Mechanisms of action and rationale for the use of checkpoint inhibitors in cancer

Clemence Granier,1,2 Eleonore De Guillebon,1,2,3 Charlotte Blanc,1,2 Helene Roussel,1,2,4 Cecile Badoual,1,2,4 Elia Colin,1,2,5 Antonin Saldmann,1,2,5 Alain Gey,1,2,5 Stephane Oudard,1,2,3 Eric Tartour1,5

ABSTRACT
The large family of costimulatory molecules plays a crucial role in regulation of the immune response. These molecules modulate TCR signalling via phosphorylation cascades. Some of the coinhibitory members of this family, such as PD-1 and CTLA-4, already constitute approved targets in cancer therapy and, since 2011, have opened a new area of antitumour immunotherapy. Many antibodies targeting other inhibitory receptors (Tim-3, VISTA, Lag-3 and so on) or activating costimulatory molecules (OX40, GITR and so on) are under evaluation. These antibodies have multiple mechanisms of action. At the cellular level, these antibodies restore the activation signalling pathway and reprogram T cell metabolism. Tumour cells become resistant to apoptosis when an intracellular PD-L1 signalling is blocked. CD8+ T cells are considered to be the main effectors of the blockade of inhibitory receptors. Certain CD8+ T cell subsets, such as non-hypermethylated (CD28+, T-bet+PD-1−), follicular-like (CXCR-5+) or resident memory CD8+ T cells, are more prone to be reactivated by anti-PD-1/PD-L1 monoclonal antibody (mAb). In the future, the challenge will be to rationally combine drugs able to make the tumour microenvironment more permissive to immunotherapy in order to potentiate its clinical activity.

The clinical benefit observed in patients with cancer after blockade of inhibitory receptors and their ligands is the fruit of a long history beginning with basic research concerning the rules of activation of T lymphocytes, followed by characterisation of the phenotype of these cells in chronic infections and in the tumour microenvironment.

A brief review of the basic knowledge about activation and regulation of T lymphocytes and the mechanisms leading to their dysfunctionality could help to elucidate and optimise these treatments.

HOW TO ACTIVATE T CELLS: THE TWO-SIGNAL HYPOTHESIS

A T cell activation depends on the interaction between the T cell receptor (TCR) and a peptide, presented by antigen-presenting cells (APCs) such as dendritic cells via major histocompatibility complex (MHC) class I or II molecules in case of CD8 or CD4 T cells, respectively. However, T cell activation also requires an appropriate cytokine environment and a ‘second signal’ in order to be effective.

In naive T cells, the interaction between the TCR and its MHC–peptide complex alone without a second signal results in an anergic state. Only memory T cells can be activated by simple recognition of a MHC–peptide complex.1

Although originally proposed in 1970 by Bretcher and Cohn, Schwartz et al were the first to validate the hypothesis of a two-signal model allowing T cell activation2: interaction between the TCR and its antigen, followed by interaction between a T cell costimulatory receptor and its ligand on the APC. The first costimulatory receptor, CD28, a member of the immunoglobulin superfamily, was discovered shortly thereafter.3 It is constitutively expressed on the membrane of naive T cells and two ligands have been identified: CD80 (B7-1) and CD86 (B7-2), both expressed by APC. TCR/CD28 engagement results in activation of several intracellular signalling pathways leading to increased production of cytokines such as interleukin (IL)-2, further supporting T cell activation.

Other positive costimulatory receptors (CD40, OX40, CD137 and so on) are also upregulated on T cells during activation allowing fine tuning of their differentiation into memory T cells and cytokine polarisation.

UPREGULATION OF COINHIBITORY RECEPTORS ALSO OCCURS DURING T CELL ACTIVATION

During T cell activation, inhibitory receptors such as CTLA-4, PD-1, Lag-3, Tim-3, Tigit and Vista are also induced to limit overstimulation...
of the immune system after antigen encounter, resulting in return to a resting state.

CTLA-4 (cytotoxic T lymphocyte associated protein 4), like CD28, is a member of the immunoglobulin superfamily. The CTLA-4 locus is very close to the CD28 locus, and they have very similar protein sequences. CTLA-4 is induced on Foxp3+ CD4+ T and CD8+ T cells after early activation, while it is constitutively expressed on regulatory T cells (Treg). Nuclear factor of activated T cells (NFAT) and Foxp3 regulate the expression of CTLA-4. CTLA-4 binds with higher avidity to the same ligands (CD80 and CD86) as CD28, leading to competitive binding between the costimulatory and coinhibitory receptors. CTLA-4 engagement inhibits T cell proliferation and IL-2 production. CTLA-4 is believed to act at the priming phase in lymph nodes, as their ligands (CD80 and CD86) are mainly expressed on APCs.

PD-1 (programmed cell death ligand 1 or CD279) is also a member of the immunoglobulin superfamily. It is more widely expressed than CTLA-4 and can be detected on activated T cells, B cells and natural killer (NK) cells, and over a longer time frame than CTLA-4 (6–12 hours on activated T cells, B cells and NK cells, more widely expressed than CTLA-4 and can be detected also a member of the immunoglobulin superfamily. It is and CD86) are mainly expressed on APCs.

CTLA-4 deficiency is lethal for mice, with early onset of aggressive lymphoproliferative disorders and multiorgan infiltration by polyclonal T cells. PD-1 deficiency induces more indolent autoimmune diseases such as rheumatoid arthritis, glomerulonephritis or dilated cardiomyopathy and is compatible with survival in mice. These findings are consistent with clinical observations of patients receiving anti-CTLA-4 or anti-PD-1 therapy, as immune adverse events are more common and often of higher grade with ipilimumab, an anti-CTLA4 antibody, than with anti-PD1 therapies.

Three other coinhibitory receptors (Tim-3, Lag-3 and VISTA) are currently under clinical investigation as potential therapeutic targets, either alone or in combination with anti-PD-1 antibodies (anti-Tim-3: NCT02817633; anti-Lag-3: NCT02488759, NCT02060188, NCT02061761, NCT01968109, NCT02658981, NCT02966548, NCT03005782; anti-Vista: NCT02812875, NCT02671955). Tim-3 (T cell immunoglobulin and mucin 3) is expressed by activated T cells, NK cells and monocytes. A majority of tumour-infiltrating lymphocytes (TILs) coexpresses PD-1 and Tim-3, and this coexpression seems to block their functionality. Tim-3 binds to Galectin-9, CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1), HMBG1 (high-mobility group box 1) and phosphatidyl serine. In the absence of ligand binding, Bat3 is bound to the cytoplasmic tail of Tim-3 and prevents inhibition of T cell signalling via recruitment of Lck. Binding of Tim-3 to its ligands leads to phosphorylation of its cytoplasmic tail, release of Bat3 and possible recruitment of Fyn, which can induce T cell anergy.

Some Tim-3 ligands (galectin-9 and HMBG1) are induced in inflammatory conditions, while Tim-3 expression is driven by Interferon (IFN)β and IL-27, suggesting a role of Tim-3 in inflammatory conditions. LAG-3 (lymphocyte-activation gene-3) is found on activated T cells, B cells, NK cells and plasmacytoid dendritic cells. Its known ligand is CMH II, and the main hypothesis regarding its mechanism of action consists of competitive inhibition of the interaction between the antigen and CD4+ T cell TCR. Recently, two other ligands of LAG-3 have been identified. LSECtin expressed by melanomas suppressed tumour-specific T cell response and Galectin-3 expressed by CD8+ T cells may inhibit antitumour T cell response via cis and trans interactions with LAG-3. LAG-3 also seems to have specific actions on CD8+ T cells and is also frequently coexpressed with PD-1 on TILs. LAG-3-deficient mice are normal under steady-state conditions but show uncontrolled expansion of T cells when challenged with antigen or staphylococcal enterotoxin B.

VISTA: programmed death-1 homologue (PD-1H, also called VISTA) is a member of the CD28 family of proteins and has been shown to act as a coinhibitory ligand on APCs that suppress T cell responses (proliferation and production of cytokines) and induce Foxp3 expression. VISTA is predominantly expressed in the haematopoietic compartment with the highest expression observed in the myeloid lineage. The VISTA receptor on T cells has not yet been identified. Anti-VISTA therapy accentuates the development of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis, in mice.

THE EXHAUSTION PHENOMENON

Chronic diseases are associated with chronic antigen exposure and inflammation and persistent T cell activation. During this persistent antigen stimulation, the effector function of CD8+ T cells gradually decreases, a phenomenon known as exhaustion. The process of...
T cell differentiation into effector and memory T cells is altered and switches towards a particular state called exhaustion. CD8+ T cells lose their IL-2 secretion and proliferative capacities, cytotoxic function and finally can no longer secrete IFN-γ or degranulate.26 Exhausted T cells express various inhibitory receptors (PD-1, CTLA-4, Tim-3, TIGIT and LAG-3) and their pattern of expression (frequency and level) correlates with different levels of exhaustion. The coexpression of many of these receptors on a single type of T cell increases their dysfunctional state.

The frequency and level of expression of PD-1 and other inhibitory receptors have been shown to be higher in intratumoural T lymphocytes than in normal tissue or peripheral blood, especially on the membrane of anti-tumour T lymphocytes.27

Exhausted T cells showed defective mitochondrial function and restoration of this function by mitochondria-targeted antioxidants improved T cell function.28 The concept of T cell dysfunction and exhaustion was first described in the setting of chronic viral infection by the groups of RM Zinkernagel29 and R Ahmed.30–31 The first molecular description of exhaustion in human cancer was reported by Rosenberg and H Zarour in melanoma patients27–32 and by Ochsenbein AF in patients with chronic myeloid leukaemia.33 In preclinical models, various groups have demonstrated the clinical value of targeting these inhibitory receptors in chronic viral infections34 and cancer.

Several factors promoting T cell exhaustion have been described. The level and duration of exposure to antigen (>2 weeks and chronic rather than acute exposure) and the absence of CD4 helper T cells are key events that induce abnormal accumulation of inhibitory receptors.35

TCR-dependent pathways, especially NFAT transcription factor, play a key role in the exhausted phenotype. NFAT has been shown to promote T cell anergy and exhaustion by binding to sites that do not require cooperation with AP-1.39 Genes directly induced by an engineered NFAT1 unable to interact with AP-1 transcription factors overlapped with genes expressed in exhausted CD8+ T cells in vivo.39 Cytokine production is severely impaired because of a selective defect in activation-induced NFAT nuclear translocation.40

Exhaustion-specific accessible regions were enriched for consensus binding sites for NFAT and Nrfa family members, indicating that chronic stimulation confers a unique accessibility profile on exhausted cells.41

Although exhausted T cells are characterised by a high level of PD-1 expression and coexpression of inhibitory receptors, all these biomarkers could be transiently expressed after activation. A recent study based on single-cell resolution of TILs reported that distinct gene modules for T cell dysfunction and activation can be uncoupled and that these modules at a single levels are exclusive. These authors showed that loss of function involved metallothioneins that upregulate zinc metabolism. In a preclinical model, they could also identify GATA binding protein 3 (GATA-3) as a driver of dysfunctionality.42 A gene profile of exhaustion can therefore be distinguished from the very similar gene activation programme.

**MECHANISMS OF ACTION OF ANTAGONISTS OF INHIBITORY RECEPTORS**

**Inhibition of the interaction between inhibitory receptors and their ligands reinvigorates intratumour CD8+ T cells**

As expected, due to their role in reversal of inhibition of tumour immunity, administration of anti-CTLA-4 or anti-PD-1/PD-L1 antibodies leads to activation of the immune system (figure 1).

An increased level of circulating IFN-γ and IFN-α-induced chemokines (CXCL-9 and CXCL-10) was observed in the serum of patients treated by both anti-PD-1 and anti-PD-L1.43–44 T cells with an activated phenotype were also induced after administration of checkpoint inhibitors. For instance, many studies have reported an increase of IFN-γ-producing CD4+ICOS+ T cells in both peripheral blood and tumour tissue after treatment with ipilimumab alone or in combination with nivolumab.45–47 Activation markers such as Ki67 and HLA-DR were also increased on T cells in patients treated by anti-PD-L1 or anti-PD-1.43–46 An influx of CD8+ T cells in the tumour microenvironment has also been reported after anti-CTLA-4 or nivolumab administration in melanoma and renal cell carcinoma.44–49 Preliminary studies have also reported that CTLA-4 blockade confers lymphocyte resistance to Treg in advanced melanoma.50 Ipilimumab also increased TCR diversity, as reflected by the number of unique TCR clonotypes, and the vast majority of changes occurred in the memory T cell pool.51 An increase of T cell reactivity against tumour antigens has also been reported after ipilimumab therapy.52

Depending on the couple of inhibitory receptors and their ligands, blockade of this interaction could alleviate an inhibitory signal on CD8+ T cells, but for other inhibitory receptors such as CTLA-4 and TIGIT, which compete with activating receptors (CD28 and CD226), blockade would also promote the positive costimulatory pathway.53–56 Moreover, CTLA-4 downregulates CD86 and CD80 expression on APCs by transcytosis and CTLA-4 blockade would inhibit this phenomenon.57

**Antagonists of inhibitory receptors lead to T cell metabolic reprogramming**

Nutrient competition between cells influences tumour cell growth, survival and function. T cell fitness and function are directly linked to metabolic activity. Activated T cells consume large quantities of glucose, amino acids and fatty acids.

Tumour cells and immune cells compete for the glucose present in the tumour microenvironment. Tumour cells have a very high glucose uptake capacity. Aerobic glycolysis, which is regulated by the bifunctional enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is required for T cells to attain full effector status.58
When glucose is present, GAPDH engages in its enzymatic function, but when cells are glucose restricted, GAPDH becomes available to bind the 3′UTR of IFN-γ messenger RNA, preventing its efficient translation. Glucose deprivation or inhibition of glycolysis by 2-deoxy-D-glucose inhibits IFN-γ production and mammalian target of rapamycin (mTOR) activity. Various molecular mechanisms can explain the glucose dependence of T cells. Phosphoenolpyruvate (PEP) is a glycolytic metabolite that plays a role in intracytoplasmic calcium mobilisation, essential for T cell activation after TCR engagement. More specifically, PEP promotes TCR-mediated Ca²⁺-NFAT signalling and effector functions by repressing sarco/ER Ca²⁺-ATPase activity. Glycolysis deprivation dampens the level of PEP, resulting in decreased levels of intracellular calcium. The downstream effect is a lack of activation of CD4⁰ TILs. EZH2 is an histone-lysine N-methyltransferase, which plays a role in DNA methylation and transcriptional repression. It binds directly to the promoter areas of Notch repressors, NUMB and FBXW7, and represses their transcription via H3K27me3, and subsequently causes Notch activation, resulting in antiapoptotic gene activation and effector cytokine expression on T cells. EZH2⁺ T cells mediate potent antitumour immunity in human cancers and are associated with long-term survival. Tumours have been shown to impair T cell EZH2 expression via glucose restriction.

Lastly, PD-L1 signalling regulates the Akt/mTOR pathway, which results in decreased translation of glycolytic enzymes and dampened glycolysis. Chang et al showed that anti-CTLA-4 and anti-PD-1 blockade therapy corrects the tumour-induced glucose restriction experienced by TILs and restores their glycolytic capacity and hence their effector function in experimental models. Other metabolic checkpoints (tryptophan, arginine, ATP and so on) are involved in T cell fitness and are lacking in the tumour microenvironment. However, the direct impact of antagonists of inhibitory receptors on these metabolites has not been demonstrated.

**Blockade of intrinsic tumour signalling mediated by PD-L1**

PD-L1 has mainly been studied as a checkpoint ligand, which delivers a negative signal to T cells leading to dysfunction and ultimately apoptosis. Previous studies have demonstrated intrinsic reverse signalling after PD-L1 binding to PD-1. Hirano et al referred to PD-L1 and PD-1 binding as a molecular shield, which can prevent tumour destruction by T cells. This finding was initially interpreted to be a negative signal of PD-L1 to T cells. However, it has subsequently been shown that loss of
the intracellular domain of PD-L1 is required to induce this molecular shield. Azuma et al reported that PD-L1 intrinsic signalling conferred an antiapoptotic property to tumour cells. After elimination of the intracellular domain of PD-L1 in tumour cells, tumour regressions were observed in mouse models. Secondary activation of the mTOR pathway and PD-L1-mediated autophagy also participate in its intrinsic role in tumour growth. Blocking the PD-1–PD-L1 interaction may affect the antiapoptotic and proliferative activities of PD-L1.64

Which cells are targeted by antagonists of inhibitory receptors
CD8+ T cells
The current dogma supported by various preclinical and clinical data argues in favour of a major role of T cells and especially CD8+ T cells to explain the therapeutic activity of antagonists of inhibitory receptors (figure 2).65 66 The presence of pre-existing T cells before therapy correlated with the clinical activity of PD-1 and CTLA-4 blockade, as TIL density in melanoma patients treated by anti-CTLA-4 correlated with good clinical response.67 Another study reported that pre-existing CD8+ T cells at the invasive tumour margin were a prerequisite for the efficacy of PD-1 blockade in a cohort of 15 patients treated for melanoma.68 In addition, during therapy, analysis of early on-treatment tumour biopsies identified a significantly higher density of CD8+ T cells in responders versus non-responders to CTLA4 and PD-1 blockade.69

Various groups, including our own, are trying to identify the subpopulations of CD8+ T cells that provide the proliferative burst after PD-1 therapy. In a model of chronic infection, Rafi Ahmed’s group identified a population of CD8+ T cells that proliferated after anti-PD-1 administration, which expressed PD-1, ICOS, CD28 and CXCR5 and with a gene signature related to CD4 T follicular helper and CD8+ T cell memory precursor. The TC1 transcription factor plays an important role to generate this population predominantly found in lymphoid tissue.70 These CXCR5-CDS+ T cells express low levels of PD-1 and control viral infection in the lymphocytic choriomeningitis virus model.71 72

In line with these results, Rafi Ahmed’s group showed that CD28 signalling is required for the efficacy of PD-1 targeted therapy.73 A population of CD8+ T cells expressing CD28 is present in the CD8 TIL population. In blood, a population of CD8+PD-1-CD28+ T cells preferentially proliferated after anti-PD-1 therapy.73 Since hyperexhausted T cells lack CD28 expression, these results support the idea that early exhausted T cells mediate the activity of PD-1–PD-L1 blockade.

It would be of interest to compare the phenotype of these responsive CD8+ T cells with that of PD-1int Tbetphil Eomeslow observed in chronic viral infection, which also proliferated, produced cytokines and exerted cytolytic function.74 75 When these cells convert into PD-1high Tbetlow and Eomeshigh with chronic exposure to antigen, they become exhausted.
We have recently shown, in a series of renal cell carcinoma patients, that the CD8+ T cells coexpressing PD-1 and Tim-3 are poorly functional after stimulation and cannot be reactivated by anti-PD-1 alone. The presence of this population correlated with poor prognosis. In mice and humans, upregulation of Tim-3 after PD-1–PD-L1 blockade was associated with clinical resistance to these drugs. Interestingly, the previous population of CXCR5+CD28−CD8+ T cells considered to be targeted by anti-PD-1 did not express Tim-3.

Finally, another population called resident memory T cells, which persist in tumour tissue, is required for the efficacy of certain immunotherapeutic approaches. This population, identified by CD103 and CD49a markers, expressed PD-1 and preferentially recognised tumour cells compared with conventional circulating effector CD8+ T cells.

Non-CD8+ T cells

CD4+CD25+Foxp3+Treg have emerged as a dominant T cell population inhibiting antitumour effector T cells. Activated and highly suppressive Tregs upregulate multiple inhibitory receptors, including PD-1, CTLA-4, Tim-3 and TIGIT, which often contribute to Treg stability and function.

Mice with Treg selective ablation of CTLA-4 developed severe autoimmune disease mimicking the off-target effects observed after anti-CTLA-4 administration in humans. The physiological function of CTLA-4 therefore appears to be to suppress T cell responses to self-antigens by controlling Treg activity.

Targeting CTLA-4 on Tregs with certain antibody isotypes (IgG1) therefore appears to deplete Tregs via antibody-dependent cellular cytoxicity and contributes to reverse tumour-induced T cell dysfunction.

CONCLUSION

Despite a clinical breakthrough following the use of antagonists of inhibitory pathways on CD8+ T cells, there was only a weak rationale for their clinical development, as T cells express many inhibitory receptors and most researchers believed that the blockade of one receptor would be compensated by the presence of other inhibitory receptors keeping the immune system in check. In addition, many other immunosuppressive mechanisms operate in the tumour microenvironment: Treg cells, myeloid derived suppressor cells, M2 macrophages, soluble immunosuppressive cytokines and enzymes (IL-10, tumor growth factor beta, indoleamine 2,3-dioxygenase, CD39 and so on), which were considered to also participate in CD8+ T cell dysfunction. To explain this poor initial judgement, it is likely that the blockade of one dominant immunosuppressive pathway has an impact on other escape mechanisms and restores the balance towards antitumour immunity. From an optimistic point of view, we could plan to target these other immunosuppressive mechanisms in combination with checkpoint inhibitor blockade in order to improve the clinical activity of immunotherapy. More than 350 clinical trials of combined therapy are ongoing, which raise new hopes and promises in the field of cancer immunotherapy. In addition, recent studies challenge the previous concept that blockade of the PD-1–PD-L1 axis reinvigorates exhausted T cells, as the epigenetic profile of exhausted T cells remains stable after anti-PD-1 therapy, and the requirement for CD28 signalling excludes a major role of terminally exhausted T cells that do not express CD28 as the effector cells mediating the clinical activity of anti-PD-1/PD-L1. These results provide a strong rationale for the combination of anti-PD-1/PD-L1 therapy with other therapeutic strategies (vaccines, oncolytic viruses and so on) designed to generate de novo induction of antitumour early memory CD8+ T cells.

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REFERENCES

1. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 2001;194:769–801.
2. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med 1987;165:302–19.
3. Harding FA, McArthur JG, Gross JA, et al. PD-L1 mediated signalling co-stimulates murine T cells and prevents induction of anergy in T cell clones. Nature 1992;356:607–9.
4. Littman DR. Releasing the brakes on cancer immunotherapy. Cell 2015;162:1186–90.
5. Gibson HM, Hedgcock CJ, Aufiero BM, et al. Induction of the CTLA-4 gene in human lymphocytes is dependent on NFAT binding the proximal promoter. J Immunol 2007;179:3831–40.
6. Zheng Y, Josefowicz SZ, Kas A, et al. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 2007;445:936–40.
7. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to Cancer therapy. Cancer Cell 2015;27:450–61.
8. Xiao Y, Yu S, Zhu B, et al. RGMb is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance. J Exp Med 2015;211:943–59.
9. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, et al. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J Exp Med 2012;209:1201–17.
10. Hui E, Cheung J, Zhu J, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science 2017;355:1428–33.
11. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Cita-4. Science 1995;270:985–8.
12. Nishimura H, Okazaki T, Tanaka Y, et al. Autoimmune mediated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001;291:319–22.

13. Nishimura H, Nose M, Hiai H, et al. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an immunoregulatory molecule. Immunity 1999;11:141–51.

14. Lesokhin AM, Callahan MK, Postow MA, et al. On being less tolerant: enhanced cancer immunosurveillance enabled by targeting checkpoints and agonists of T cell activation. Sci Transl Med 2015;7:293sr6.

15. Fourcade J, Sun Z, Pagliano O, et al. PD-1 and Tim-3 regulate the expansion of tumor antigen-specific CD8 T cells induced by melanoma vaccines. Cancer Res 2014;74:1045–55.

16. Granier C, Arancane D, Combe J, et al. Tim-3 expression on tumor-infiltrating PD-1+CD8+ T cells correlates with poor clinical outcome in melanoma. Cancer Res 2017;77:1075–82.

17. Rangachari M, Zhu C, Sakuishi K, et al. Inhibition of PD-L1 by MPDL3280A and clinical activity in pts with metastatic urothelial bladder Cancer (UBC). ASCO annual meeting. J Clin Oncol 2014.

18. Immunomodulatory activity of nivolumab in metastatic renal cell carcinoma (mRCC): Association of biomarkers with clinical outcomes. ASCO annual meetingChicago. J Clin Oncol 2015.

19. Peripheral and tumor immune correlates in patients with advanced melanoma treated with combination nivolumab (anti-PD-1, BMS-936558, ONO-4538) and ipilimumab. ASCO Chicago (Illinois). USA. J Clin Oncol 2013.

20. Ng Tang D, Shen Y, Sun J, et al. Increased frequency of ICOS+ CD4 T cells as a pharmacodynamic biomarker for anti-CTLA-4 therapy. Cancer Res 2013;73:1229–34.

21. Li J, Ni L, Dong C. Immune checkpoint receptors in cancer: redundant by design? Curr Opin Immunol 2017;45:37–42.

22. Zhu C, Sakushi K, Xiao S, et al. An IL-7/Notch signalling axis drives Tim-3 and IL-10 expression and T-cell dysfunction. Nat Commun 2015;6:6072.

23. Xu F, Liu J, Liu D, et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. Cancer Res 2015;75:5118–28.

24. Kouo T, Huang L, Pucsek AB, et al. Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. Cancer Immunol Res 2015;3:412–23.

25. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. Eur J Immunol 2003;33:970–9.

26. Lines JL, Sempere LF, Broughton T, et al. VISTA is a novel broad-spectrum negative checkpoint regulator for cancer immunotherapy. Cancer Immunol Res 2014;2:510–22.

27. Wang L, Rubinstein R, Lines JL, et al. VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses. J Exp Med 2011;208:577–92.

28. Wherry EJ, Teichgräber V, Becker TG, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol 2003;4:225–34.

29. Ahmadzadeh M, Johnson LA, Heemskerk B, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood 2009;114:1537–44.

30. Fisicaro P, Barili V, Montanini B, et al. Targeting mitochondrial inhibition results in distinct stages of functional impairment. Exp Med 1998;188:2205–13.

31. Rangachari M, Zhu C, Sakuishi K, et al. Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8+ T cell functions during chronic viral infection. Proc Natl Acad Sci U S A 2007;104:4565–70.

32. Scott-Browne JP, López-Moyado IF, Trifari S, et al. Dynamic changes in chromatin accessibility occur in CD8(+) T cells responding to viral infection. J Exp Med 2016;263:1327–40.

33. Singer M, Wang C, Cooke L, et al. A distinct gene module for dysfunction uncoupled from activation in Tumor-Infiltrating T cells. Cell 2016;166:1500–11.

34. Inhibition of PD-L1 by MPDL3280A and clinical activity in pts with metastatic urothelial bladder Cancer (UBC). ASCO annual meeting. J Clin Oncol 2014.

35. Martinez GJ, Pereira RM, Aijó T, et al. The transcription factor NFAT promotes exhaustion of activated CD8 T cells. Immunity 2015;42:265–78.

36. Agnellini P, Wolint P, Rehr M, et al. Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8+ T cell functions during chronic viral infection. Proc Natl Acad Sci U S A 2007;104:4565–70.

37. Dong H, Strome SE, Salomao DR, et al. T cell exhaustion. Nat Rev Cancer 2002;2:1039–800.

38. Wherry EJ. T cell exhaustion. Nat Rev Cancer 2005;6:1089–96.
64. Clark CA, Gupta HB, Sareddy G, et al. Tumor-Intrinsic PD-L1 signals regulate cell growth, pathogenesis, and Autophagy in ovarian cancer and melanoma. *Cancer Res* 2016;76:6964–74.

65. Badoual C, Hans S, Merillon N, et al. PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. *Cancer Res* 2013;73:128–38.

66. Roussel H, De Guilhbon E, Biard L, et al. Composite biomarkers defined by multiparametric immunofluorescence analysis identify ALK-positive adenocarcinoma as a potential target for immunotherapy. *Oncoimmunology* 2017;6:e1286437.

67. Ji RR, Chasalow SD, Wang L, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunol Immunother* 2012;61:1019–31.

68. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014;515:568–71.

69. Chen PL, Roh W, Reuben A, et al. Analysis of immune signatures in longitudinal tumor samples yields Insight into biomarkers of response and mechanisms of resistance to immune checkpoint blockade. *Oncoimmunology* 2017;6:e1286437.

70. Im SJ, Hashimoto M, Gerner MY, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* 2016;537:417–21.

71. Leong YA, Chen Y, Ong HS, et al. CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles. *Nat Immunol* 2016;17:1187–96.

72. He R, Hou S, Liu C, et al. Follicular CXCR5- expressing CD8(+) T cells curtail chronic viral infection. *Nature* 2016;537:412–28.

73. Angelosanto JM, Blackburn SD, Crawford A, et al. Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *J Virol* 2012;86:8161–70.

74. Nizard M, Roussel H, Diniz MO, et al. Induction of resident memory T cells enhances the efficacy of cancer vaccine. *Nat Commun* 2017;8:15221.

75. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* 2010;236:219–42.

76. Karaki S, Anson M, Tran T, et al. Is there still room for cancer vaccines at the era of checkpoint inhibitors. *Vaccines* 2016;4:37.