly enhances the immunogenicity of the antigen[65–68]. Members of the HSP family were first used as tumour vaccines by purifying them from tumour cells, followed by immunization. HSPs isolated from tumours are complexed naturally with an array of tumour-associated peptides. Other approaches to link antigens to HSPs have included the production of recombinant fusion proteins, in which antigenic peptides are covalently or noncovalently linked to the HSP[69,70], as well as DNA-based vaccines, in which fusion genes are incorporated between the genes that encode antigen and HSP. In one direct comparative study using the HPV E7 antigen as a model, it was shown that a DNA vaccine encoding an E7–HSP70 fusion protein was 30-fold more effective than the wild-type E7 protein in generating a CD8$^+$ T-cell response[71]. Immunogenic HSPs complexed with antigenic peptides have been shown to load the MHC class I processing pathway efficiently (so called *in vitro* CROSS-PRESENTATION)[72]. Although the intracellular pathway by which HSPs effectively load MHC class I molecules with HSP-associated peptides has not been determined yet, Srivastava and colleagues have identified CD91, the α_2-macroglobulin receptor, as an important receptor for several HSPs (gp96, HSP70 and HSP90)[73]. Ultimately, it is proposed that the immunogenicity of HSPs results from their ability to activate APCs and target antigens to MHC processing pathways[64]. One report has suggested that HSP70 can activate macrophages through CD14/TLR4 (LPS receptor)-dependent and -independent pathways[74]. HSPs have been reported to activate DCs also[75], but the receptors that mediate these putative activation functions have yet to be elucidated.

Enhancing T-cell activation

Because virtually all signals to T cells begin at the cell membrane, the efficacy of vaccines and other immunotherapies can be enhanced by the inclusion of ligands that bind to these cell-membrane receptors. The enhanced versatility of new vector systems allows the combinatorial construction of immunotherapies that contain elements that target several points in the pathway of T-cell activation.

Enhancement of signal 1. In considering the immunogenicity of various antigenic formulations, it is commonly assumed that alterations in the immune response will depend strictly on the set of co-stimulatory signals (signal 2) provided to T cells at the time of antigen recognition. However, it is now clear that both qualitative and quantitative characteristics of the peptide–MHC interaction with T-cell receptor (TCR) (signal 1) are equally important in determining the outcome of T-cell responses. The two most well-defined parameters of TCR engagement are ligand density and TCR affinity. Low-affinity ligands have partial agonist properties and, ultimately, antagonist properties. The favoured model to explain these findings is the kinetic proof-reading model, which indicates that the TCR must be engaged by peptide–MHC for long enough to initiate the complete set of intracellular biochemical signalling events that are required for T-cell activation[76]. Even for high-affinity ligands, it has been shown that the exposure of T cells to ligand densities below the activation threshold can result in the induction of T-cell unresponsiveness. A fundamental corollary of the immune tolerance hypothesis — that endogenous tumours can induce the tolerance of T cells that are specific for neoantigens — is that the residual repertoire of tumour-antigen-specific T cells will be either of low affinity or specific for epitopes that are presented at low density. Similar mechanisms might operate when viruses evade immune elimination and establish a chronic carrier state.

Indeed, the analysis of T-cell responses that are specific for defined tumour antigens has provided experimental evidence for this idea. Most melanoma-specific T cells that have been grown in culture recognize melanocyte-specific differentiation antigens, such as MART1/melan-A, gp100 and tyrosinase[77,78]. A surprisingly large number of the specific MHC class-I- and class-II-restricted epitopes that have been identified seem to have extremely low affinities for their presenting MHC molecule, which results in a low density of peptide–MHC complexes on both the tumour and APCs that are loaded with the antigen. This low affinity for MHC is associated generally with the presence of undesirable residues at crucial anchor positions in the antigen. In other cases, tumour peptides bind well to MHC molecules but the available T cells have low affinities for the peptide–MHC complex.

As tumour antigens continue to be identified (BOX 1), there will be important opportunities to modify epitopes so that they are presented to T cells in a manner that most effectively transmits the TCR signal (signal 1) (FIG. 2). For antigens that have poor affinity for MHC, several groups have shown that the alteration of MHC anchor residues to more favourable amino acids can result in a marked enhancement of the binding of antigen to MHC, and the peptide–MHC complex

CROSS-PRESENTATION
The presentation of exogenous antigen by MHC class I molecules.

IMMUNODOMINANT
Refers to the antigen(s) in a complex mixture (such as a whole virus or tumour cell) that are recognized preferentially during an immune response.

retains the capacity for enhanced activation of T cells that are specific for the original wild-type epitope[79,80]. This results in a heteroclitic response, in which vaccines that contain the anchor-modified epitope can produce stronger immune responses against the wild-type peptide than are elicited by the wild-type peptide itself *in vivo*, which results in enhanced antitumour immunity. Epitope engineering can also generate enhanced immunity at the level of TCR affinity for peptide–MHC. For example, for the low-affinity response to the gp70-derived peptide that is IMMUNODOMINANT in mouse CT26 colon cancer, a single amino-acid alteration of the gp70 epitope did not affect binding to MHC but increased the affinity of peptide–MHC for the TCR by threefold. Immunization with DCs that are loaded with this altered peptide resulted in a pronounced enhancement of the *in vivo* expansion of T-cell populations that are specific for the original wild-type peptide and the enhancement of systemic antitumour immunity[81].

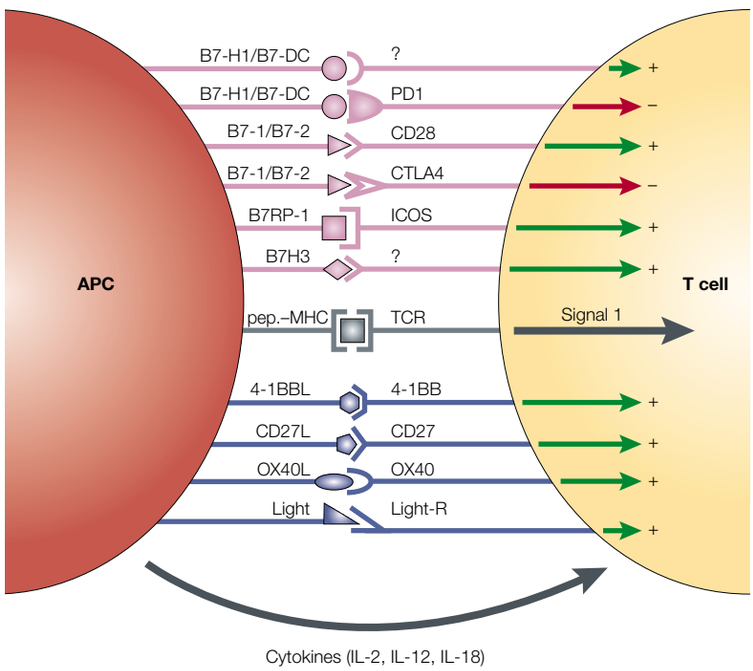
The density of peptide–MHC ligand on the surface of APCs can be enhanced by targeting antigens to the MHC processing pathways. Two approaches have been used to accomplish this goal. One approach links targeting signals to the antigen to target it more effectively into MHC processing compartments and pathways. Strategies for antigen targeting to the MHC class II processing pathway have used the invariant-chain targeting signal and the endosomal/lysosomal targeting signal of the cytoplasmic tail of lysosome-associated membrane protein 1 (LAMP1). The fusion of genes that encode the HPV E7 antigen and LAMP1 sorting signal resulted in the increased targeting of E7 into the MHC class II processing pathway and enhancement of presentation to E7-specific MHC class-II-restricted $CD4^+$ T cells. Incorporation of the LAMP1 targeting signal into the gene that encodes E7 enhanced $CD4^+$ T-cell responses and the antitumour potency of both recombinant vaccinia and recombinant DNA vaccines[82–84].

Several approaches that target the MHC class I pathway have also resulted in enhanced immunization potency (FIG. 2). The antigen–HSP70 fusions described previously are an example of the selective enhancement of antigen-specific $CD8^+$ T-cell responses[71]. Other validated targeting signals that enhance $CD8^+$ T-cell priming include calreticulin, VP22 (a herpesvirus-encoded protein) and the endoplasmic reticulum (ER) translocation subunit of *Pseudomonas* exotoxin[85,86]. Another strategy for enhanced MHC class I processing is the construction of 'epitopes on a string'[87]. This approach separates out individual epitopes from a given antigen and strings them together, separated by linkers that encode basic amino acids that are good substrates for proteasome-mediated cleavage. As several of these strategies probably function through distinct mechanisms, the maximal loading of MHC on APCs is most likely to be achieved by combining different targeting signals.

Enhancement of signal 2 — co-stimulation. Qualitative and quantitative elements of T-cell activation and differentiation are determined, in large part, by signals delivered by co-stimulatory molecules. As the number of known co-stimulatory molecules increases, a picture is emerging in which T-cell activation requires the integration of a large number of different signals. The most well-characterized co-stimulatory signals fall into three families: the B7 family, the TNF family and cytokines (BOX 2).

Box 2 | The expanding families of co-stimulatory molecules

At its simplest level, T-cell activation requires two signals. Signal 1 is delivered through the T-cell receptor (TCR) after engagement by a peptide–MHC (pep.–MHC) complex. Signal 2 — the co-stimulatory signal — was thought originally to result from CD80 (B7-1) interacting with CD28 on T cells. Over the past ten years, many additional co-stimulatory signals have been identified (see figure). These fall broadly into three families — the B7 family, the tumour-necrosis factor (TNF) family and cytokines. The B7 family currently comprises six members with defined co-stimulatory activity. Two of the founding members of the B7 family, CD80 (B7-1) and CD86 (B7-2), each bind to an activating receptor, CD28, and a counter-regulatory inhibitory receptor, cytotoxic T-lymphocyte antigen 4 (CTLA4). Two newer B7 family members, B7-H1/PDL1 and B7-DC/PDL2, have been reported to have both co-stimulatory and inhibitory activities. Their inhibitory activity is probably mediated through the programmed cell death 1 (PD1) receptor, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM). The putative activating receptor for B7-H1 and B7-DC has not been identified as yet. B7RP-1, which binds to the activating receptor inducible co-stimulatory molecule (ICOS), seems to be particularly important in T-cell–B-cell interactions and antibody production. The receptor for B7H3 has not been indentified yet. Several TNF family members that are expressed by antigen-presenting cells can co-stimulate T-cell activation by binding to specific TNF receptor family members expressed on T cells. Most of these TNF receptor family members are upregulated on T-cell activation, indicating that the role of this family might be to amplify responses once initial T-cell activation has occurred. Finally, cytokines such as interleukin-12 (IL-12) are very important in determining the direction of T-cell differentiation — that is, T helper 1 (T_H1) versus T_H2 — as a consequence of T-cell stimulation. Although several ligand–receptor pairs have been validated individually as providing co-stimulatory or inhibitory signals, the complex integration of co-stimulatory and/or inhibitory signals together with signal 1 has yet to be delineated. Once these complex interactions and signalling outcomes are defined, it should be possible to build multiple co-stimulatory signals into vaccines in an appropriate combinatorial fashion.



Of these three categories of co-stimulatory molecules, the B7 family seems to be the only one that can signal unidirectionally from APCs to T cells. Tremendous effort has been directed towards engineering co-stimulatory molecules into vaccines and other immunotherapies to enhance their activity. In the case of the B7 family members, most work has focused on CD80 (B7-1) and CD86 (B7-2). It will be interesting to see how the four new members of the B7 family (B7h/B7RP1, B7H1/PDL1, B7DC/PDL2 and B7H3) will fit into the scheme, because they bind different receptors from CD80 and CD86 and have only partially overlapping biological activity.

Two primary methods have been used to incorporate CD80 and/or CD86 into immunotherapeutic approaches. One approach involves the transduction of tumour cells with genes that encode B7 molecules to enhance their immunogenicity as vaccines[88,89]. This concept arose from the idea that tumours fail to stimulate immune responses under normal circumstances because they do not express sufficient co-stimulatory molecules. However, a more detailed analysis of the mechanisms of rejection and immune priming by B7-transduced tumours supports the emerging view that the direct presentation of antigen by tumour cells to the immune system is a relatively minor pathway compared with the indirect presentation pathway of bone-marrow-derived APCs[90].

A more promising application of the B7 molecules to vaccine design has been the inclusion of genes that encode B7 molecules into recombinant DNA and viral vaccine vectors for antigen-specific vaccination[91,92]. This is based on the idea that these vaccines immunize through the direct transduction or infection of APCs. Theoretically, even though DCs naturally express B7 molecules, the increased level of expression of B7 due to genes engineered into recombinant vaccines, as well as altered patterns or ratios of expression of the different B7 family members, could significantly modify the outcome of T-cell priming *in vivo*. Several studies have shown that the incorporation of either CD80 or CD86 into recombinant vaccines enhances the generation of CTL responses and, in some cases, antibody responses. Interestingly, these studies have, in some cases, shown differential activity of CD80 and CD86, with CD86 typically generating superior CTL responses *in vivo* to CD80.

The list of potential TNFR family molecules expressed by T cells that might participate in modifying T-cell responses continues to grow. Two very interesting candidate co-stimulatory receptors of the TNFR family are 4-1BB (also known as TNFR superfamily member 9 (TNFRSF9)) and OX40 (also known as TNFRSF4). The administration of putative agonistic antibodies specific for 4-1BB and OX40 has been shown to induce antitumour immunity when administered alone or together with a vaccine[93,94]. Recent evidence indicates that OX40 might even be able to break anergic tolerance[95]. 4-1BB ligand and OX40 ligand are expressed on APCs, and their ligand–receptor pairs could, therefore, provide co-stimulatory signals from APC to T cell. CD27 and CD30 are two additional TNFR family members expressed by T cells that might be interesting targets for the activation or inhibition of antigen-specific responses.

Cytokines are the largest category of immunoregulatory molecules and they have been used as systemic agents, local agents and components of genetically modified cell-based and recombinant antigen-specific vaccines. The principal clinical application of cytokines has been in the form of systemic administration of the recombinant cytokine protein. In general, the systemic administration of cytokines as single agents in cancer immunotherapy has produced disappointing results. Of the many cytokines tested, IL-2 has an established track record against metastatic renal cancer and melanoma, and has been shown to induce durable complete responses for these two types of cancer in 3–10% of patients[96]. Unfortunately, the toxicity of systemically administered cytokines (including IL-2) is quite high and significantly limits their widespread clinical use. There are vast differences in the tolerability of systemic cytokines, such as IL-2, TNF-α and IL-12, between mice and humans, with mice tolerating 50–300-fold higher serum concentrations. So, doses of these cytokines that induce tumour regression in mouse models are lethal in humans.

As virtually all cytokines behave physiologically as autocrine and/or paracrine factors, it is not surprising that applying them as systemic agents results in unacceptable levels of toxicity. Indeed, the primary motivation for incorporating cytokines into tumour-cell vaccines, as well as recombinant DNA or viral vectors, is to maximize the expression of the cytokine at the site of antigen delivery. In the case of cytokine-encoding gene-transduced tumour vaccines, the introduction of genes encoding cytokines that target T cells has been less successful in priming immune responses than the transduction of cytokines aimed at APCs, particularly DCs (see previously). Considering that the vaccines are typically administered intradermally, subcutaneously or intramuscularly, whereas T-cell priming generally occurs in the draining lymph node, it is not surprising that tumour-cell vaccines engineered to produce DC-targeted cytokines might ultimately be more effective.

Another important approach to cytokine-based therapy is the targeting of cytokines to areas of tumour metastasis, to which T cells would subsequently traffic and carry out their effector functions. Proof of principle for the efficacy of such an approach has come from studies by Reisfeld and colleagues, who have observed antitumour responses after the administration of chimeric antibody–cytokine fusion molecules, in which cytokines such as IL-2 are linked to the Fc regions of antitumour antibodies[97].

Possibly the most promising application of T-cell co-stimulatory or proliferative cytokines is the incorporation of their encoding genes into recombinant DNA and viral vaccines. Several studies have shown that the incorporation of cytokine-encoding genes into recombinant vaccines of this sort can not only quantitatively enhance T-cell responses, but also alter the differentiation pattern of antigen-specific T cells. For example, Berzofsky and

Table 1 | **Comparison of different vaccine formulations**

Formulation	Advantages	Disadvantages
Peptide + adjuvant	Very easy to produce Safe	Must be matched to patient's HLA Weakly immunogenic
Protein + adjuvant	Easy to produce Many possible adjuvants	Generally good for inducing antibody production but less potent for CTL induction
Ex-vivo-loaded DCs	Potential for high immunogenicity Multiple means of Ag loading Potency can be enhanced by transducing DC-activating genes	Individualized cell processing required Variabilities in quality control Most DCs do not home to draining LNs after *in vitro* culture/loading
DNA	Easy to produce Versatile engineering by building in targeting and/or co-stimulatory genes	Intrinsically weak immunogen
Recombinant virus	Potential for high immunogenicity Many different viruses with natural immunological properties Versatility in construction	Safety is a significant issue
Recombinant bacterium	Potential for high immunogenicity Can carry engineered plasmids Toxicity manageable by antibodies	Perceptions of safety are a significant issue

Ag, antigen; CTL, cytotoxic T lymphocyte; DC, dendritic cell; HLA, human leukocyte antigen; LN, lymph node.

colleagues found that when the gene encoding IL-12 was incorporated into DNA vaccines, antigen-specific responses were predominantly T_H1 in nature, whereas when IL-4 or IL-10 was used, T_H1 responses were inhibited and the predominant response was of the T_H2 type. By contrast, GM-CSF, which acts on APCs, generated the greatest level of T-cell immunity, although the response was of a mixed T_H1 and T_H2 type[98].

The growing armamentarium of vaccine vectors

For all of the added value that recombinant DNA technology provides in engineering elements into vaccine constructs that enhance their potency, nature itself provides a virtually limitless array of delivery systems, in the form of diverse microbes with potent intrinsic immunological properties. These immunogenic properties of microbes derive from their expression of PAMPs (which activate DCs through TLRs), their ability to induce the expression of pro-inflammatory cytokines by infected cells and ability to target intracellular MHC processing compartments. Of the three main classes of microbes — virus, bacterium and fungus — viruses and bacteria have been investigated most intensively (TABLE 1). A recent report of engineered yeast vaccines emphasizes the potential immunological use of the third microbial class.

Engineered viruses. Viruses are the most diverse and efficient gene-transfer agents; their natural cell tropism and biological features can significantly enhance the immunogenicity of antigens carried within them (TABLE 1). Using standard recombination approaches, Moss and Paoletti[99,100] were the first to explore recombinant viruses as vaccine vectors. They used vaccinia virus, a highly immunogenic virus related to the smallpox virus that is relatively nonvirulent in immunocompetent individuals. In most cases, a single immunization with recombinant vaccinia that carry a gene encoding an antigen will generate significantly greater immune responses against that antigen than will immunization with the corresponding protein or peptide epitopes mixed with standard ADJUVANTS. This is particularly true for the generation of CTLs. So far, many viruses have been explored as recombinant vaccine vectors, including attenuated, replication-deficient poxviruses (such as modified vaccinia Ankara, fowlpox and canarypox), adenovirus, herpesviruses and Venezuelan equine encephalitis virus[99–105]. Each of these viruses has various advantages and disadvantages, and no clear 'winner' has emerged as the absolute vector of choice. Features of viruses that can enhance their potency as vaccine vectors include their ability to induce immunological 'danger' signals at sites of infection and directly infect APCs. Features of viruses that can diminish their potency as vaccine vectors include the presence of virally encoded inhibitors of immunity. These include molecules that block the processing and presentation of antigen by the MHC class I pathway (such as inhibitors of transporter for antigen processing (TAP) and MHC class I traffic out of the ER) and cytokine decoys, to mention a few[106]. Deleting immunological inhibitory genes from recombinant viruses might further enhance their vaccine potency and simultaneously attenuate their virulence.

An important barrier to virus-based vaccination is the presence of neutralizing antibodies in pre-exposed or prevaccinated individuals, which inhibit the initial round of virus infection and replication, thereby reducing the ability of the virus to immunize. Individuals who have never been previously exposed to the vaccinating virus generate neutralizing antibody after the first vaccination, thereby precluding subsequent vaccination with the same vector. This finding has led to the concept of cycling different viral vectors in PRIME–BOOST formats. A marked enhancement of immunization potency has been observed with prime–boost formats using different viruses, such as fowlpox followed by vaccinia, as well as DNA vaccines and recombinant viral vaccines[107,108]. Another theoretical concern about the use of recombinant viral vaccines as immunogens against tumour antigens is that the strong viral antigens will dominate, thereby inhibiting responses to the weaker tumour

ADJUVANT
An agent mixed with an antigen that enhances the immune response to that antigen on immunization.

PRIME–BOOST
When a single application of a vaccine is insufficient, repeated immunizations are performed using the same vaccine preparation (homologous prime–boost) or using different vaccine preparations (heterologous prime–boost) to sequentially stimulate a better immune response.

antigen. This concern arises from the ability of immunodominant epitopes in antigens to quench responses to subdominant or CRYPTIC EPITOPES. However, such a quenching phenomenon is, in fact, rarely observed with recombinant viral vaccines. The cycling of different viral vectors would certainly diminish the probability of such a phenomenon, because the recombinant tumour antigen would be the only one being repetitively boosted in such an immunization strategy.

Engineered bacteria. Genetic engineering of intracellular bacteria, such as *Mycobacterium bovis* Bacillus Calmétte–Guerin (BCG), *Salmonella*, *Shigella* and *Listeria*, has produced several interesting and promising vaccines[109–112]. In principle, bacteria that enter APCs might be a good vehicle for the delivery of recombinant antigens. Certain bacteria, such as *Listeria*, have complex life cycles that involve both phagolysosomal and cytoplasmic stages. So, recombinant *Listeria monocytogenes* engineered to secrete antigens will load the MHC class II processing pathway during the phagolysosomal phase of its life cycle and the MHC class I pathway during the cytoplasmic phase. In addition, several recombinant bacteria actively induce infected APCs to secrete pro-inflammatory cytokines, such as IL-12. More recently, recombinant bacteria have been used as vectors for the delivery of DNA vaccines[113–115]. Bacterial vaccines containing plasmids with eukaryotic promoter and enhancer elements that drive the expression of the antigen gene result in potent immunization. These results indicate that the bacteria can transfer plasmids directly into eukaryotic transcriptional compartments within infected APCs.

Blockade of immunological checkpoints

As engineered immunotherapeutics continue to improve, a potency 'ceiling' will be reached owing to the presence of hardwired inhibitory pathways that negatively regulate lymphocyte responses. It is now clear that the quantitative response to an antigen is balanced by both positive (co-stimulatory) and negative (checkpoint) signalling pathways. In the case of T-cell responses, several of these pathways seem to have components that are either exclusively, or at least selectively, expressed by T cells. So, immunological checkpoints are a main target for pharmacological intervention. Past efforts in the development of pharmacological agents that target the immune system have identified drugs that either inhibit or activate immune responses in a non-specific, antigen-independent fashion. The discovery of specific, negative regulatory signalling pathways that check immune responses by dampening TCR or co-stimulatory signalling pathways provides an exciting opportunity to develop drugs or antibodies that block these pathways, thereby amplifying antigen-specific activation stimuli.

Among the best studied of these counter-regulatory pathways is that initiated by the engagement of cytotoxic T-lymphocyte antigen 4 (CTLA4)[116]. Naive T cells express the co-stimulatory B7 receptor CD28, the engagement of which amplifies TCR-dependent responses. After T-cell activation, a second B7 receptor, CTLA4, is expressed. CTLA4 has a much higher affinity than CD28 for CD80 and CD86. CTLA4 delivers inhibitory signals to T cells that oppose the co-stimulatory signals delivered by CD28. *Ctla4*-knockout mice die at a relatively young age owing to 'hyperimmune' infiltrates in several organs, indicating that Ctla4 is a crucial negative regulator of T-cell activity. Allison and colleagues have shown that the transient *in vivo* blockade of Ctla4 with a blocking antibody administered at the time of vaccination with a GM-CSF-transduced tumour vaccine can significantly enhance vaccine potency, which leads to the regression of established tumours[117,118]. Although the vaccine–Ctla4 combination approach induced autoimmune disease, the autoimmunity was confined to the tissue from which the tumour vaccine was derived. So, the treatment of mice with a B16-melanoma–GM-CSF vaccine plus anti-Ctla4 antibody resulted exclusively in vitiligo — patchy depigmentation due to an autoimmune response restricted to melanocytes. Mice that received the prostate-cancer–GM-CSF vaccine plus anti-Ctla4 antibody developed prostatitis, but no other signs of autoimmunity[119]. These findings show that there is a hierarchy of tolerance induction, in which tolerance to tissue-specific antigens might be maintained less stringently than tolerance to more-ubiquitous self-antigens. This hierarchy provides a therapeutic window for cancers derived from dispensable tissues, in which tissue-specific antigens expressed by both the cancer and normal tissue are viable immunological targets.

The dissection of signalling pathways in T cells has revealed several additional potential targets for inhibitors of immunological checkpoints. The membrane molecule programmed cell death 1 (PD1), expression of which is induced after T-cell activation, is a CTLA4-like inhibitory molecule that decreases cytokine responses in T cells and might enhance their activation-induced cell death. PD1 is a receptor for two of the newer B7 family members, B7-H1/PDL1 and B7-DC/PDL2 (REFS 120–123). Given that both B7-H1/PDL1 and B7-DC/PDL2 can co-stimulate enhanced cytokine production by naive T cells, it is probable that PD1 is a counter-regulatory inhibitory receptor paired with an as yet unidentified co-stimulatory receptor on naive T cells. *Pd1*-knockout mice do not develop the broad hyperimmune organ infiltrates that *Ctla4*-knockout mice develop but, rather, they have a more focal autoimmunity. Therefore, PD1 is an interesting potential target for blockade in the context of immunization, analogous to CTLA4 blockade.

Several intracellular inhibitory signalling pathways in T cells are additional targets for pharmacological intervention. Some of the best candidates include Cbl-b, Cabin and certain protein tyrosine phosphatases (PTPs), as well as the tyrosine kinase Csk. Among the phosphatases, Src-homology 2 (SH2)-domain-containing inositol polyphosphate 5′ phosphatase 1 (SHIP1), SH2-domain-containing protein tyrosine phosphatase 1 (SHP1) and SHP2 have all been implicated in downmodulating signalling pathways that are activated by TCR engagement[124]. More recently, the

CRYPTIC EPITOPE
An antigenic peptide that is generated at sub-threshold levels. When cryptic epitopes become visible to the immune system they can elicit an immune response that is responsible for autoimmune disease.

CD45 PTP has been shown to regulate immune responses negatively by inhibiting the activation of Janus family kinase 1 (JAK1) and JAK2, thereby down-modulating responses to certain cytokines[125]. Downstream of the JAKs, activation of signal transducer and activator of transcription (STAT) factors is inhibited by the CIS/SOCS family[126]. Csk has been shown to inhibit or downmodulate TCR signalling by the phosphorylation of regulatory tyrosines of Src family tyrosine kinases, which are crucial for T-cell activation[127]. Cbl-b is an adaptor protein that seems to negatively regulate T-cell activation by antagonizing CD28-mediated co-stimulatory pathways. So, T cells from *Cbl-b*-knockout mice are hypersensitive to low doses of T-cell stimulatory ligands and are, furthermore, relatively independent of CD28 in their activation[128,129]. Cabin is a molecule that seems to have several functions, including forming a scaffold for the coordination of transcription factors. Cabin was identified originally as a molecule that binds to and inhibits calcineurin, a crucial serine phosphatase that mediates TCR-dependent cytokine activation through the dephosphorylation of nuclear factor and activator of transcription c (NFAT-c), which is crucial for nuclear translocation[130]. The calcineurin-inhibiting portion of Cabin has been localized and is an interesting target for pharmacological intervention.

New frontiers in molecular immunotherapy

The accessibility of the immune system, together with its central role in so many disease processes, makes it highly appropriate for therapeutic intervention. Until recently, immunotherapies tested clinically have been fairly crude, failing to take advantage of our knowledge of the molecular pathways that regulate immunity. Not surprisingly, these approaches have largely failed to eliminate cancers or persistent pathogenic infections, which become established because they have developed mechanisms to render tolerant, inactivate or escape detection by natural immune responses. As is the case with infectious organisms, different tumours will probably be susceptible to different immune effector mechanisms. So, qualitative, as well as quantitative, elements of activation must be engineered into immunotherapies that match the susceptibility of the tumour or pathogen being treated. The robust efforts in the molecular profiling of cancers and pathogens, undertaken largely to define susceptibilities to drug therapy, should also provide invaluable information for the design of immunotherapies.

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