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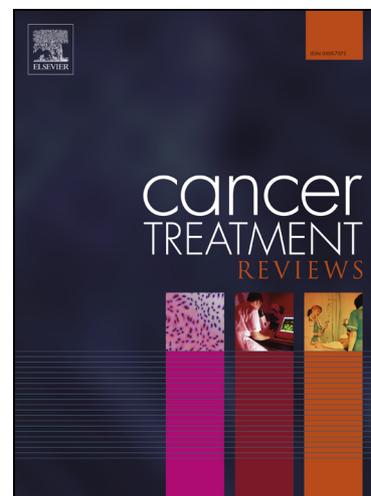
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Targeting immune checkpoints: new opportunity for mesothelioma treatment?

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Abstract

Malignant pleural mesothelioma is an aggressive cancer linked to asbestos exposure in most patients. Due to the long latency between exposure and presentation, incidence is expected to further increase in the next decade, despite the ban on asbestos import which occurred at the end of last century in industrialized countries. Platinum-based palliative chemotherapy is the only treatment with proven benefit on outcome, resulting in selected patients in a median overall survival of about 1 year. Therefore, there is room for therapeutic improvement using a new strategy to prolong survival. Dealing with cancer cell induced immunosuppression is a promising approach. Reactivating immune responses that are silenced by immune checkpoints recently gained a lot of interest. Checkpoint blockade has already shown promising preclinical and clinical results in several cancer types and is currently also being investigated in mesothelioma. Here, we discuss the expression patterns and mechanisms of action of CTLA-4 and PD-1 as the two most studied and of TIM-3 and LAG-3 as two interesting upcoming immune checkpoints. Furthermore, we review the clinical results of molecules blocking these immune checkpoints and point out their future opportunities with a special focus on mesothelioma.

Keywords

Immunotherapy

Programmed death-1

Cytotoxic T-lymphocyte antigen-4

Mesothelioma

Immune checkpoint

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive and nearly always fatal cancer, causally linked to previous, mostly professional, exposure to asbestos [1]. The highest incidence rates, around 30 cases per million inhabitants, are reported for Australia, Belgium and the UK [2,3]. The incidence of MPM is still expected to increase over the next decades due to the long latency between exposure to asbestos and diagnosis and because asbestos is still being used in developing countries [4]. The prognosis of MPM patients remains poor with a median overall survival time in untreated patients of about 10 months and a 5 year survival rate of less than 5% [4,5]. Palliative platinum-antifolate chemotherapy is the only treatment with proven improvement of outcome in MPM, resulting in a median survival of about 1 year. There is therefore a need for new therapeutic strategies. The discovery of immune checkpoint receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and more recently programmed death-1 (PD-1) introduced a new, exciting era in cancer immunotherapy [6]. Immune checkpoints are responsible for controlling and inactivating the immune system in order to avoid autoimmunity and prevent collateral tissue damage [7]. The new paradigm consists of reactivating silenced immune responses by neutralizing molecules that induce T-cell exhaustion and immune tolerance. Immune checkpoint blocking antibodies have already shown promising results in several cancer types [8-13]. Recently antibodies blocking immune checkpoints are being investigated in mesothelioma patients. In this review, we discuss the expression pattern and mechanisms of action of CTLA-4 and PD-1 as the two most studied checkpoint receptors and of T-cell immunoglobulin mucin-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) as two interesting upcoming immune checkpoints. Furthermore, we review the clinical results of therapeutic molecules blocking these immune checkpoints with primary focus on CTLA-4 and PD-1 since FDA approved antibodies are available for both of them. Future opportunities of immune inhibitory molecules will be pointed out, with a special focus on MPM.

CTLA-4 and PD-1: high priority targets

CTLA-4: the first clinically targeted immune checkpoint receptor

CTLA-4 is an immune inhibitory receptor that is mainly found on T-cells and to a lower extent on activated B-cells, monocytes, dendritic cells and granulocytes [14-17]. Its primary role is to regulate T-cell activation upon antigenic stimulation of the T-cell receptor (TCR). T-cell activation can be

explained by the two-signal model. The first signal is provided when an antigen, presented by an antigen presenting cell (APC) in combination with a major histocompatibility complex (MHC) molecule, interacts with the T-cell receptor (TCR) and a CD4 or CD8 co-receptor. Secondly, interactions between co-stimulatory molecules on the T-cell and APC will then result in priming and differentiation of naïve T-cells or reactivation of effector T-cells to exert their function [18].

CTLA-4 is a transmembrane protein that is retained in intracellular vesicles [19]. The intracellular and surface expression are induced by T-cell activation, after which the vesicles travel to the cell surface where CTLA-4 expression is then upregulated [20]. Wang et al.[21] described that interferon (IFN) γ induces CTLA-4 expression in human T-cells in the presence of APC, suggesting that the effect of IFN γ might be exerted via monocyte activation resulting in T-cell stimulation and hence the expression of CTLA-4.

CTLA-4 exerts its modulatory function by competing with the CD28 molecule for the B7 ligands CD80 and CD86, expressed on APC [22] (*Fig.1*). Engagement of CTLA-4 by CD80 and CD86 limits and decreases T-cell activation. Under physiological conditions the immune inhibitory effect of CTLA-4 is involved in provoking an effective immune response without causing excessive damage to the normal surrounding tissue. However, tumor cells can stimulate abnormal expression of CTLA-4 by secreting transforming growth factor- β (TGF- β), an immunosuppressive cytokine that induces CTLA-4 overexpression, resulting in T-cell exhaustion [23-25]. T-cell exhaustion is a state of T-cell dysfunction which represents a mechanism of immunosuppression [26]. Exhausted T-cells fail to proliferate and are no longer able to exert their effector functions. Among the different suppressive mechanisms and pathways by which CTLA-4 modulates T-cell activation are: (i) expression of CTLA-4 on the surface of T-cells outcompetes the CD28 co-stimulation by higher overall affinity for both CD80 and CD86 [27]; (ii) through activation of protein phosphatases SHP2 and PP2A, CTLA-4 transduces co-inhibitory signals in the T-cell kinase signaling pathway by inhibiting Akt phosphorylation [28,29]; (iii) CTLA-4 is constitutively expressed on regulatory T-cells (Tregs) that are activated upon CTLA-4 ligation to CD28, resulting in secretion of the immunosuppressive cytokine TGF- β [23,30].

Due to its immunosuppressive effects, CTLA-4 is an interesting target for enhancing the anti tumor activity of T-cells. Allison and colleagues were the first to discover that CTLA-4 is vital for maintaining host immune tolerance to established tumors. Melanoma and colon cancer mouse models showed consistent and durable anti tumor responses following systemic treatment with CTLA-4 monoclonal

blocking antibodies [12,13]. Promising preclinical findings were the impetus to test CTLA-4 immunotherapy in patients leading to FDA approval of the human IgG1 monoclonal antibody 'ipilimumab' (Yervoy[®], BMS) for late-stage melanoma in 2011. In addition to ipilimumab, another human anti CTLA-4 antibody, called 'tremelimumab', is under clinical investigation. While ipilimumab is an IgG1 isotype antibody, tremelimumab is an IgG2 antibody. This difference in isotype class might explain the variation in clinical effectiveness of both antibodies. Antibodies with an IgG1 isotype have been described to better induce antibody dependent cell-mediated cytotoxicity (ADCC) and fixing complement compared to IgG2 [31]. ADCC is mediated via binding to activating $F_c\gamma$ receptors expressed on immune cells, natural killer (NK) cells among them. Laurent et al.[32] described that activated T-cells are not killed by ADCC probably due to transient CTLA-4 expression upon activation and that blocking CTLA-4 even puts off the brake on effector T cells. On the other hand, ADCC does result in depletion of Tregs through activating $F_c\gamma$ receptors as described by Selby et al.[33]. So at least for ipilimumab, ADCC is part of the working mechanism of the antibody to induce strong anti tumor immune responses. Cancer patients treated with anti CTLA-4 therapy initially showed disease progression followed by disease regression that can be delayed up to 6 months after treatment initiation [34-36]. These kinetics are an interesting feature described for immunotherapies. While early clinical effects are mostly observed using cytotoxic agents, immunotherapeutic agents often demonstrate delayed clinical effects [37]. This difference in response pattern can be explained by the dynamics of the immune system: due to T-cell expansion and infiltration the tumor lesion initially increases in size [12]. According to the Response Evaluation Criteria in Solid Tumors (RECIST), this is considered as disease progression and treatment should be stopped. However, response to immunotherapy may occur after progressive disease and therefore immune related response criteria (irRC) were developed. For the irRC, radiographic measurements, such as helical computer tomography, are used to assess the tumor burden and the size of new lesions. If there is an increase in tumor burden of at least 25% compared to baseline after 2 consecutive measurements (at least 4 weeks apart) it is defined as progressive disease and treatment cessation is recommended [37]. Immunotherapeutic treatments are associated with immune-related adverse events. Diarrhea, fever and rash have been reported for anti CTLA-4 treatment but most of them are reversible after corticosteroid treatment. However, 10-15% of patients show severe adverse events, such as colitis, that can be long lasting, difficult to treat and even lethal [38].

PD-1: cancer breakthrough target of the year 2013

In the nineties, the discovery of another T-lymphocyte-associated immune checkpoint receptor, PD-1, and its ligands PD-L1 and PD-L2 triggered a major breakthrough in oncology research. The PD-1 protein has a structure similar to CTLA-4 but it has a distinct biological function and ligand specificity. It is hypothesized that CTLA-4 is responsible for modulating central T-cell activation in the lymph nodes, while PD-1 in contrast is responsible for controlling peripheral T-cell activation at the tumor site [39]. Like CTLA-4, PD-1 is a transmembrane protein, mainly expressed on activated T-cells, B-cells and macrophages [40]. In analogy with CTLA-4, PD-1 also binds to inhibitory molecules of the B7 family, more specifically to PD-L1 (also known as B7-H1) and PD-L2 (also known as B7-DC) thereby preventing autoimmunity and avoiding tissue damage. As for CTLA-4, PD-1 its homeostatic immune function is regulated by a feedback loop via IFN γ [36]. T-cell activation not only induces the expression of PD-1 but it also results in secretion of several cytokines, IFN γ among them, which in turn causes an upregulation of PD-L1 and/or PD-L2 that can interact with PD-1. In this way, immune response are attenuated and the extent of immune mediated tissue damage is controlled.

PD-L1, is constitutively expressed on various hematopoietic cells (T cells among them), some parenchymal cells and tumor cells of many tumor types such as melanoma, lung cancer, breast and ovarian cancer, pancreatic and esophagus adenocarcinoma, renal cell carcinoma and bladder cancers as well as in hematopoietic malignancies [41-44]. In addition to binding PD-1, PD-L1 can also bind CD80 [45]. PD-L2, has a more narrow inducible expression profile restricted to macrophages, dendritic cells, mast cells and also tumor cells such as pancreatic, ovarian and esophageal cancer cells [18,46]. However, compared to PD-L1, it binds to PD-1 with a much higher affinity [47]. PD-L2 expression on tumor or on stromal cells (including fibroblasts) can impair effector T-cell activity in the tumor microenvironment [18,46,48], making it an attractive candidate for immunotherapy as well. Interaction of PD-1 with one of its ligands results in decreased peripheral T-cell activity. In cancer this means there will be a less efficient anti tumor immune response.

The mechanisms by which PD-1 exerts its inhibitory effect can partially be explained by its intracellular signaling pathway [40]. The PD-1 protein has a cytoplasmic tail, a transmembrane and extramembrane part. Analogous to CTLA-4, the PD-1 protein is also stored in vesicles which will translocate to the cell surface upon antigen recognition. Binding of PD-L1 or PD-L2 to PD-1 results in

phosphorylation of two signaling motifs, immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM), that are conserved amino acid sequences in the PD-1 cytoplasmic tail, responsible for recruiting enzymes involved in the cell signaling pathway. This phosphorylation will lead to the recruitment of the protein phosphatases SHP1 and SHP2 which in turn will dephosphorylate intracellular molecules of the TCR signaling pathway and thereby inhibit the induction of the phosphatidylinositol-3-kinase (PI3K) activity and downstream activation of Akt [40]. This results in transcription blockade of genes responsible for: (i) interleukin (IL)-2 secretion that causes T-cell activation; (ii) protein synthesis such as initiation, termination and elongation factors needed for protein mRNA translation and; (iii) cell survival (*Fig.1*). Since PD-1 expression hinders productive anti tumor immunity, it has been identified as a marker for exhausted T-cells [49,50]. A role for the PD-1/PD-L1 pathways in T-cell exhaustion in cancer has already been demonstrated in several studies. First, PD-1 expression was found on CD8+ tumor infiltrating lymphocytes (TILs) in many tumor types. Second, PD-1+ T-cells have lost their effector function. Third, high PD-L1 expression was found in several cancer types and was strongly associated with a poor prognosis in some of them [51-53]. And finally, blockade of PD-1/PD-L1 interactions has been shown to improve clinical outcome and partially restore T-cell responses in several cancer types [54-56].

Interaction of PD-1 with either PD-L1 or PD-L2 has several consequences, including inhibition of T-cell proliferation, survival and effector functions. Therefore, blocking this ligand-receptor interaction with monoclonal antibodies can result in a beneficial anti tumor immune response. Noteworthy, the molecular interactions targeted by PD-1, PD-L1 or PD-L2 blocking antibodies are not identical [10,11] (*Fig.2*). Anti PD-L2 antibodies block the interaction between PD-1 and PD-L2 [46,47]. Anti PD-1 antibodies block interactions between PD-1 and its ligands PD-L1 and PD-L2, while anti PD-L1 antibodies can block interactions of PD-L1 with both PD-1 and the B7 molecule CD80. Data show that the latest seems to function as a 'second receptor' for PD-L1, which means that PD-L1 can exert its inhibitory function via two receptors [45]. Stimulation of the PD-1 pathway not only results in a reduced effector T-cell function, it also increases the immunosuppressive function of Tregs [40,57]. PD-1 is highly expressed on Tregs, as is the case for CTLA-4 [58]. Ligand binding to the PD-1 receptor on the cell surface of Tregs contributes to an upregulation of the cellular phosphatase PTEN that antagonizes PI3K signaling and thus the Akt-mTOR pathway [59]. Akt-mTOR signaling normally controls FoxP3 expression, which is restricted to Tregs, and thereby exerts immunotolerance via homeostasis

between effector T-cells and Tregs. If this signaling is inhibited, FoxP3 expression is no longer controlled and naïve CD4⁺ T-cells are converted into Tregs eventually leading to more immunosuppression. So, blockade of the PD-1 pathway may not only enhance anti tumor responses by switching off the brakes on effector T-cell activation, but also by reducing the number and suppressive activity of intratumoral Tregs [58].

Blockade of the PD-1 pathway leads to drug related adverse events, as also reported for anti CTLA-4 treatment. Most adverse events are generally of low grade toxicity (grade 1 or 2), such as fever, fatigue, diarrhea, headache and nausea [8,10,11,60]. More severe immune related adverse events (grade 3 or 4 toxicity) have been reported for anti PD-1 by Hamid et al.[8] and Topalian et al.[10]. They observed vitiligo, pneumonitis, renal failure and hepatitis in some of the treated patients. Also anti PD-L1 has been associated with grade 3 or 4 toxic effects such as asthenia, thrombocytopenia, sarcoidosis and renal insufficiency [11,60]. However, grade 3 and 4 immune related adverse events are rare for anti PD-1 and PD-L1 treatment and if they appear treatment is stopped immediately and glucocorticosteroids are given to the patient.

Other interesting immune checkpoints: LAG-3 and TIM-3

Current research is mainly focused on CTLA-4, PD-1 and their ligands. However, also other molecules on the surface of T lymphocytes can exert inhibitory functions, such as LAG-3 and TIM-3. These two 'neglected' immune inhibitory molecules are now gaining more interest since they have been described to be related to T-cell tolerance and exhausted T-cells that are infiltrating the tumor microenvironment [61-63]. The hypothesis that also other molecules are involved in T-cell exhaustion is supported by the observation that targeting PD-1 or its ligands does not always restore T-cell function [64] and that PD-1 expression is not always associated with an exhausted phenotype [65,66].

LAG-3 is a cell surface molecule expressed on activated CD4⁺ and CD8⁺ T lymphocytes [67]. Its structure is quite similar to the CD4 molecule and therefore it is also categorized as a CD4-like protein. As CD4, LAG-3 also binds to MHC class II molecules. However, these molecules are bound with a higher affinity and at a distinct site than CD4 [68]. Besides, CD4 and LAG-3 have different locations and distinct functions. CD4 is mainly expressed on the cell surface while most of the LAG-3 molecules are retained intracellularly and only expressed after lymphocyte activation [69]. While CD4 acts as a positive co-stimulatory molecule, LAG-3 has a negative regulatory effect on T-cell function. It has been

identified in a study by Blackburn et al.[65] as being expressed on exhausted T-cells, similarly to CTLA-4 and PD-1. The mechanisms by which LAG-3 negatively modulates T-cell function are still under investigation. However, LAG-3 has a unique cytoplasmic tail that contains the KIEELE motif. Workman et al.[70] have shown in a mouse model that LAG-3 regulates the expansion of activated T-cells and that its function depends on binding to MHC class II molecules and signaling through its cytoplasmic KIEELE motif. When LAG-3 interacts with MHC class II molecules there is no TCR signaling that normally leads to activation and proliferation (*Fig.3A*). In contrast, signaling occurs through the cytoplasmic tail preventing the entry of T-cells into the growth phase of the cell cycle ultimately leading to inhibition of T-cell expansion [69,71]. In another in vivo study, Workman et al.[72] saw that there was increased expansion of both CD4+ and CD8+ T-cells in mice lacking LAG-3 expression, clearly showing that both cell types were equally affected by the absence of LAG-3. Interestingly, although MHC II is classically considered as a ligand for CD4+, it also influences expansion of CD8+ T-cells via LAG-3. In cancer, this means that LAG-3 expression will negatively influence the anti tumor response which could be detrimental. Interfering with the LAG-3-MHC II interaction may help priming or potentiating pre-existing T-cell responses to tumoral antigens. Interestingly, in contrast to anti CTLA-4 and anti PD-1, PD-L1 or PD-L2 treatment, nearly no drug related adverse events have been reported to date for the LAG-3 antibody IMP321 [73], except for some grade 1 local reactions [74]. Nearly ten years ago TIM-3 was discovered as a specific cell surface molecule expressed on IFN γ -secreting CD4+ T helper 1 cells (Th1) and cytotoxic T-cells type 1 (Tc1) [75]. Its ligand, galectin-9, is widely expressed on several cell types and upregulated in various types of cancer as well. Under physiological conditions, interaction of TIM-3 with its ligand galectin-9 silences Th1 immune responses by inducing a death signal in the Th1 cells in order to prevent auto-immunity and undesirable immunopathology [76]. Due to its negative regulatory function on the immune system TIM-3 can be classified as an immune checkpoint, along with CTLA-4, PD-1 and LAG-3. While CTLA-4, PD-1 and LAG-3 are upregulated on all activated T-cells, TIM-3 expression is restricted to Th1 and Tc1 cells [75]. Over the years, TIM-3 expression has also been found on innate immune cells such as monocytes, macrophages, dendritic cells and mast cells [77]. When there is no TIM-3 signaling, stimulation of these cells will result in the activation of other immune cells and the production of several cytokines. Depending on the cytokines, different immune responses can be elicited such as CD4+, CD8+ T-cell and B-cell responses. IL-12 promotes Th1 and Tc1 responses

resulting in secretion of IFN γ . This upregulates galectin-9 in Th1 and Tc1 cells that will interact with TIM-3+ Th1 and Tc1 cells, inducing cell death or decreased cell functioning (*Fig.3B*). The underlying molecular mechanism is not known yet and needs to be further elucidated. Several in vivo preclinical studies showed that blocking TIM-3 restores T-cell function, which is supported by remarkable clinical effects in cancer patients [62,78,79]. Similar results were found by an in vitro study showing restored functioning of melanoma patients' T-cells after TIM-3 blockade [80]. TIM-3 regulates responses that are critically important in fighting cancer. Recent studies have addressed a key role to TIM-3 in T-cell dysfunction or exhaustion that occurs in cancer [62,78-80]. Sakuishi et al.[62] described TIM-3 expression on a large fraction of CD8+ TILs in mice bearing solid tumors. All the TIM-3+ cells co-expressed PD-1 and those TIM-3+PD-1+ TILs failed to proliferate and produce IL-2, tumor necrosis factor (TNF) and IFN γ . These cells exhibited the most profound defects in T-cell effector function and were categorized as the most severe exhausted phenotype. The authors showed that single targeting of the PD-1 and TIM-3 pathway has variable effects on tumor growth, whereas combined treatment with anti PD-1 and anti TIM-3 was highly effective in controlling tumor growth and restoring IFN γ production by T-cells.

In summary, TIM-3 and LAG-3 are attractive candidates for cancer immunotherapy. Achieving a full understanding of their role in regulating immune response might help the development of new anti cancer (combination) treatments.

Clinical results of blocking antibodies

Different PD-1 blocking compounds are available, such as nivolumab (Opdivo[®], BMS-936558, Bristol-Meyers Squibb), pembrolizumab (Keytruda[®], MK-3475, Merck) and pidilizumab (Cure-Tech). The latter is the first PD-1 blocking agent that entered clinical trials [81]. It is a humanized (i.e. a mixed human and murine antibody) IgG1 monoclonal antibody, initially investigated during a phase I trial in patients with advanced hematological malignancies. Durable responses of more than 60 weeks were noted, the drug was well tolerated and a maximum tolerated dose was not reached. Second, anti PD-1 immunotherapy with pembrolizumab has already shown prolonged clinical activity in phase I/II trials in melanoma, kidney cancer and lung cancer [8,9,11]. In 2014, the FDA approved pembrolizumab for the treatment of advanced or inoperable melanoma in patients who have failed prior treatment, followed by the approval of nivolumab for the same group of patients. While pidilizumab and pembrolizumab

are humanized antibodies, nivolumab is a fully human IgG4 PD-1 targeting monoclonal antibody. It was first investigated in a phase I trial with advanced solid tumors (NCT00441337)[82]. No maximum tolerated dose was reached and there seemed to be a correlation between PD-L1 expression and response to therapy. Nivolumab was further studied in other phase I and phase II trials, showing durable responses. In March 2015, FDA also approved nivolumab as second line treatment for advanced squamous non-small cell lung carcinoma (sourced from www.fda.gov). Compared to the chemotherapeutic agent docetaxel, nivolumab improved overall survival in previously treated patients with 3.2 months [83].

Besides blocking PD-1 directly, the pathway can also be interrupted by inhibiting interaction of one of its ligands with the PD-1 receptor. PD-L1 expression is broadly found across different human tumor types such as melanoma, ovarian cancer and renal cell carcinomas [41-44]. Promising results with anti PD-L1 immunotherapy (BMS-936559) have been described in a phase I trial that included several advanced cancers [11]. MPDL3280A (Atezolizumab[®], Genentech) and MEDI4736 (Durvalumab[®], AstraZeneca) are both human IgG1 monoclonal antibodies recognizing PD-L1. Complement mediated cytotoxicity and ADCC are eliminated since they both have mutations in their F_c tail. This prevents immune cells to be eliminated by the own immune system when the blocking antibody binds to it. An acceptable safety profile and durable clinical activity of MEDI4736 has been reported [71]. A phase I trial investigating another PD-L1 compound, avelumab (MSB0010718C, Pfizer), is currently ongoing (NCT01772004, *table 1*).

Interestingly, avelumab, as opposed to atezolizumab and durvalumab, has no mutations in its F_c tail thereby allowing ADCC and thus depletion of PD-L1 expressing cells.

Until now, only limited information on clinical trials related to the PD-L2 blockade can be found. However, since PD-L2 binds with a higher affinity to PD-1 compared to PD-L1 [47] and because expression of PD-L2 on tumor cells or in the stroma can impair effector T-cell activity within the tumor microenvironment, it is also an attractive candidate for targeted immunotherapy. An ongoing phase I trial tests a PD-L2 cross linking antibody, rHlgM12B7, in patients with metastatic melanoma (NCT00658892).

As an alternative strategy to monoclonal blocking antibodies, AMP-224 (GSK) was developed. It is a recombinant fusion protein comprised of the extracellular domain of human PD-L2 and the F_c domain of human IgG1. The mode of action of AMP-224 is distinct from direct blocking of PD-1/PD-L1

interaction. The hypothesis for AMP-224 mode of action is depletion of dysfunctional CD8+PD-1^{high}+ TILs. Data was sourced from a presentation given at the TAT congress in 2013 (<http://tatcongress.org/wp-content/uploads/2014/05/I02.04-smothers-tat-2013-final.pdf>). A phase I trial to study the safety, tolerability and pharmacokinetics of AMP-224 in patients with advanced cancer has been completed (NCT01352884). Varying doses of AMP-224 were given to 42 patients with advanced solid tumors. No drug-related inflammatory adverse events were observed. Immunohistochemical analysis of biopsies showed that 31% of the baseline tumors were PD-L1⁺. Paired biopsies (at baseline and following treatment) from individuals with partial or mixed responses or stable diseases showed a reduction of PD-1+ cells and the development of functional T-cell responses based on the presence of IFN γ +, IL-2+, CD4+ and CD8+ T-cells [84]. Like for CTLA-4 blockade, also immune-related adverse events such as fever, fatigue and rash are reported for anti PD-1/PD-L1/PD-L2 treatment. The fact that anti PD-1 therapy seems to exert less immune related adverse events compared to anti CTLA-4 is remarkable but might be explained by their possible difference in site of action (blocking central versus peripheral T-cell activation). This may also explain the higher response rate and more rapid tumor response associated with PD-1/PD-L1 blocking antibodies [6].

In a phase I dose escalation study the anti LAG-3 blocking antibody IMP321, in combination with chemotherapy, more specifically paclitaxel, was investigated in metastatic breast cancer patients. 30 patients were included, divided into 3 cohorts. Objective tumor response rates were seen in 50% of the patients compared to 25% in a historical control group of patients derived from a phase III study with a similar dosing schedule for chemotherapy [85]. During follow up further tumor regression was observed, something that is not seen with chemotherapy alone (NCT00349934) [86]. Also trials with IMP321 in renal cell cancer, breast cancer and melanoma patients [44,69] showed enhanced anti tumor immune responses and improved tumor control [78]. Additional trials investigating IMP321 are underway or ongoing in several chronic diseases and cancer types.

BMS-986016 is another anti LAG-3 monoclonal antibody currently being tested in patients with advanced solid tumors (NCT01968109)(*table 1*). Its safety and tolerability, as well as the maximum tolerated dose is being assessed alone and in combination with nivolumab. Results are awaited. A lot of in vivo research on blocking TIM-3 has been described yet. Unfortunately, till now no clinical grade antibodies targeting TIM-3 are being tested.

Immune checkpoints as biomarker

The value of PD-L1 as a prognostic biomarker has been addressed in many cancer types. Data on correlation between expression and overall survival remain controversial. Hino et al.[87] reported PD-L1 expression as a poor prognostic factor in malignant melanoma, while the opposite seems to be suggested by data from Gadiot et al.[88]. Similar discrepancy was also found for NSCLC, ovarian cancer and renal cell carcinoma [42,89-91] and might be explained by the use of different sample sizes and staining protocols.

Whether PD-L1 is a good predictive marker for response to anti PD-1/PD-L1 therapy is still unclear. While Topalian et al.[10] state that PD-L1 expression is a good predictive marker for anti PD-L1 immunotherapy in melanoma patients, Wolchock et al.[92] suggest that patients can have a response regardless of their PD-L1 status at baseline.

Taken together, PD-L1+ patients show increased response rates but patients with PD-L1- tumors do respond as well and they have the same overall survival rate as PD-L1+ patients. The prognostic and predictive discrepancy can be partially explained by: (i) the absence of standardized kits or clear cut off values for immunohistochemistry to determine PD-L1 positivity, (ii) heterogeneous PD-L1 expression within or between tumor lesions and (iii) variation of PD-L1 expression over time during changes in the tumor microenvironment, resulting in variable expression data. Other techniques to analyse PD-L1 expression that might be more sensitive, such as in vivo imaging, can offer a solution. Heskamp et al.[93] reported about a non-invasive imaging technique with radiolabeled PD-L1 antibodies that has several advantages compared to the immunohistochemical analysis of PD-L1 expression. Since it allows monitoring of expression throughout the course of disease no repetitive biopsies are required and the problem of heterogeneous expression is also solved because expression of the whole tumor lesion can be determined using this technique. On the other hand, it should be noted that anti PD-1/PD-L1 therapy can not only interfere at the tumor cell/immune cell interface, but also with the interaction between APC and T-cells, which might result in an alleviated brake on effector T-cell activation. This might also be an explanation why patients with PD-L1- tumors can respond to anti PD-1/PD-L1 therapy.

Importantly, a search on other possible biomarkers with good predictive value such as the mutational load, smoking status of the patient, line of treatment and T-cell infiltration of the tumor is ongoing.

Immune checkpoints in mesothelioma: a new treatment opportunity?

Clinical evidence suggests that the immune system plays a critical role in protection against MPM [5,94,95]. TILs play an important role in anti tumor immune responses by recognizing tumor-specific antigens and T-cell infiltration has already been associated with a good prognosis in many cancers, such as ovarian and colon cancer. In MPM, high number of CD8+ TILs has shown to be beneficial for prognosis [96-98], while others reported no association between TILs and survival [99]. Although current data about the role of TILs in MPM are controversial, it is clear that T lymphocytes are important for anti tumor immunity and that activating them by inhibiting immune checkpoints might be an efficient strategy to improve MPM prognosis.

Tremelimumab, a CTLA-4 inhibitor, is currently undergoing evaluation in malignant mesothelioma. There is preclinical evidence of a synergistic effect between CTLA-4 blocking therapy and chemotherapy in murine models of malignant mesothelioma [100,101] and targeting CTLA-4 has already shown promising results in mesothelioma patients. In 2013, Calabrò et al.[12] reported a phase II trial (MESOT-TREM-2008, NCT01649024, *table 1*) using tremelimumab in patients with chemotherapy-resistant advanced MPM. Results of this study showed that tremelimumab has encouraging clinical activity and an acceptable safety profile in treated patients [12]. Disease control was noted in 31% of the patients and the median overall survival was 10.7 months, which is more favorable compared to 8.7 months reported in a retrospective review about second line treatment with chemotherapy in MPM patients [102]. Those findings correlate to the ones from the MESOT-TREM-2012 study (NCT01655888), in which a lower tremelimumab concentration and more doses were given and a maintenance phase was included [103]. 29 patients were enrolled in this study whereby tremelimumab showed an acceptable safety profile and a median overall survival of 11.3 months. A randomized phase II double blind trial comparing tremelimumab treatment to placebo as second or third line treatment in patients with unresectable mesothelioma has recently closed its accrual and its results are awaited (NCT01843374)[104,105].

In MPM, PD-L1 expression by immunohistochemistry in formalin-fixed paraffin embedded material was reported, ranging between 20% and 70% [104,106-109] (*table 2*). Most studies reported the expression data per MPM subtype (*table 2*). PD-L1 expression was significantly associated with a worse survival and overexpression was more common in non-epitheloid histology. To date, in one

publication only researchers also looked at cell subtypes, reporting expression both on tumor cells and TILs. They showed that PD-L1+ tumors had more PD-L1+ TILs and lymphoid aggregates than PD-L1-MPM tumors.

Recently, preliminary data of the phase I trial, KEYNOTE-028, were presented (NCT02054806)[110]. This trial investigated pembrolizumab in an extension cohort of 25 MPM patients at a dose of 10mg every 2 weeks. Response to treatment was evaluated every 8 weeks. Partial responses were observed in 7 patients (28%) and stable disease in 12 patients (48%). The overall disease control rate was 76%. A phase II trial investigating pembrolizumab in mesothelioma (NCT02399371) will explore to which extent pembrolizumab is active in MPM, as well as if PD-L1 expression could be a good predictive biomarker for response to PD-1 inhibitors. Taken together, results show that PD-1 and PD-L1 blocking antibodies might be a good approach for MPM treatment. About PD-L2, as well as LAG-3 and TIM-3 nothing has been described in mesothelioma patients to date. It would be worth further investigating the second PD-1 ligand and those rather 'unknown checkpoints'. Furthermore, new studies trying to define the synergistic mechanisms of combined approaches will lay the foundation for selecting optimal combination therapies.

Discussion

Inhibiting immune checkpoints with blocking antibodies has already shown promising results in several cancer types [111] and encouraging clinical data with blocking CTLA-4, as well as expression data of PD-1 and PD-L1 in MPM support further investigation of immune checkpoint targeted therapy for MPM treatment. Thorough investigation of the expression of immune checkpoints, also the 'neglected' ones, in MPM and the effect of their blockade would help to identify new targets for immunotherapy and to find a predictive biomarker for patients. For the latter, it should be kept in mind that PD-L1 expression can vary over time due to up- or downregulation and that it is so far unknown whether it is a conclusive biomarker for PD-1 and/or PD-L1-targeting immunotherapy.

Another important aspect to judge eligibility of cancers for immunotherapy is the concept of mutational load. Immune checkpoint blocking antibodies might be more effective in tumor types that have a lot of neoantigens, since in these cases previously induced immune responses can be potentiated by the antibodies [112]. This might be an explanation for the high susceptibility of melanoma, known as a strongly mutated cancer, to immune checkpoint blockade [113]. Based on exome sequencing data,

mesothelioma is estimated to have on average 5 somatic mutations per megabase (data presented by A. Nowak at the IASLC 16th Annual Meeting in Denver, USA 2015). Although to date the number of neoantigens has not been described for MPM, preclinical data [100,101] as well as (preliminary) clinical results [12,103,110] support its susceptibility to immune checkpoint blockade.

Given the fact that multiple pathways are involved in inhibiting anti tumor responses it is likely that blockade of multiple inhibitory pathways will generate a more potent immune response to cancer. For the checkpoint inhibitors, combined CTLA-4 and PD-1 blockade showed preclinical synergistic anticancer activity without overt toxicity [114]. Wolchock et al.[92] were the first to report on the combination of anti PD-1 (nivolumab) and anti CTLA-4 (ipilimumab) antibodies in melanoma patients. Combined blockade improved response rates even further compared to stand alone treatment with ipilimumab, but also increased cytotoxicity. These data are supported by a randomized double blind study in patient with metastatic melanoma (NCT01927419)[115]. The objective response rate and progression free survival were significantly greater in patients receiving the combined therapy compared to ipilimumab monotherapy. Also an acceptable safety profile has been reported for the combination strategy. Besides melanoma, combination of nivolumab with ipilimumab also showed promising results as first-line treatment in patients with NSCLC (NCT01454102)[116]. The combination therapy was reported to be feasible and anti tumor activity was seen in PD-L1+ as well as PD-L1- patients.

Enhanced anti tumor responses were also reported when PD-1 blockade was combined with LAG-3 or TIM-3 blocking antibodies [63,117,118] compared to modest effects of all three when used as monotherapy. In MPM, so far nothing has been described on combined blockade. Blocking inhibitory receptors in combination with active immunotherapy or chemotherapy already showed promising preclinical and clinical results in several cancers, including prostate cancer, melanoma and breast cancer [100,119-124]. However, the optimal timing of such a combination therapy remains to be elucidated and so far nothing has been described about the effects of combining chemotherapy with immune checkpoint blockade in MPM patients.

Preclinical studies are necessary to unravel working mechanisms of different immune checkpoint inhibitors in mesothelioma and to investigate a possible synergy of their combination with other therapies (chemotherapy, other immune checkpoints,...). Preclinical proof-of-concept that immune

checkpoint blockade has anti tumoral effects in MPM as stand-alone treatment or in combination with other strategies would guide the rational design of future clinical studies.

Conclusion

PD-1 and PD-L1 were awarded as cancer breakthrough targets of the year 2013 evolving in a booming attention for immune checkpoints in oncology research. Whilst MPM has interesting immunological features, only few studies until now have focused on this cancer. Research on the expression of immune checkpoints in mesothelioma might help to identify new biomarkers thereby improving patients selection and avoiding toxic side effects in non-responders. Better understanding of the mechanisms of action of immune checkpoint blocking antibodies will provide the rationale to select optimal combination therapies.

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Tables

Antibody	Company	Target	Phase	Cancer	NCT number	Status
MONOTHERAPY						
Nivolumab	Bristol-Meyers Squibb	PD-1	I	Solid tumors	NCT00441337	Completed
Pembrolizumab	Merck	PD-1	II	Mesothelioma	NCT02399371	Recruiting
Pembrolizumab	Merck	PD-1	I	Mesothelioma	NCT02054806	Active, not recruiting
Avelumab	Pfizer	PD-L1	I	Solid tumors	NCT01772004	Recruiting
AMP-224	GlaxoSmithKline	PD-L2	I	Advanced cancers	NCT01352884	Completed
Tremelimumab	MedImmune LLC	CTLA- 4	II	Malignant mesothelioma	NCT01655888	Unknown, ref [103]
Tremelimumab	MedImmune LLC	CTLA- 4	II	Malignant mesothelioma	NCT01649024	Unknown, ref [12]
Tremelimumab	MedImmune LLC	CTLA- 4	II	Unresectable pleural or peritoneal malignant mesothelioma	NCT01843374	Active, not recruiting
COMBINATION THERAPY						
BMS-986016 and BMS-936558	Bristol-Meyers Squibb	LAG-3 and PD-1	I	Solid tumors	NCT01968109	Recruiting

Table 1. Overview of clinical trials mentioned in this review and registered on ClinicalTrials.gov of blocking agents targeting immune checkpoints as monotherapy or in combination with other agents in studies open for mesothelioma patients (09/09/2015).

Reference	Method	Antibody clone	Sample size	Cut off	% PD-L1+
Mansfield et al.[106]	Immunohistochemistry	5H1-A3	106	≥ 5%	40% (E 21%, S 94%, B 57%)
Cedres et al.[108]	Immunohistochemistry	E1L3N	119	> 1%	20% (E 13%, NE 38%)
Kindler et al.[125] <i>(and personal communication)</i>	Immunohistochemistry	5H1-A3	45	≥ 50%	27% (E 17%, B 7%)
Cowan et al.[109]	Immunohistochemistry	5H1-A3	33	≥ 5%	70%
Alley et al. <i>(data presented at the 16th World Conference on Lung cancer in Denver, USA)</i>	Immunohistochemistry	22C3	80	> 1%	45,2%

Table 2. Summary of PD-L1 expression in mesothelioma reported by four different groups using immunohistochemistry. E, epitheloid subtype; S, sarcomatoid subtype; B, biphasic subtype; NE, non-epitheloid subtype.

Figure legends

Fig. 1. PD-1 and CTLA-4 immune inhibitory pathways. Interaction between TCR and MHCII molecule followed by a co-stimulatory signal via CD28-CD80/CD86 results in normal TCR signaling and translocation of intracellular vesicles with PD-1 and CTLA-4 to the cell surface. Upon binding PD-L1 or PD-L2, the ITIM and ITSM of PD-1 its cytoplasmatic tail get phosphorylated (①). In turn, these signaling motifs will recruit protein phosphatases, SHP-1 and SHP-2 (②), that will dephosphorylate PI3K thereby inhibiting Akt activation downstream (③). Binding of CTLA-4 to CD80/CD86 (①) also blocks Akt activation by preventing its phosphorylation via the protein phosphatases SHP-2 and PP2A (②). In the end, both signaling pathways result in transcription blockade of several genes important for cell survival and protein synthesis.

Abbreviations: PD-1, programmed death-1; CTLA-4, cytotoxic T lymphocyte antigen-4; TCR, T-cell receptor; MHC, major histocompatibility complex; CD, cluster of differentiation; PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; PI3K, phosphatidylinositol-3-kinase.

Fig. 2. Molecular interactions blocked by monoclonal antibodies targeting PD-1, PD-L1 or PD-L2.

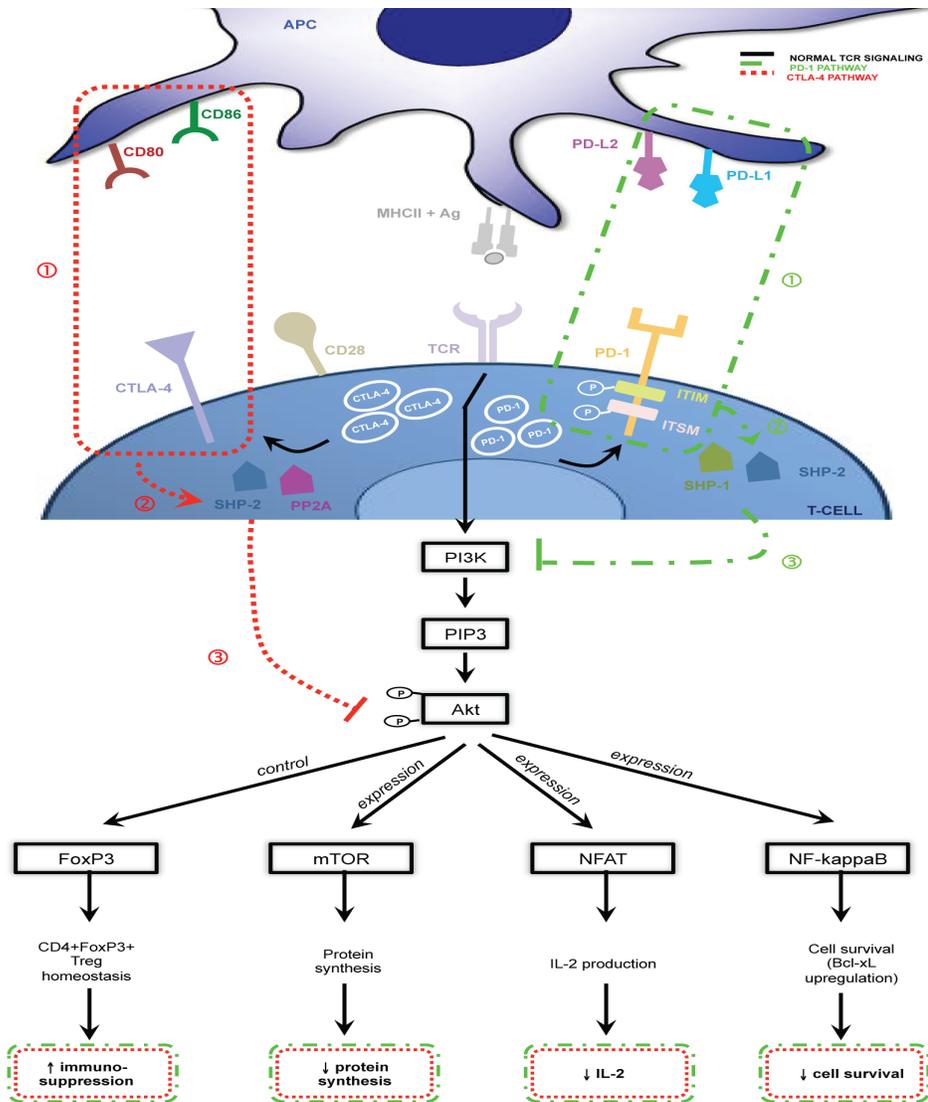
Blocking PD-1 prevents interaction with its 2 ligands, PD-L1 and PD-L2. PD-L1 blockade avoids binding of PD-L1 to PD-1, as well as to CD80, which has shown to be a 'second receptor' for PD-L1. Anti PD-L2 antibodies block ligation of PD-L2 to PD-1.

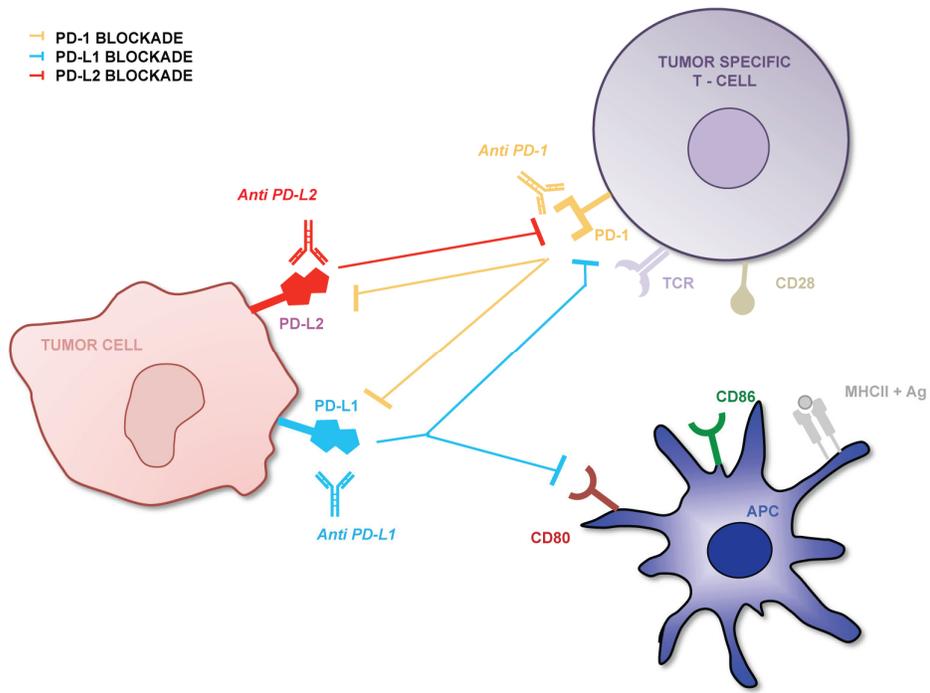
Abbreviations: PD-1, programmed death-1; PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; CD, cluster of differentiation.

Fig. 3. Immunoregulation by LAG-3 and TIM-3. (A) Binding of LAG-3 to an MHCII molecule prevents interaction of CD4 and the TCR with the same MHC molecule, thereby inhibiting normal TCR signaling. Via the KIEELE motif on its cytoplasmatic tail cell proliferation is blocked which eventually results in a less efficient antitumor response. (B) Upon interaction with galectin-9 which is expressed on different cell types, TIM-3 induces a death signal in Th1 and Tc1-cells. By decreasing IL-12 production a brake is put on the Th1 and Tc1 differentiation which leads to less IFN γ secretion that normally stimulates immune activation.

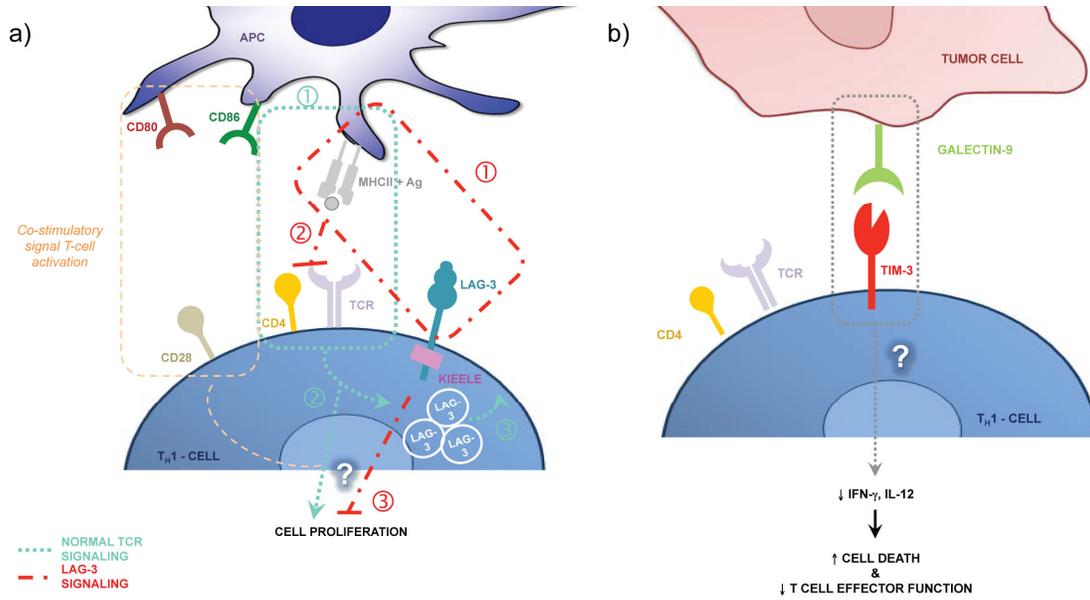
Abbreviations: LAG-3, lymphocyte antigen-3; TIM-3, T-cell immunoglobulin mucin-3; MHC, major histocompatibility complex; TCR, T-cell receptor; IL, interleukin; Th1, T helper 1; Tc1, cytotoxic T cell type 1; IFN, interferon.

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Highlights review**“Targeting immune checkpoints: new treatment opportunity for mesothelioma?”**

- ❖ Working mechanism and expression pattern of immune checkpoints
- ❖ Clinical data of immune checkpoint blocking antibodies are very promising
- ❖ Immune checkpoint blockade deserves further investigation in mesothelioma
- ❖ Future opportunities for research on immune inhibitory molecules

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