

Evolving synergistic combinations of targeted immunotherapies to combat cancer

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Abstract | Immunotherapy has now been clinically validated as an effective treatment for many cancers. There is tremendous potential for synergistic combinations of immunotherapy agents and for combining immunotherapy agents with conventional cancer treatments. Clinical trials combining blockade of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) may serve as a paradigm to guide future approaches to immuno-oncology combination therapy. In this Review, we discuss progress in the synergistic design of immune-targeting combination therapies and highlight the challenges involved in tailoring such strategies to provide maximal benefit to patients.

Immune synapses

Transient cell–cell interactions of T lymphocytes that are highly structured to favour the organized interaction of surface receptors and are sustained by adhesion molecules and reorganization of the cytoskeleton and a centriole.

Surface receptors of immune cells mediate intercellular communication and frequently operate in transient but well-structured cell–cell contacts known as immune synapses¹ (FIG. 1). A major class of these receptors, checkpoint receptors², inhibits the development or execution of effector functions of killer and pro-inflammatory lymphocytes. These mechanisms probably evolved to limit damage to non-infected cells in virally infected tissues and to attenuate excessive systemic inflammation and prevent the development of autoimmunity. However, these mechanisms are also being hijacked by tumours to avoid immune attack.

As well as mediating intercellular communication, surface receptors can also stimulate killer lymphocytes in coordination with clonally distributed antigen receptors (T cell receptors (TCRs)). Monoclonal antibodies (mAbs), through their bivalency and their ability to interact with receptors for their Fc fragments on neighbouring cells, can crosslink activating receptors and act as agonists^{3–5}.

The approval of ipilimumab (a mAb that inhibits the checkpoint receptor cytotoxic T lymphocyte-associated antigen 4 (CTLA4)) for advanced-stage melanoma has validated the concept of using mAbs⁶ to activate antitumour immunity^{7,8}. Agonistic mAbs are also being developed for cancer immunotherapy and have shown some success in early-phase clinical trials.

In fact, multiple strategies for eliciting and enhancing antitumour immunity have been developed and are in the clinic^{9,10} (BOX 1). There is currently much focus on synergistically combining cancer immunotherapies

to modulate immune outcomes. In pharmacology, the concept of synergy refers to a combination of agents having effects that are superior to what would be expected, in the dose–response curve, from adding their separate effects. Owing to mechanistic interactions, drugs with no single-agent activity can cooperate with other interventions to improve efficacy. In cancer immunotherapy, two parameters can help to define synergy: the intensity of the elicited measurable immune response against cancer; and the actual reduction (or disappearance) of tumour lesions.

Synergistic combinations of immunotherapies have largely been empirically developed by translating results from mouse models into clinical trials, the key example being the effective treatment of patients with melanoma by combining antibodies against the checkpoint receptors CTLA4 and programmed cell death protein 1 (PD1)¹¹. Preclinical and clinical synergies have also been observed for immuno-oncology agents in combination with chemotherapy¹², targeted therapies¹³, radiotherapy^{14,15}, anti-angiogenic agents^{16,17} and partial surgical resections¹⁸. The field is developing rapidly, and the goal is to move from an era of empirical combinations to one of rational design by considering the compatibility of mechanisms that interact synergistically, either to mediate antitumour efficacy or to reduce on-target side effects¹⁹. In addition, tackling immune-escape strategies evolved by the tumour may necessitate adding agents to the combinations²⁰. New combinations are being identified almost monthly, to the point that the colour-coding diagram included in various previous

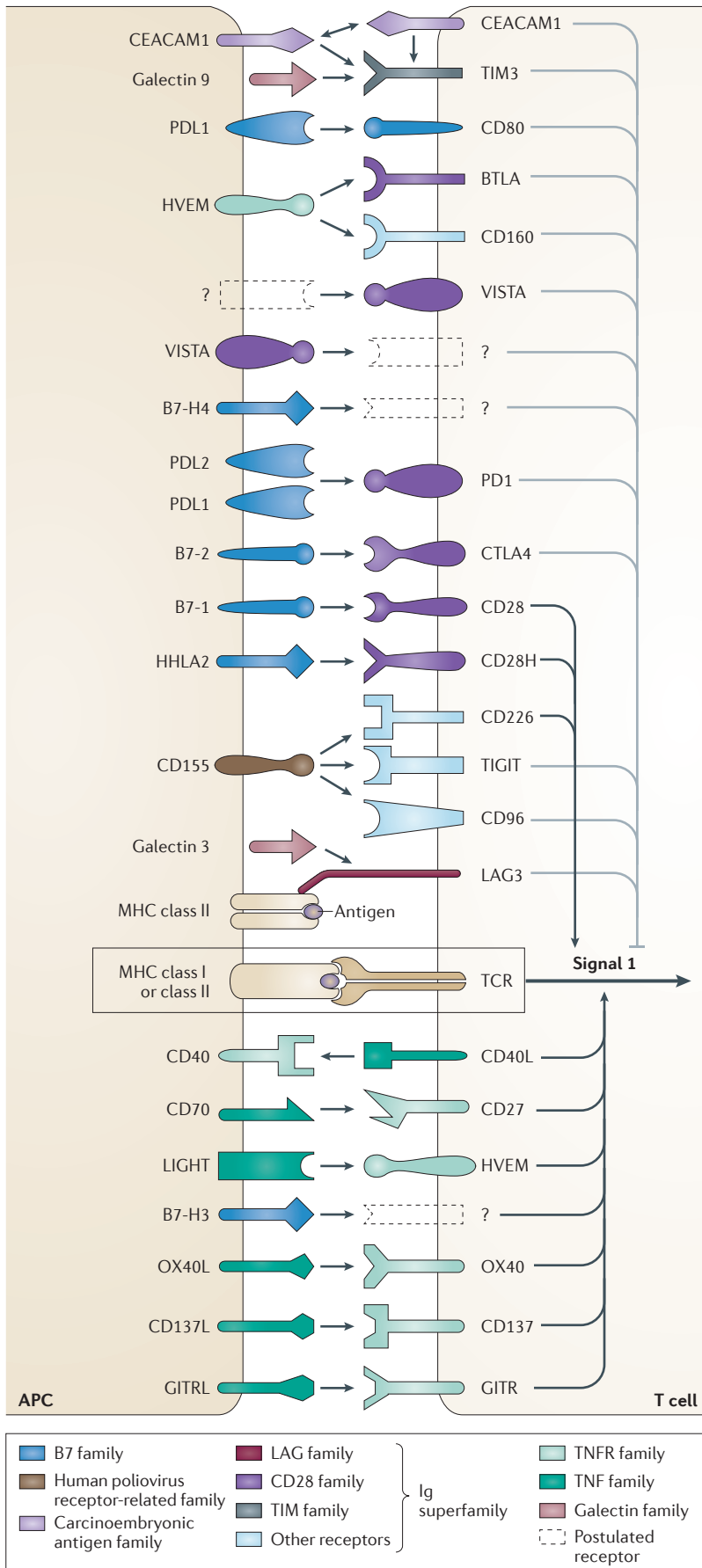
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reviews^{6,21} becomes almost instantly outdated⁶ (FIG. 2). The other field that is revolutionizing immunotherapy of cancer is adoptive T cell therapy^{22,23}, mainly in the form of T cells that are genetically modified to express chimeric antigen receptors against malignancies derived from transformed B cells, such as leukaemia and lymphoma²³. Notably, there is preclinical evidence that adoptive T cell therapy and immunostimulatory mAbs can be synergistically combined^{24,25}. This Review summarizes the key elements and concepts of immunotherapy combinations and the challenges to the rational design of synergistic combination strategies involving immune modulators.

Targeting immunomodulatory pathways

The multiple natural negative feedback mechanisms that fine-tune the adaptive immune response, by activating or inhibiting T cell or natural killer (NK) cell function, provide pathways by which tumours can escape from the immune system. However, they also offer multiple opportunities for therapeutic intervention^{1,9}.

Inhibitory immune checkpoints. CTLA4 is expressed by activated T cells and regulatory T cells (T_{Reg} cells). Binding of CTLA4 to its ligands (B7-1 (also known as CD80) and B7-2 (also known as CD86)) on antigen-presenting cells (APCs) leads to inhibition of T cells^{26,27}. CTLA4-specific mAbs may bias the competition for ligands between CTLA4 and its co-stimulatory homologue, CD28, towards the latter.

◀ **Figure 1 | Receptor–ligand pairs of the immune system that are amenable to pharmacological manipulation with immunostimulatory monoclonal antibodies.** This is a representation of the receptor and cognate ligands on juxtaposed cells forming immune synapses (antigen-presenting cell (APC) on the left and T cell on the right). The co-stimulatory (black pointed arrows) or co-inhibitory (grey inhibitory arrows) outcome of receptor ligation is provided for each interaction. Signal 1 refers to antigen-specific recognition by the T cell receptor (TCR). It is of note that these interactions take place in transient cell-to-cell interactions, named immune synapses¹, in which the surface of interaction between cells is highly structured, permitting complex levels of crosstalk among receptors. B7-H3, B7 homologue 3 (also known as CD276); B7-H4, B7 homologue 4 (also known as VCTN1); BTLA, B lymphocyte and T lymphocyte attenuator; CD28H, CD28 homologue; CD40L, CD40 ligand; CD137L, CD137 ligand (also known as TNFSF9); CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CTLA4, cytotoxic T lymphocyte-associated antigen 4; GITR, glucocorticoid-induced TNFR family-related protein; GITRL, GITR ligand; HHLA2, HERV-H LTR-associating 2 (also known as B7-H7); HVEM, herpes virus-entry mediator (also known as TNFRSF14); Ig, immunoglobulin; LAG3, lymphocyte activation gene 3 protein; LIGHT, HVEM ligand (also known as TNFSF14); MHC, major histocompatibility complex; OX40L, OX40 ligand (also known as TNFSF4); PD1, programmed cell death protein 1; PDL, PD1 ligand; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM3, T cell Ig mucin domain-containing 3; TNF, tumour necrosis factor; TNFR, TNF receptor; VISTA, V-domain Ig suppressor of T cell activation (also known as PD1 homologue). Updated figure from REF. 6, Nature Publishing Group.

T cell receptors

(TCRs). Antigen-specific surface receptors on T lymphocytes that recognize peptides (antigens) bound to self-antigen-presenting molecules of the major histocompatibility complex. Clonally distributed repertoires of TCRs result from random gene rearrangement and are shaped by processes of positive and negative selection in the thymus.

Fc

Crystallizable fragment of immunoglobulins that corresponds to the constant regions of the heavy chains, which convey biological properties to the antibody but are not involved in antigen recognition.

Regulatory T cells

(T_{Reg} cells). A special subset of T cells that function to repress the activation of other T lymphocytes. The main T_{Reg} cell subset is characterized by expression of the transcription factor forkhead box P3.

They may also function through other mechanisms, such as partially depleting T_{Reg} cells in the tumour microenvironment^{28,29} and interfering with the CTLA4-mediated sequestration of co-stimulatory ligands at immune synapses³⁰. As well as in metastatic melanoma^{7,31}, CTLA4 checkpoint inhibition with ipilimumab has confirmed clinical activity for other tumours³². Tremelimumab³³ is another CTLA4-specific antibody that is in development for melanoma and other malignant diseases.

The receptor PD1 is expressed on activated T cells, B cells, NK cells and T_{Reg} cells³⁴. PD1 has two identified ligands: PDL1 (also known as B7-H1; also has affinity for CD80 (REF. 35)) and PDL2 (also known as B7-DC). PD1 signalling contributes to T cell exhaustion³⁶, and tumours may exploit this pathway, as PDL1 is expressed on many solid tumours and is associated with a poor outcome^{37–39}. Antibodies that inhibit the interaction between PD1 and its ligands have shown promising results in various tumour types. PD1-specific agents include nivolumab³⁸, pembrolizumab^{40–43} and pidilizumab (CT-011)⁴⁴; PDL1-specific agents include atezolizumab (MPDL3280A)⁴⁵, MEDI4736 (ClinicalTrials.gov identifiers NCT02087423 and NCT01693562) and MSB0010718C (NCT01772004, NCT01943461 and NCT02155647); and PDL2-specific agents include rHlgM12B7 (NCT00658892).

Lymphocyte activation gene 3 protein (LAG3; also known as CD223) and other inhibitory receptors are progressively expressed on T cells during exhaustion^{46,47}. Until recently, the only ligands identified for LAG3 were major histocompatibility complex (MHC) class II molecules, but evidence has shown that it can also be bound by galectin 3 with functional consequences⁴⁸. The expression of LAG3 on tumour-infiltrating T_{Reg} cells

and CTLs suggests that it may be involved in immune evasion by tumours; thus, blocking LAG3 may reverse T cell exhaustion and enhance antitumour immunity^{47,49}. BMS-986016, a LAG3-specific mAb, is in clinical development (NCT01968109 and NCT02061761).

T cell immunoglobulin and mucin domain-containing 3 (TIM3; also known as HAVCR2)⁵⁰ is expressed on T helper 1 ($T_{\text{H}}1$) cells and CTLs, but also on innate immune cells such as dendritic cells (DCs)⁵¹; its function differs depending on the cell type in which it is expressed. Expression of TIM3 by tumour-infiltrating lymphocytes (TILs) is common in melanoma⁵² and non-small-cell lung cancer (NSCLC)⁵³, and it is thought to keep the lymphocyte status inactive or even to induce apoptosis upon ligation to galectin 9 or other, as yet undefined, ligands⁵⁴. A functional interaction with carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) has also been described⁵⁵.

Co-stimulatory receptors. CD137 (also known as 4-1BB and TNFRSF9) is a potent T cell and NK cell co-stimulatory receptor that is expressed at the cell surface following lymphocyte activation⁵⁶. Promoting its signalling improves cytotoxic antitumour responses⁵⁷ and T cell survival^{58,59}. Urelumab and PF-05082566 (REF. 60) are agonistic CD137-specific mAbs that are under evaluation for various malignancies. Importantly, agonistic CD137-specific mAbs may enhance NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC)⁶¹. Data from mouse models has shown that tumour cells coated with tumour-targeted mAbs induced the upregulation of CD137 expression on NK cells^{62–64}. Subsequent addition of an agonistic CD137-specific mAb increased NK cell degranulation and tumour lysis. Therefore, pre-clinical evidence predicts powerful synergistic effects of CD137-specific mAbs when combined with rituximab (a mAb specific for CD20), trastuzumab (a mAb specific for human epidermal growth factor receptor 2 (HER2; also known as ERBB2)) or cetuximab (a mAb specific for epidermal growth factor receptor (EGFR))^{62–64}.

Glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR; also known as TNFRSF18) is a co-stimulatory molecule that reverses T_{Reg} cell-mediated suppression of T cells and activates proliferation and effector functions in CD4⁺ and CD8⁺ T cells^{65,66}. Activating GITR may overcome self-tolerance, reverse T_{Reg} cell-mediated suppression and enhance antitumour immune responses^{67–70}. The agonistic GITR-specific mAbs TRX518 and MK-4166 are undergoing Phase I evaluation (NCT01239134 and NCT02132754).

OX40 (also known as TNFRSF4) is a co-stimulatory receptor expressed primarily on activated CD4⁺ and CD8⁺ T cells; it enhances antitumour immune responses by promoting T cell proliferation and survival^{71–73}. OX40 agonists enhanced antitumour immunity by inhibiting T_{Reg} cells and promoting T cell survival in preclinical mouse models^{71,72}. Several agonistic OX40-specific mAbs are under clinical evaluation: namely, MEDI6469 (NCT01862900 and NCT01303705)⁷⁴, MEDI6383 (NCT02221960), MEDI0562 (NCT02318394) and MOXR0916 (NCT02219724).

Box 1 | Immunotherapies that are approved or in development**Vaccines**

- Dendritic cell-based vaccines
- Autologous granulocyte–macrophage colony-stimulating factor (GM-CSF)-transfected vaccines
- Viral vector vaccines
- mRNA-based vaccines
- Multi-peptide-based vaccines
- Locally released virotherapy

Targets of modulatory monoclonal antibodies

- Cytotoxic T lymphocyte-associated antigen 4 (CTLA4)
- Programmed cell death protein 1 (PD1)
- PD1 ligand 1 (PDL1)
- CD137
- OX40
- Lymphocyte activation gene 3 protein (LAG3)
- T cell immunoglobulin and mucin-domain containing 3 (TIM3)
- Glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR)
- CD27

Adoptive T cell therapy

- Tumour-infiltrating lymphocytes
- Chimeric antigen receptors (CARs)
- CAR-transduced T lymphocytes

Antigen-presenting cells (APCs). Cells that have the main function of presenting antigens to T lymphocytes. The most efficient APCs belong to the various lineages of dendritic cells.

T cell exhaustion
A dysfunctional state of T cells characterized by the inability to proliferate and exert effector functions while still viable.

CD40 (also known as TNFRSF5) is a stimulatory surface receptor of the tumour necrosis factor receptor (TNFR) family; it promotes the activation of APCs and enhances their co-stimulatory and antigen presentation activities, leading to T cell activation^{75,76}. CD40 enables APCs to prime CD8⁺ T cells that then differentiate into CTLs. An agonistic CD40-specific antibody, CP870,893, is under evaluation⁷⁷.

CD27 is a receptor of the TNFR superfamily that is expressed on resting and naive T lymphocytes but not on fully differentiated effector T cells. It is also expressed

on a subset of NK cells⁷⁸. Binding of CD27 to its ligand, CD70, on activated APCs enhances T cell activation, effector function, maturation, survival and long-term memory of the CD27-expressing cell. CD27 has a role in enhancing NK cell proliferation and cytotoxicity, and in B cell activation and immunoglobulin synthesis^{79–85}. CDX-1127, a CD27-directed mAb, is under clinical development (NCT01460134).

Killer inhibitory receptors. Inhibitory killer cell immunoglobulin (Ig)-like receptors (KIRs) negatively regulate the cytotoxic activities of NK cells⁸⁶ and some T cell subsets. KIRs recognize self-MHC class I molecules on cells and can inhibit NK cell activation. The loss or downregulation of self-MHC class I molecules (as occurs in most tumour cells) is sufficient to induce NK cell sensitivity: this is known as the ‘missing self-recognition model’ (REFS 87,88). However, tumour cells that retain proper MHC class I expression can evade immune surveillance by NK cells and thereby escape subsequent immune-mediated destruction. The use of KIR-specific mAbs for cancer therapy is under evaluation: lirilumab (also known as BMS-986015 and IPH2101) is a pan-specific KIR mAb designed to block multiple KIR family members⁸⁹. NK cells and certain T lymphocyte subsets express other cytotoxicity-inhibiting receptors, such as NKG2A (also known as KLRC1)⁹⁰ and CD96 (also known as TACTILE)⁹¹, which are also potential targets for cancer immunotherapy.

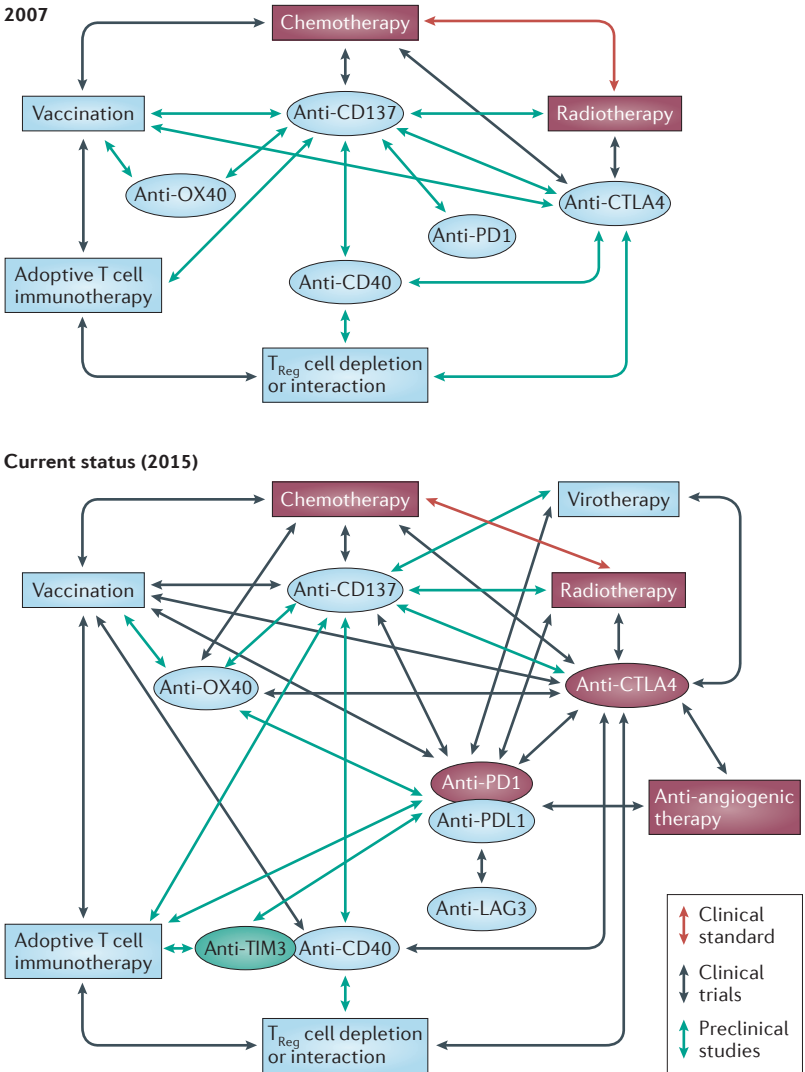


Figure 2 | The rapid evolution of combination immunotherapy. Schemes representing combinations tested in immunotherapy involving immunostimulatory monoclonal antibodies. Combinations are represented by colour-coded, two-headed arrows. Green arrows represent preclinical evidence of synergy in mouse models, blue arrows represent agents or combinations under evaluation in clinical trials and red specifies agents approved for at least one indication. The schemes compare the current statuses of immunotherapy combinations with those in 2007, illustrating the fast progress that is being made. CTLA4, cytotoxic T lymphocyte-associated antigen 4; LAG3, lymphocyte activation gene 3 protein; PD1, programmed cell death protein 1; PDL1, PD1 ligand 1; TIM3, T cell immunoglobulin and mucin domain-containing 3; T_{Reg} cell, regulatory T cell. Scheme shown for 2007 (upper part of figure) from REF. 6, Nature Publishing Group.

Combination immunotherapies for cancer
The breadth of immunotherapies in development provides multiple opportunities for combining — simultaneously or sequentially — therapies with distinct but potentially complementary mechanisms of action^{8,32,92–99} (FIG. 3; TABLE 1). Successes of PD1 and PDL1 blockade in monotherapy regimens suggest that these agents will be preferred as the primary building blocks for combinations (FIG. 4). The discovery of synergistic combinations stems from careful preclinical appraisal^{100,101} (FIG. 5a) and leads to stepwise testing of the simultaneous or sequential combinations in humans^{94,99} (FIG. 5b,c,d).

Combining immune checkpoint inhibitors. Combination therapy to block more than one immunomodulatory pathway may further enhance the antitumour efficacy of each individual treatment²¹.

The best-studied combination is that of CTLA4-specific and PD1-specific inhibitory mAbs, which have shown marked antitumour activity in many, but not all, tumour models^{102–104}. These negative regulators affect different signalling pathways within T cells^{105,106}, suggesting that these therapeutic mAbs could synergize (for example, through enhancing CTL effector activity). Recent data have shown that ipilimumab treatment may lead to broadening of the melanoma-specific killer T cell response¹⁰⁷, pointing towards a role in the priming of cancer-specific T cell immunity. In addition, evidence is emerging from mouse models showing that CTLA4-specific antibodies can promote the depletion of T_{Reg} cells specifically in tumours^{28,29,108}, as well as repress T_{Reg} cell functions¹⁰⁹.

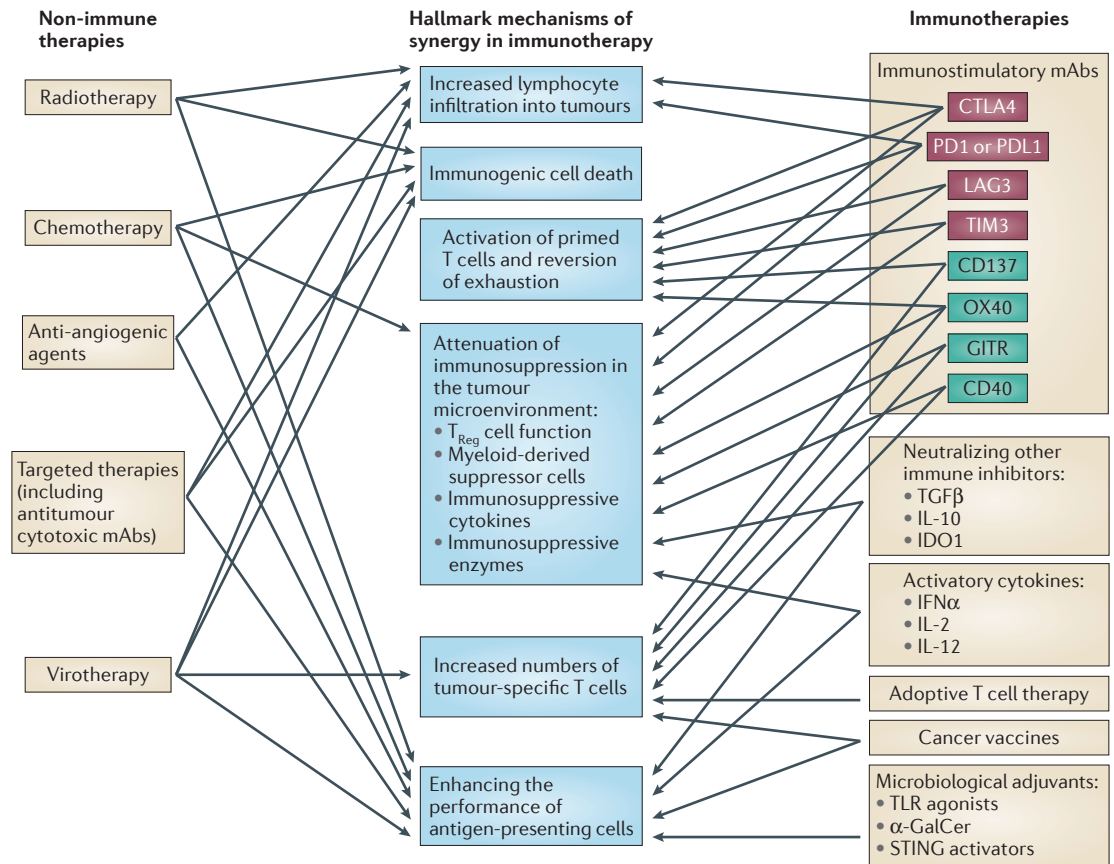


Figure 3 | Schematic representation of the main mechanisms of action postulated to mediate synergistic effects of combined immunotherapies. Non-immune cancer therapies (left) and the main immunotherapies under clinical development (right) are represented. Such therapies are linked with arrows to six mechanisms of action (centre) that are postulated to be involved in mediating synergistic effects. Immunogenic cell death is defined as a series of mechanisms set in motion by tumour cells undergoing apoptosis or necrosis that result in enhanced immunity. This enhanced immunity is due to the release of antigens and pro-inflammatory factors that dendritic cells interpret to activate T cell responses (reviewed in REF. 92). Infiltration of effector and suppressive leukocytes into tumours is controlled by the vascular endothelium, and these migratory functions are affected by immune and non-immune therapies (reviewed in REF. 93). Activation of effector T cells to proliferate and exert their functions is counteracted by suppressive mechanisms in the tumour microenvironment that include regulatory T (T_{Reg}) cells, pro-tumoural macrophages and myeloid-derived suppressor cells, as well as a milieu shaped by immunosuppressive cytokines and metabolites. The performance and activation of professional antigen-presenting cells is amenable to enhancement by systemic and locally released treatments. Increasing the number of effector T lymphocytes that recognize tumour antigens is achieved by promoting their proliferation or inhibiting their attrition by apoptosis. Numbers of these antigen-specific T cells can be grossly increased artificially by the adoptive transfer of cultured T lymphocytes. The number of arrows is testament to the strong potential for synergistic effects involving various complementary mechanisms. Green boxes show targets of co-stimulatory monoclonal antibodies (mAbs) and red boxes show targets of inhibitory mAbs. α GalCer, α -galactosylceramide; CTLA4, cytotoxic T lymphocyte-associated antigen 4; GITR, glucocorticoid-induced tumour necrosis factor receptor family-related protein; IDO1, indoleamine-2,3-dioxygenase 1; IFN α , interferon- α ; IL, interleukin; LAG3, lymphocyte activation gene 3 protein; PD1, programmed cell death protein 1; PDL1, PD1 ligand 1; STING, stimulator of IFN genes; TGF β , transforming growth factor- β ; TIM3, T cell immunoglobulin and mucin domain-containing 3; TLR, Toll-like receptor.

Major histocompatibility complex (MHC). A genomic locus encoding the main transplant antigen molecules that function to present peptide antigens to T lymphocytes. MHC class I molecules present antigens to CD8⁺ T lymphocytes, and MHC class II molecules present antigens to CD4⁺ T lymphocytes.

Autologous
Obtained from the same individual.

Antibody-dependent cellular cytotoxicity (ADCC). An immune mechanism of cell killing that is mediated by natural killer cells and macrophages and that leads to the destruction of antibody-coated target cells.

Interference with inhibitory pathways in effector T cells and the elimination of immune-suppressive cells such as T_{Reg} cells are currently thought to be the dominant mechanisms for enhanced antitumour activity by combined targeting of CTLA4 and PD1. However, the central role of inhibitory effects of PD1 expression in T cells that are chronically stimulated by antigen (along with the possible co-expression of CTLA4 on these cells) suggests that the combination has other possible mechanisms of action. Multiple repressive receptors such as CTLA4, TIM3 and

LAG3 may cooperate with PD1 to maintain an exhausted phenotype of T cells, and thus combinations of blocking antibodies will be required to overcome this cooperation.

A Phase I clinical trial of ipilimumab and nivolumab in patients with metastatic melanoma⁹⁴ explored a dose escalation of nivolumab (starting at 0.3 mg per kg) added to the standard dose of ipilimumab (3 mg per kg once every 3 weeks for 4 doses). The maximum tolerated dose was declared at 3 mg per kg of each antibody, and impressive clinical responses were observed in patients

Table 1 | Outcomes from key clinical trials of immunotherapies in combination regimens

Agents	Indication	Regimen or design	n	Overall response (CR and PR)	Survival	Refs
Ipilimumab and nivolumab	Advanced-stage untreated melanoma	Nivolumab alone versus nivolumab plus ipilimumab versus ipilimumab alone	945	<ul style="list-style-type: none"> • 44% nivolumab • 58% ipilimumab plus nivolumab • 19% ipilimumab 	NA	99
Ipilimumab and nivolumab	Advanced-stage melanoma	Concurrent or sequential combination with dose escalation	53	42%	OS rate: <ul style="list-style-type: none"> • 85% 1-year • 79% 2-year 	94
Ipilimumab and nivolumab	Advanced-stage untreated melanoma	Ipilimumab plus nivolumab versus ipilimumab alone	142	<ul style="list-style-type: none"> • 61% ipilimumab plus nivolumab • 11% ipilimumab 	Median PFS: <ul style="list-style-type: none"> • 4.4 months for ipilimumab • Not reached for ipilimumab plus nivolumab 	98
Ipilimumab and bevacizumab	Advanced-stage melanoma	Concurrent combination with escalating doses	46	19.6%	Median OS: 25.1 months	95
Ipilimumab and GP100 vaccine	Previously treated advanced-stage melanoma	Ipilimumab alone versus ipilimumab plus vaccine versus vaccine alone	676	<ul style="list-style-type: none"> • 10.9% ipilimumab alone • 5.7% ipilimumab with vaccine • 1.5% vaccine alone 	Median OS: <ul style="list-style-type: none"> • 10.1 months for ipilimumab alone • 10.0 months for ipilimumab plus vaccine • 6.4 months for vaccine alone 	7
Ipilimumab and dacarbazine	Treatment-naive advanced-stage melanoma	Ipilimumab plus dacarbazine versus dacarbazine alone	502	<ul style="list-style-type: none"> • 15.2% ipilimumab with dacarbazine • 10.3% dacarbazine alone 	Median OS: <ul style="list-style-type: none"> • 11.2 months for ipilimumab plus dacarbazine • 9.1 months for dacarbazine alone 	8
Ipilimumab and radiotherapy (single fraction)	Post-docetaxel CRPC	Ipilimumab plus radiotherapy versus placebo plus radiotherapy	799	NA	Median OS: <ul style="list-style-type: none"> • 11.2 months for radiotherapy followed by ipilimumab • 10.0 months for radiotherapy followed by placebo 	32
Carboplatin plus paclitaxel and ipilimumab	NSCLC	Sequential or concurrent schedule versus control	204 (68 sequential and 136 concurrent)	<ul style="list-style-type: none"> • 32% irBORR ipilimumab • 18% sequential chemotherapy control 	Median irPFS: <ul style="list-style-type: none"> • 5.7 months for ipilimumab • 4.6 months sequential chemotherapy control (P=0.05) 	96
Carboplatin plus paclitaxel and ipilimumab	ED-SCLC	Sequential or concurrent schedule	130 (42 sequential)	<ul style="list-style-type: none"> • 71% irBORR ipilimumab • 53% sequential chemotherapy control 	Median irPFS: <ul style="list-style-type: none"> • 6.4 months for ipilimumab • 5.3 months for sequential chemotherapy control (P=0.03) 	97

CR, complete response; CRPC, castrate-resistant prostate cancer; ED-SCLC, extensive-disease small-cell lung cancer; irBORR, immune-related best overall response rate; irPFS, immune-related progression-free survival; NA, not available or not presented; NSCLC, non-small-cell lung cancer; OS, overall survival; PR, partial response.

treated with the lowest dose levels. In the combination group receiving nivolumab at a dose of 1 mg per kg and ipilimumab at a dose of 3 mg per kg, 40 patients were treated and 53% of these patients achieved objective responses, with 28% of patients showing an 80% or greater tumour reduction after the first scans (within 12 weeks)⁹⁴. For patients who received concurrent ipilimumab and nivolumab across all doses, the 1-year and 2-year overall survival rates were notable at 85% and 79%, respectively⁹⁴, suggesting that the activity of the combination was superior to that of either agent alone.

Combination therapy also led to an increase in the frequency of adverse events compared with prior experience with either antibody alone, in particular with nivolumab (for example, 61% of patients reported grade III adverse events in the group treated with nivolumab 1 mg per kg in combination with ipilimumab 3 mg

per kg)⁹⁴. Nevertheless, the variety of adverse events observed (including gastrointestinal effects such as diarrhoea and pathological inflammation of gut tissues) was within prior experience with these antibodies and consistent with a toxicology study of the combination in non-human primates (M. Selby, J. Engelhardt, L.-S. Lu, M. Quigley, C. Wang, B. Chen and A.J.K., unpublished observations). Interestingly, many of the grade III adverse events — in particular, those that led to establishing the maximum tolerated dose — were laboratory abnormalities without clinical correlates, such as asymptomatic elevation of lipase, amylase, alanine aminotransferase and/or aspartate aminotransferase.

Recently, the first results from a randomized, placebo-controlled Phase II study comparing ipilimumab plus nivolumab with ipilimumab alone were reported⁹⁸. A total of 142 patients without prior

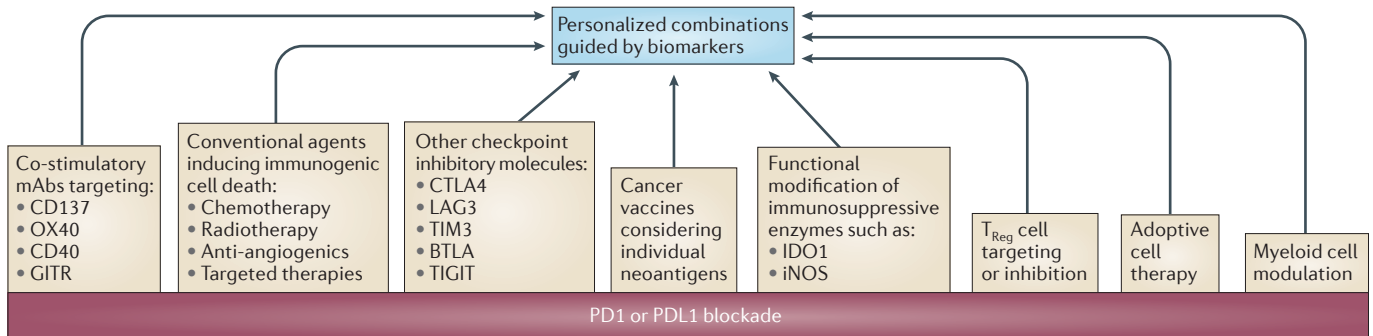


Figure 4 | Building immunotherapy combinations on the pillar of PD1 or PDL1 blockade, and steps in the development of an immunotherapy combination. A schematic representation of potential treatment combinations involving programmed cell death protein 1 (PD1) or PD1 ligand (PDL1) blockade. Given the clinical success of PD1–PDL1 blockade in multiple diseases, this pathway will probably become the foundation for immunotherapy combinations. Importantly, data from preclinical models show that PD1–PDL1 blockade acts synergistically with most, if not all, of the other immunotherapy modalities shown. Development and personalization of these PD1–PDL1 blockade combinations should be

guided by biomarkers. Additionally, triplets or higher order-of-magnitude combinations have the potential to further increase efficacy. BTLA, B lymphocyte and T lymphocyte attenuator; CTLA4, cytotoxic T lymphocyte-associated antigen 4; GITR, glucocorticoid-induced tumour necrosis factor receptor family-related protein; IDO1, indoleamine-2,3-dioxygenase 1; iNOS, inducible nitric oxide synthase; LAG3, lymphocyte activation gene 3 protein; mAb, monoclonal antibody; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains; TIM3, T cell immunoglobulin and mucin domain-containing 3; T_{Reg} cell, regulatory T cell.

systemic treatment were randomly assigned to the two groups, and results in patients with *BRAF* wild-type tumours (the largest group in this study) showed an objective response rate of 61% for the combination treatment and 11% for ipilimumab alone. Of the 72 patients with *BRAF* wild-type tumours assigned to the combination treatment, 22% experienced a complete remission, whereas there were no complete responders in the ipilimumab-alone arm. Clinical benefit in the combination arm was independent of PDL1 expression by tumour cells before treatment. By contrast, ipilimumab-induced responses were significantly more common in PDL1-positive tumours, similar to observations with PD1-specific therapy. The progression-free survival for patients treated with ipilimumab was 4.4 months and not yet reached for patients treated with ipilimumab plus nivolumab therapy (hazard ratio (HR) 0.40; $P < 0.001$). The adverse events observed in this trial were similar to those observed with the combination in the Phase I trial¹¹. These efficacy and biomarker results have recently been confirmed in a randomized, three-arm Phase III clinical trial in which patients received ipilimumab, nivolumab or the combination regimen of ipilimumab plus nivolumab⁹⁹. Although 945 patients were enrolled, the study was not statistically powered to compare progression-free survival and response rate between nivolumab alone and the combination. When the data are mature, comparative overall survival results from this clinical trial will be of paramount importance to demonstrate the full effect of combination immunotherapy. Adverse events of grade III or above were experienced by 68% of patients receiving the combination, compared with 43.5% and 55.6% of those receiving nivolumab alone and ipilimumab alone, respectively⁹⁹.

On the basis of these extremely encouraging efficacy results, the combination of ipilimumab and nivolumab has been or is being explored in Phase III studies in renal

cell carcinoma and NSCLC^{110,111}, and earlier-phase clinical trials are being conducted in various other tumour types (small-cell lung, triple-negative breast, pancreatic, gastric and bladder cancer). The combination of tremelimumab and a PDL1-specific antibody, MEDI4736, has also recently entered clinical testing (see [Supplementary information S1](#) (table)).

PD1–PDL1 pathway blockade may be viewed as a pillar for future immunotherapy combinations (FIG. 4), and the promising results of combining ipilimumab and nivolumab have spurred the combinations of other immune checkpoint inhibitors preclinically and clinically⁶ (FIG. 2). Attractive combinations include PD1-specific antibodies with specific antibodies against LAG3 or TIM3, both of which have shown synergism with PD1 blockade in mouse tumour models^{49,54}. The rationale underlying synergistic effects is that each dysfunctional antitumour T cell could be kept unresponsive by more than one repressor and could only be fully capable to exert its functions when completely released from checkpoint inhibition¹¹².

Combining checkpoint inhibitors and immunostimulatory mAbs. Another interesting combination approach involves delivering immunostimulatory mAbs with immune checkpoint inhibitors. These combinations pose new challenges for the clinical management of patients, and safety needs to be carefully addressed. A Phase I–II trial is underway to evaluate a combination of PD1-blocking agents with CD137-specific mAbs (NCT02253992). Although CD137-specific and PD1-specific mAbs are well tolerated as single agents, these studies will still need to be followed carefully for synergistic toxicity (for example, hepatitis). The combination of tremelimumab and the CD40-specific superagonist antibody CP-870,893 is also being examined in patients with metastatic melanoma (NCT01103635). In addition, trials are ongoing in

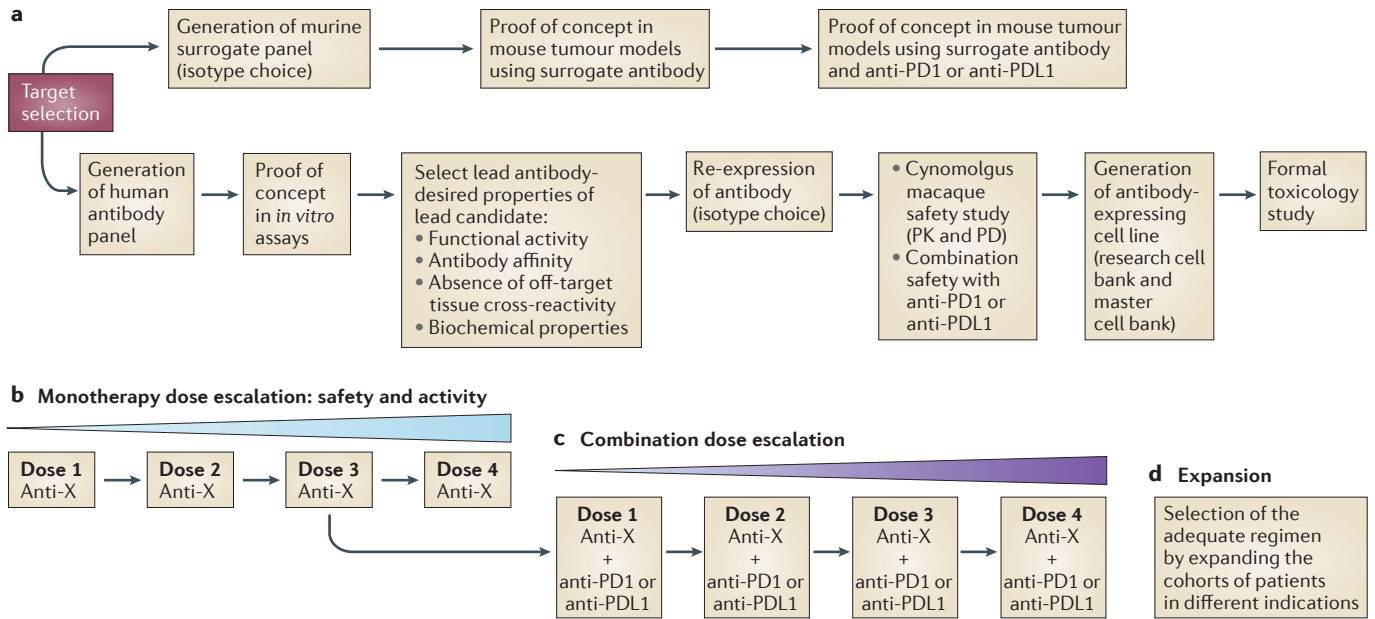


Figure 5 | Preclinical and clinical development of combinations of immunostimulatory monoclonal antibodies. The flow chart in part **a** shows the development of a therapeutic antibody to be used in combination therapy. Once a potential molecular target is identified, antibodies against the human molecule and the murine homologue are generated. *In vitro* functional assays demonstrating the desired activity of the antibody (for example, co-stimulation of T cell responses) are used to select a functional antibody. For testing in mouse models, various antibodies with different Fc regions would ideally be developed to allow evaluation of the importance of the Fc region in functional activity *in vivo*¹⁰⁰. Although transplantable subcutaneous tumour models may have limitations as a model system, such as the inability to mimic the specific tumour indication, these models do provide a measure of activity in immunological functions of the target, and efficacy can frequently be observed. Importantly, antitumour activity may not be observed when an agent is used as monotherapy, but combinations with programmed cell death protein 1 (PD1)–PD1 ligand (PDL1) pathway blockers may provide evidence of synergy. In parallel with or subsequent to proof of concept of the value of a target in mouse models, a panel of antibodies to the human molecule is evaluated. Aside from the desired functional activity to select a potential lead molecule, other essential qualities are required of a lead antibody for its selection in development, including the selection of the desired isotype choice. Toxicology testing is typically performed in cynomolgus macaques. Interestingly, cynomolgus macaques (and frequently the murine system) have not been informative for the ability to foretell adverse events for immuno-oncology products in humans¹⁰¹. Combination toxicology with PD1–PDL1 pathway blockers may be carried out to see whether the combination results in novel or apparent toxic effects that are not observed with either drug alone. These studies, along with pharmacokinetic (PK) and pharmacodynamic (PD) assessments (target engagement and functional activity *in vivo*), form the basis for selecting the initial dose in humans and proceeding to clinical studies. Panels **b**, **c** and **d** show how trial design could be standardized for combination evaluation^{94,99}. Once a dose level is declared safe, combinations with PD1–PDL1 blockade could be started in cohorts of patients entering at lower dose levels of the new agent.

patients with advanced-stage solid tumours to evaluate the combination of OX40-specific mAbs with checkpoint inhibitors (MEDI6469) plus tremelimumab or MEDI4737 (NCT02205333), and that of lirilumab with nivolumab (NCT01714739) or ipilimumab (NCT01750580). In these trials, additional clinical caution is recommended, as unexpected severe reactions could occur.

Our current knowledge of the crosstalk among co-inhibitory and co-stimulatory receptors is limited. However, multiple mechanisms for synergy arise when considering that the functions repressed by checkpoints are frequently induced by co-stimulatory molecules on the same target cells. Figuratively, it would be like releasing the brakes while acting on the gas pedal of a car^{92,93,113} (FIG. 3). Although combinations of mAbs targeting co-stimulatory molecules (such as CD137-specific

and GITR-specific antibodies) with PD1-specific or PDL1-specific antibodies work synergistically in mouse models, it is difficult to predict whether — and, if so, how — this would translate to the human situation. Therefore, these combinations should be evaluated carefully in early clinical trials.

Combining immunomodulation with conventional therapies. Conventional treatment regimens — such as chemotherapy, radiotherapy, targeted therapy and ADCC mediated by tumour-targeting antibodies — may, in causing tumour cell death, allow the release of tumour antigens for presentation and thus prime the immune system^{92,93,114–117} (FIG. 3). Therefore, it will be important to test the effects of combining immunomodulatory mAbs with these therapies. Reducing tumour burden using conventional therapies may also allow immunotherapy

to be more effective. A few key trials have been completed^{7,8,32,94–99} (TABLE 1), and numerous trials are ongoing (see [Supplementary information S1–S3](#) (tables)).

In the pivotal trials, ipilimumab was combined either with a GPI00 peptide vaccine or with the chemotherapeutic agent dacarbazine^{7,8}. Whereas the first combination did not add toxicity or benefit over ipilimumab alone, the dacarbazine and ipilimumab combination conferred a survival benefit over dacarbazine alone. Although mechanistically, dacarbazine, as a DNA-alkylating agent, could lead to tumour cell destruction, release of antigens and sensing by immune cells of tumour-derived DNA, its response rate is too low to expect an important synergistic effect. Therefore, the improved overall survival achieved by the combination compared with dacarbazine alone has been attributed solely to ipilimumab. However, this combination had a high rate of treatment discontinuations, mostly owing to increased levels of liver enzymes. This was considered to be the result of synergistic toxicity, as both drugs have intrinsic hepatotoxicity via different mechanisms: direct liver toxicity by dacarbazine and liver inflammation mediated by ipilimumab. Although the exact mechanism has not been clearly elucidated, this synergistic dose-limiting toxicity is a cautionary note for immuno-oncology combinations. Interestingly, data presented at the 2014 American Society of Clinical Oncology (ASCO) annual meeting showed that a combination of ipilimumab with temozolomide (a drug similar in structure to dacarbazine) did not have such a high rate of dose-limiting liver toxicity, as unlike dacarbazine, temozolomide is not metabolized by the liver¹¹⁸.

Hepatotoxicity resulting from combination therapy was also reported when the BRAF-V600E inhibitor vemurafenib was combined with ipilimumab in a small 10-patient Phase I study¹¹⁹. The rationale for this combination was that MAPK inhibitors not only induce tumour cell death but also lead to the upregulation of MHC class I molecules and melanocyte differentiation antigens, such as melanoma antigen recognized by T cells 1 (MART1), GPI00 and tyrosinase¹²⁰. In addition, BRAF inhibitors and ipilimumab monotherapy had been shown to improve presentation of tumour-specific antigens and increase the infiltration of lymphocytes into the tumour^{120,121}, also providing a rationale for this combined approach. Preclinical data in mice had shown that inhibiting wild-type BRAF in T cells has no detrimental effect on their viability and function, and even seemed to improve T cell function¹²². Although the BRAF-V600E inhibitor vemurafenib is known to induce skin toxicity and, more rarely, liver toxicity, grade III elevation of liver enzymes was observed in six of ten patients, and the study was stopped¹¹⁹. As ipilimumab has a half-life of 15 days, administration of vemurafenib within 4 weeks of the last dose of ipilimumab can be considered combination therapy. This may have resulted in an unexpectedly high and severe (grade III) skin rash in a small, sequential therapy study (all of the three treated patients showed skin rashes)¹²³. On biopsy, the rash was consistent with a drug reaction but not autoimmune dermatitis, so the exact pathogenesis remains unresolved. There is currently intensive investigation into PD1 blockade in combination with BRAF inhibitors

alone or with BRAF inhibitors and MEK inhibitors (see [Supplementary information S1](#) (table)). In biopsy samples taken shortly after the initiation of BRAF-V600E inhibition in patients with melanoma, there were clear signs of an influx of TILs¹²², providing a rationale for combining BRAF inhibitors with immunotherapies such as PD1 blockade.

Ipilimumab has also been combined with the vascular endothelial growth factor (VEGF) inhibitor bevacizumab, which inhibits tumour angiogenesis. In addition to its pro-angiogenic function, VEGF has immunomodulating properties, which include decreasing the influx of lymphocytes and DCs into the tumour, while increasing the intratumoural frequencies of T_{Reg} cells and myeloid-derived suppressor cells (MDSCs). Hodi *et al.*¹⁷ recently reported a trial combining ipilimumab and bevacizumab in patients with metastatic melanoma. In total, 46 patients were treated with this combination, and the efficacy was remarkably good, resulting in a median overall survival of more than 2 years. High-grade toxicity was more common than expected for either drug alone, but it was manageable and included hypophysitis, temporal arteritis, dermatitis and hepatitis. Interestingly, the combination led to an accumulation of CD8⁺ T cells and DCs in the tumour microenvironment — suggesting synergism of immunotherapeutic effector mechanisms — and warrants further investigation of this combination.

In radiotherapy, the *abscopal* effect describes a rare event of regression of metastatic lesions distant and outside of the irradiation field. A patient with metastatic melanoma who was treated with ipilimumab and then showed tumour progression at several sites was given radiotherapy to target one painful metastasis. Thereafter, the patient responded not only at the irradiated site but also at other lesions¹²⁴. The patient's tumour expressed the cancer-testis antigen NYESO1, and during ipilimumab treatment, the levels of NYESO1-specific antibodies in their serum slowly increased. After radiotherapy, antibody titres against various antigens rose rapidly concurrently with tumour regression, suggesting that an immunological response had been triggered by the radiotherapy while on ipilimumab maintenance treatment. This observation has recently been confirmed by others¹²⁵. Mechanistically, the combination of stereotactic radiotherapy followed by CTLA4 blockade was dependent on CD8⁺ TILs; resistance to the combination was predicted by a low CD8⁺ T cell/T_{Reg} cell ratio, expression of PDL1 by tumour cells and the presence of CD8⁺ T cells with an exhausted phenotype¹²⁶. Part of the resistance could be overcome by adding PDL1-specific antibodies to the combination of stereotactic radiotherapy and CTLA4 inhibition. In support of this, a study of a small number of patients receiving ipilimumab and radiotherapy showed evidence of benefit when some tumour lesions were irradiated with hypofractionated doses¹²⁶.

Managing combination-associated toxicity

Checkpoint-targeted immunotherapies can induce a new class of adverse effects as a result of supraphysiological immune activation that may overwhelm key organ tolerance mechanisms^{127–129}. These immune-mediated adverse

Hypophysitis

The anterior pituitary gland (adenohypophysis), when inflamed, can cause dysfunction and require substitutive therapy of the relevant hormones owing to insufficient function (panhypopituitarism).

Abscopal effect

A therapeutic effect of radiotherapy achieved outside the irradiated field in a patient with metastatic cancer. The term is also generalized to other local treatments with systemic effects.

Stereotactic radiotherapy

The precise delivery of ionizing radiation to tumour lesions by means of computer-assisted spatial localization to avoid damage to the surrounding non-tumoural tissue.

Commensal flora

Microbiota (for example, bacteria and fungi) that live on epithelial surfaces of an organism without causing harm or even exerting symbiotic functions.

Ectopic

In the context of this Review refers to the presence or expression of an antigen in an organ or cell type that does not normally express it.

Immune editing

The selection of less immunogenic cancer cell variants as a consequence of the evolutionary pressure of immune surveillance.

Neoantigens

Antigens of tumours that result from a mutation in an exon sequence, thus giving rise to a peptide presented by a self-human leukocyte antigen (HLA) molecule. These antigens are individual to the tumour of each patient and are not shared with other cases.

events mimic autoimmune diseases (such as dermatitis, inflammatory colitis, thyroiditis, hypophysitis and autoimmune hepatitis). However, they lack the chronicity that is often associated with true autoimmunity and, at least for gastrointestinal adverse events, seem to have different pathophysiology from classic inflammatory bowel disease¹³⁰. Clinically apparent dermal and mucosal inflammation might also result from overactive immune responses to antigens of the commensal flora. Each immunotherapeutic class of drugs is associated with adverse events¹⁰ that can be clinically managed but that in certain combinations might surpass the threshold of tolerability.

Studies of combined PD1 and CTLA4 blockade in melanoma and other tumour types suggest that, with sufficient clinical experience and appropriate management algorithms, immune checkpoint inhibitors can be safely given to patients^{11,110,111}. Little is currently known about the long-term effects of combination therapy, and whether a different range of immune-mediated toxic effects will manifest with chronic exposure. In the case of CTLA4 blockade, unexpected ectopic expression of CTLA4 has been reported in the adenohypophysis. This expression may explain the hypophysitis cases described during CTLA4-specific mAb treatment that occurred, at least in part, as a result of complement fixation by the mAb¹³¹.

Immunotherapy combinations can also be used to reduce the adverse events caused by monotherapy. Based on preclinical mouse models, it was hypothesized that combining granulocyte-macrophage colony-stimulating factor (GM-CSF) with ipilimumab may result in synergistic antitumour activity through increased inflammation of the tumour. In a recent Phase II clinical trial that assessed the combination of ipilimumab and subcutaneous GM-CSF, the incidence and severity of immune-mediated adverse events associated with ipilimumab were unexpectedly mitigated by this combination. Results from this randomized study showed improved 1-year survival and overall survival in patients with metastatic melanoma when compared with ipilimumab monotherapy⁹⁵. Interestingly, gastrointestinal toxic effects in particular were observed to be significantly less frequent and less severe in the combined treatment group compared with in patients treated with ipilimumab alone. As GM-CSF is important for the induction of immune regulation in the gut, the recombinant GM-CSF (sargramostim) may have functioned to protect the gut mucosa from ipilimumab-induced mucositis in this trial.

Biomarkers for combination immunotherapies

Challenges to identifying biomarkers. Predicting optimal immunotherapy combinations accurately and confirming their clinical activity in cancers will require the identification and validation of reliable surrogate biomarkers. Several approaches and data sources can be used for identifying and prioritizing immunotherapy combinations, but there are important considerations for each of them^{100,101} (FIG. 5a). Animal models have advantages in terms of their tractability and can be useful for identifying combination therapies. For example, syngeneic mouse tumour models showed synergy of CTLA4-specific and PD1-specific antibodies¹³² (M. Selby, J. Engelhardt, L.-S.

Lu, M. Quigley, C. Wang, B. Chen and A.J.K., unpublished observations) and, in a transplanted murine melanoma model, CTLA4-specific antibodies induced an increase in the number of PD1⁺ and PDL1⁺ TILs^{103,132}, providing a rationale for combining ipilimumab and nivolumab in patients¹¹. However, these models are unlikely to recapitulate the complex interactions between the human immune system and a heterogeneous tumour that has undergone immune editing¹³³.

The general consensus holds that many immunotherapy biomarkers are in the tumour microenvironment^{134–136} (FIG. 6a) and thus may require invasive repeated biopsies^{45,137–140}. Relying on immune parameters from peripheral blood is attractive owing to the ease of sampling, but it may not reflect the local tumour microenvironment where the immune contexture is crucial. In addition, potential biases may be introduced by the site of tumour sample collection. For example, samples of superficial cutaneous and lymph node metastases may be the easiest to collect; however, their biology may differ from the primary tumour or from metastases at other sites¹⁴¹. In melanoma tumour staging, the location of metastases is itself prognostic: visceral metastases are associated with a worse prognosis than non-visceral metastases¹⁴². In addition, PDL1 expression varies by melanoma location¹⁴³.

Evaluating post-treatment tumour samples is also crucial for identifying pharmacodynamic changes that are induced by immune-checkpoint inhibition. However, the timing of the biopsy may influence the results. Sampling at a fixed time point early after treatment may not allow enough time for immune activation, and bias may be introduced by the sample location, although the data could offer insight into the early steps in immune activation. An alternative approach would be to identify and sample lesions that are regressing or progressing regardless of time since therapy. Such an approach may capture the ultimate mechanisms of antitumour inflammation and/or tumour resistance.

Assessment of tumour samples by flow cytometry and functional analyses on cell suspensions are very difficult to carry out, primarily owing to the limitations of sample volume. However, these analyses may prove to be most informative, as they can provide functional data on specific immune cell types, rather than on the tumour as a whole), and they allow more accurate quantification than is generally achievable by using immunohistochemistry (IHC) and gene expression profiling. Technological developments using multicolour tissue immunofluorescence should be incorporated to maximize the information obtained from limited biopsy specimens. Recent examples include: a functional evaluation of the TIL response to neoantigens in a patient with advanced-stage melanoma who had a clinical response to ipilimumab¹⁴⁴; and the observation that ipilimumab treatment induced a significant number of newly detected T cell responses but only infrequently boosted pre-existing responses¹⁰⁷. More recently, similar correlative data have been found that indicate an association between the number of non-synonymous mutations giving rise to self-human leukocyte antigen (HLA)-presented tumour neoantigens and the response to pembrolizumab in patients with NSCLC¹⁴⁵ (BOX 2).

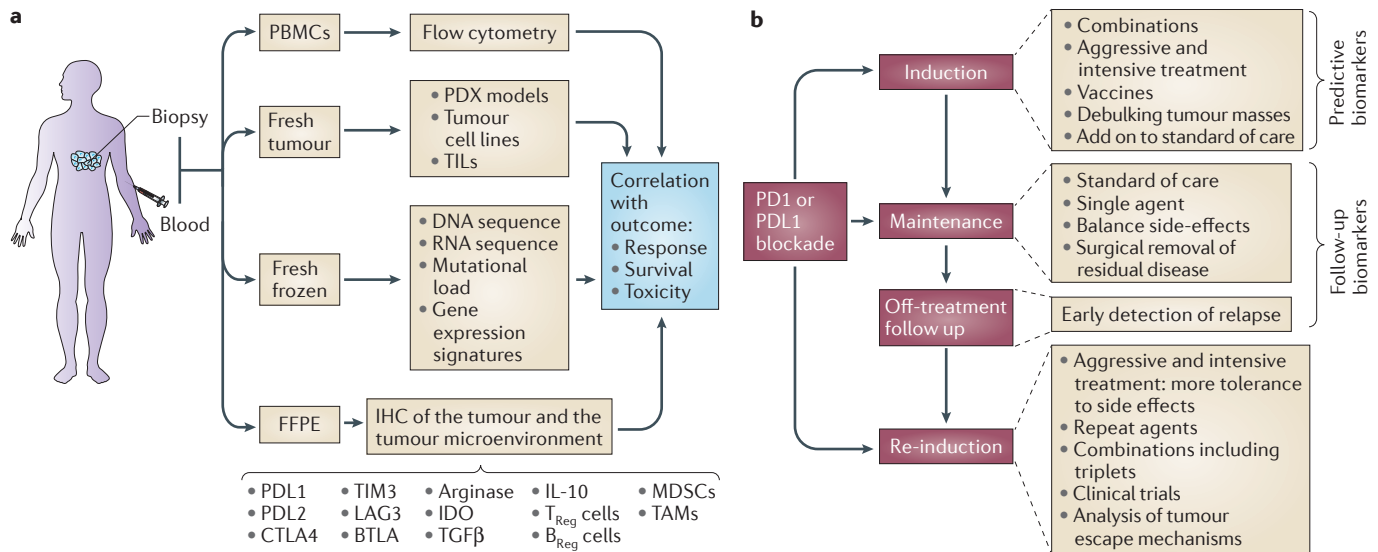


Figure 6 | Biomarker discovery for combination immunotherapy and proposed new concepts for clinical management with immunotherapy based on biomarkers. **a** | For biomarker studies, excisional or needle biopsy samples are taken from patients together with blood samples. Tumour tissue is processed either by enzymatic digestion followed by dimethyl sulfoxide freezing, by snap-freezing or by being formaldehyde-fixed and paraffin-embedded (FFPE). Blood samples undergo Ficoll density centrifugation for peripheral blood mononuclear cell (PBMC) isolation. Serum and granulocytes will be frozen. The digested tumour material will be used to grow patient-derived xenografts (PDXs) in non-obese diabetic (NOD) mice that have severe combined immunodeficiency and are deficient for the common γ -chain (also known as IL-2RG) to grow tumour cell lines and to isolate tumour-infiltrating lymphocytes (TILs). TILs will be subjected to flow cytometry studies, co-culture with tumour cell lines or tumour tissue digest, or used for adoptive transfer in PDX models. The fresh-frozen tumour tissue will be used for deep sequencing (both whole-exome DNA and whole-exome RNA sequencing, and for the development of gene expression signatures), and the FFPE material for analyses by immunohistochemistry (IHC) of the tumour and the tumour microenvironment (which involves staining for the receptors that are shown on the T cell in FIG. 1 and other cells in the microenvironment). PBMCs will be used for flow cytometry analyses. Results obtained from these analyses will be correlated with patient outcomes to find predictive biomarkers. **b** | Proposed new concepts for clinical management with immunotherapy. As we progress with immunotherapy, we face paradigm shifts regarding clinical management. Patients experiencing durable responses that are sustained even off treatment require new concepts in risk management and mitigation, while making the most of the clinical benefit. Overall, a phased approach can be envisioned in which aggressive combination regimens should achieve frequent clinical responses, to be followed by maintenance with less aggressive and safer regimens, reaching the point of weaning patients off treatment. Immunotherapy has the peculiarity of keeping patients in response off treatment^{134,135} and the possibility of re-induction¹³⁶, even with the same agents that were used in induction, with an important proportion of patients responding again. Identifying biomarkers will be crucial for optimal clinical management. BTLA, B lymphocyte and T lymphocyte attenuator; CTLA4, cytotoxic T lymphocyte-associated antigen 4; IDO1, indoleamine-2,3-dioxygenase 1; IL-10, interleukin-10; LAG3, lymphocyte activation gene 3 protein; MDSC, myeloid-derived suppressor cell; PD1, programmed cell death protein 1; PDL1, PD1 ligand; TAM, tumour-associated macrophages; TGFβ, transforming growth factor-β; TIM3, T cell immunoglobulin and mucin domain-containing 3; T_{Reg} cell, regulatory T cell.

In one of the responders to PD1 blockade, CD8⁺ T cells against a neoantigen expressed by the tumour became detectable after initiation of treatment, and the frequency measured in peripheral blood was highly correlated with the developing objective antitumour response, strongly suggesting a role for these T cells in the clinical benefit observed.

As detailed in BOX 2, the presence and abundance of non-synonymous somatic mutations that give rise to MHC-presented antigens are crucial to the effect of immunotherapies. This is the interpretation of the positive results from a recent Phase II clinical trial that was designed to evaluate the PD1-specific mAb pembrolizumab in a relatively small, but very informative, series of patients with colorectal and those with non-colorectal cancer whose tumours harboured mismatch-repair

deficiencies¹⁴⁶. In such mutation-prone cases, PD1-specific therapy achieved a much more superior overall response rate and higher rates of progression-free survival than in cases without mismatch-repair deficiencies.

Therapeutic biomarkers in the tumour microenvironment. For many cancer types, the presence of immune infiltrates — especially T cells — is associated with improved survival, as it illustrates the presence of an ongoing immune response^{147–151}. An inflammatory tumour microenvironment, especially the presence of CD8⁺ T cells at the invasive tumour margins, may predict a response to immunotherapy with PD1-specific or PDL1-specific antibodies^{45,138}. Other immune cell types recruited to the tumour can suppress an anti-tumour immune response. Tumours are rendered

resistant to attack owing to the expression of PDL1 and the production of transforming growth factor- β (TGF β), indoleamine 2,3-dioxygenase 1 (IDO1) and other immunosuppressive compounds and molecules by tumour-associated macrophages (TAMs), immature tumour-associated DCs (TADCs), T_{Reg} cells, interleukin-10-producing regulatory B cells, MDSCs and tumour cells themselves^{152–154}. The presence of some of these immune-inhibitory molecules — in particular, high expression of PDL1 by tumour immune infiltrates (as well as high expression of CTLA4 and fractalkine (also known as CX3CL1)) — seem to predict responses to PD1- or PDL1-specific treatment in several tumour types, including bladder cancer, NSCLC, melanoma and renal cell carcinoma; it also reflects interferon- γ (IFN γ) expression by tumour-specific T cells, hence representing an ongoing immune response^{45,155}. IHC and genetic profiling of the tumour microenvironment may be used to categorize cancers according to their immunosuppressive mechanisms and thus rationally treat them.

Tumour samples taken sequentially, before and after treatment, have been analysed to elucidate the mechanisms of action of CTLA4 and PD1–PDL1 inhibition¹⁰⁷, and such approaches of single immunotherapy may help to guide the selection of combination immunotherapies^{45,107,138,144,145,156–161} (TABLE 2). In these studies, the collection of baseline tumours was generally proximal to

administration of the CTLA4-, PD1- or PDL1-specific therapy. A randomized Phase II study of 82 patients with melanoma treated with 3 mg or 10 mg per kg ipilimumab showed that patients presenting objective responses or stable disease for ≥ 6 months tended to have higher expression of forkhead box P3 (FOXP3) and IDO1 at baseline, as determined by IHC, than patients without clinical activity^{156,157}. Gene expression profiling of the tumour samples showed that patients with clinical activity had higher baseline expression of immune-related genes than those without clinical activity¹⁵⁷. The association between IDO1 expression and benefit from ipilimumab may be validated by two ongoing clinical trials that are evaluating IDO1 inhibitors in combination with ipilimumab in patients with advanced-stage melanoma (NCT01604889 and NCT02073123).

Clinical studies have also shown that CTLA4 inhibition leads to increased numbers of TILs, as assessed by IHC^{156,160,162} and gene expression profiling¹⁵⁷. These TILs include memory T cells¹⁶² and probably result in an increase in the expression of the genes encoding IFN γ and other T_H1 cell-associated proteins¹⁵⁷. In addition, these studies also showed possible associations with clinical activity relative to baseline biopsies in TILs¹⁵⁶, CD8⁺ T cell/FOXP3⁺ T cell ratios¹⁶⁰ and the expression of immune-related genes¹⁵⁷. However, these studies have generally only identified associations and do not constitute identification of a definitive biomarker. Furthermore, the intratumoural spatial distribution of inflammation may be an important feature to characterize^{138,139}.

The combination of ipilimumab and nivolumab was supported by preclinical studies with surrogate antibodies^{103,132} and by the assessment of human tumours following treatment with ipilimumab or PD1-specific therapy, which showed an increase in the number of TILs and in the expression of IFN γ -inducible genes^{156,157}. The expression of CTLA4 in pretreated tumours also correlated with more frequent clinical responses to PDL1-specific treatment⁴⁵. In addition, analysis of metastatic melanoma samples showed that PDL1⁺ tumour cells colocalize with both TILs and IFN γ expression¹⁵³, and that pretreated tumours with elevated baseline expression of IFN γ -inducible genes — including *IDO1* — seem to respond to PDL1 blockade⁴⁵; this further supported clinical trials with combination ipilimumab and nivolumab therapy¹⁵³.

Although biomarkers can be used to develop algorithms that predict the probability of responses to immunotherapies (for example, in PDL1 and PD1 blockade in melanoma¹³⁸), the value of predictive markers may change markedly with combination therapy. For instance, in melanoma biopsy samples taken from patients before treatment PDL1 expression lost its partial predictive value at least in estimating overall response rate and progression-free survival in patients undergoing concomitant CTLA4 and PD1 blockade^{11,98,99}. Therefore, using biomarkers to guide the clinical management of combinations^{134–136} (FIG. 6b) is uncharted territory that remains to be developed, but it will probably represent a dramatic shift in our clinical strategies. The value of PDL1 expression in tumour tissue determined by IHC as a predictor of response is also challenged by a recent report of the

Epitopes

Minimal elements of an antigen that are recognized by an antibody, or the peptide within a protein sequence that is specifically recognized by a given T cell receptor.

Box 2 | Tumour antigenicity and immunotherapy

Mutational load in cancer correlates with its response to immunotherapy. Mutated antigens (referred to as neoantigens) may be presented by the major histocompatibility complex (MHC) and are recognized by the immune system. Autologous neoantigen-specific T cells have been identified among tumour-infiltrating T cells isolated from melanomas and other cancers^{144,145,167,177}. In most cases, these T cells are not specific for epitopes resulting from mutations in known oncogenic drivers but are mostly directed towards products from bystander somatic mutations. However, T cell targeting of antigens from genes indispensable for cancer survival may result in more durable clinical responses. Little is known about the function of these passenger neoantigens, as only a limited number of tumours that have responded to immunotherapy with durable responses have been studied¹⁶⁸. In most of the patients who have been studied, few neoantigen-specific T cell populations, both CD4⁺ and CD8⁺, were found^{144,145,167,177,178}. This observation suggests that in the case of a durable complete response or partial response, the neoantigen targeted by the T cells is indispensable for the tumour for a reason that is, as yet, unexplained. Alternatively, destruction of tumour cells expressing the neoantigen may lead to the death of surrounding tumour cells that do not express the antigen (bystander killing).

Neoantigen-specific tumour-infiltrating lymphocytes may have a major role in clinical responses to checkpoint blockade. Patients with melanoma treated with cytotoxic T lymphocyte-associated antigen 4 (CTLA4)-specific antibodies who were shown to have CTL immunity against tumour-specific neoantigens had an improved outcome compared with patients lacking these T cell responses¹⁷⁹. Intriguingly, in durable responses to CTLA4 blockade, patterns of tetrapeptide strings within the neoantigens were homologous with epitopes derived from microbial pathogens, suggesting that pre-existing pathogen-specific T cells are cross-reactive to these antigens¹⁷⁹ or that there is a pre-immune T cell receptor repertoire biased to deal with such epitopes¹⁸⁰. Recently, data from a preclinical model showed that tumour rejection following programmed cell death protein 1 (PD1) blockade was dependent on CTLs specific for a few tumour-specific neoantigens, rather than for shared tumour antigens¹⁸¹. T cell responses against neoantigens may be specifically boosted by vaccination strategies^{182,183}, or the adoptive transfer of neoantigen-specific T cells or their receptors may be selected for cell therapy alone or in combination with immune checkpoint blockade or other immunostimulatory drugs.

Table 2 | Immunotherapy biomarkers in the tumour microenvironment

Immunohistochemistry	Flow cytometry and functional assay	RNA and DNA
Baseline		
<ul style="list-style-type: none"> Ipilimumab: higher levels of IDO1 and FOXP3 at baseline were associated with clinical activity¹⁵⁶. Pembrolizumab: density of CD8⁺ T cells at the invasive margin was associated with response. Other potential predictors include PD1⁺ T cells in proximity to PDL1⁺ cells, and clonal T cell repertoire observed in responders¹³⁸. 	NA	<ul style="list-style-type: none"> Ipilimumab: high expression of immune-related genes (including <i>IDO1</i>) was associated with clinical activity¹⁵⁷. Atezolizumab: expression of CTLA4 and fractalkine was associated with response and progression, respectively. Expression of IFNγ-inducible genes in melanoma was associated with response⁴⁵.
Post-treatment		
<ul style="list-style-type: none"> Tremelimumab: increased infiltration of CD8⁺ T cells; increased numbers of HLA-DR⁺ and CD45RO⁺ memory T cells. No change in FOXP3⁺ T cells or IDO1-expressing cells post-treatment versus pretreatment¹⁵⁸. Ipilimumab: possible association between clinical activity and an increase in total TILs by week 4 relative to baseline; CD8⁺ T cell/FOXP3⁺ T cell ratio post-treatment is associated with the degree of necrosis^{156,160}. Pembrolizumab: a significantly higher level of expression of the IFNγ-inducible protein STAT1 was seen in responders at baseline and after PD1 inhibition¹³⁸. Atezolizumab: a decrease in tumour size seems to be associated with an increase in PDL1 expression on tumour and immune cells. Progressing lesions seem to have no intratumoural inflammation and no PDL1 expression⁴⁵. 	<ul style="list-style-type: none"> Tremelimumab: one patient had >40% CD3⁺; TILs were CD4⁺ with HLA-DR⁺, CD45RO⁺, CD27²⁺ and CCR7⁻ (memory phenotype)¹⁵⁸. Ipilimumab: one patient with a regressing lesion had a higher number of a subset of NK cells (CD56⁺CD16⁻) than of lymphocytes¹⁵⁹. One patient showed an autologous T cell (TIL) response to neoantigens that increased after treatment¹⁴⁴. An expansion of the CD8⁺ T cell population specific for shared tumour antigens in peripheral blood was found after treatment¹⁰⁷. Pembrolizumab: in blood from a patient with NSCLC, a neoantigen-specific T cell response was observed that increased during tumour regression¹⁴⁵. 	<ul style="list-style-type: none"> Ipilimumab: general increase in the expression of immune-related genes associated with clinical activity; general increase in the expression of IFNγ and T_H1 cell-associated markers¹⁵⁷. Nivolumab: there was an increase in the expression of MIG and IP10 (REF. 161). Atezolizumab: the RNA expression pattern from regressing lesions was consistent with CD8⁺ T cell and T_H1 cell phenotypes, but there was no change in FOXP3 expression⁴⁵.

CCR7, C-C chemokine receptor type 7; CTLA4, cytotoxic T lymphocyte-associated antigen 4; FOXP3, forkhead box P3; HLA, human leukocyte antigen; IDO1, indoleamine-2,3-dioxygenase 1; IFN γ , interferon- γ ; IP10, IFN γ -induced protein 10 (also known as CXCR3); MIG, monokine induced by IFN γ (also known as CXCL9); NA, not applicable; NK, natural killer; NSCLC, non-small-cell lung cancer; PD1, programmed cell death protein 1; PDL1, PD1 ligand 1; STAT1, signal transducer and activator of transcription 1; T_H1 cell, T helper 1 cell; TIL, tumour-infiltrating lymphocyte.

extraordinary clinical benefit with nivolumab as second-line monotherapy for patients with squamous NSCLC¹⁶³. In this study, the expression of PDL1 was neither prognostic nor predictive for benefit with nivolumab. In addition, for PDL1 expression in the microenvironment to become of value to clinical decision making, effort is needed to harmonize and validate the different IHC assays developed by academia and by pharmaceutical companies.

Further studies of tumour samples at baseline and after treatment, including lesions that progress with other immunotherapeutic agents, may continue to help to prioritize optimal combinations, providing that relevant targets are expressed. A crucial unmet need is the development of non-invasive imaging techniques designed to assess immune infiltrates.

Conclusions

Two main types of combination regimens are under investigation and will provoke much activity in the near future: combinations of immunotherapies with standards of care (see [Supplementary information S2](#) (table)) and, more excitingly, combinations of immunotherapies among themselves, chiefly involving PD1–PDL1 blockade as a partner (see [Supplementary information S1–S3](#) (tables)). The art of finding markedly synergistic effects at present is empirical rather than rational, mainly because the complexity of the mechanisms of action involved means that the overall effect is difficult to predict and model in experimental systems.

Decisions to move new combinations into the clinic are to be informed by preclinical data in animal models, mechanistic evidence for pharmacodynamic interactions and selection of patients based on biomarkers primarily found in malignant tissue biopsies. However, clinicians should acknowledge that the ability to predict which combination is the best for a given specific malignant indication, or for a given patient, is currently rather limited.

To make the most of this landscape of opportunities^{135,136}, scientists and clinicians need to work together to actively discover and develop new agents as partners for combination and take advantage of biotechnological advances to produce improved, next-generation immunology agents. Consideration needs to be given to delivery routes of immunotherapies to maximize their bioavailability in tumours and tumour-draining lymph nodes^{164,165}. Agents with low efficacy as monotherapy, but with multiplicative potential for synergistic effects, should also be tested. In addition, it will be important to consider the optimal types of trial design for combination therapies^{94,99} (FIG. 5b) (for example, it may be necessary to extend signal-seeking trials to demonstrate efficacy)^{155,166}. Revisions of regulatory rules may be needed to evaluate and demonstrate the efficacy of combinations in clinical trials for approval; for example, more meaningful end points should be considered, such as overall survival at a given milestone following treatment (that is, overall survival at 1 year or 2 years post-treatment).

Energy

An epigenetically induced dysfunctional state in which there is no immune response by T lymphocytes to a specific antigen.

On the basis of currently available efficacy data, we predict that most immunotherapy combinations will be built on PD1–PDL1 blockade (FIG. 4). Therefore, thorough study of the limiting factors for this molecular strategy will help in the quest for selection of optimal combination partners. These may include strategies to prime T cell responses, including vaccines¹⁰³ and adoptive T cell therapy directed to neoantigens presented by tumour cells^{167,168}; inducers of immunogenic cell death in strategies of *in situ* vaccination^{92,169,170}; simultaneous targeting of checkpoint

inhibitors responsible for T cell energy and/or exhaustion^{11,113,171,172}; and simultaneously providing artificial co-stimulatory^{132,173,174} and/or local pro-inflammatory agents^{175,176}. Ideally, these therapies should be selectively targeted to the tumour microenvironment and tumour-draining lymphoid tissue^{164,165}.

Regardless of their complexity and the issues associated with designing optimal combinations, immunotherapy combinations may be perceived as a leading way to increase therapeutic success across a whole range of tumour types.

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Competing interests statement

The authors declare [competing interests](#): see Web version for details.

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