NOVEL DEVELOPMENTS IN IMMUNE CHECKPOINT THERAPY

Targeting the Adenosine A2 receptor: an immune checkpoint that inhibits the anti-tumour immune response

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Abstract

Immune checkpoints consist of inhibitory pathways that regulate the immune system in the event of an active immune response. Tumour cells abuse these checkpoints to influence the immune system and thereby interfere with the anti-tumour response. Targeting these immune checkpoints composed a potential target for cancer immunotherapy; which has shown promising responses and has been a focal point in cancer research. Therapeutic agents targeting CTLA-4 and PD-1/PD-L1 have shown success in clinical trials and have been approved for treatment in a variety of advanced cancers. Research into other checkpoints has been conducted and the adenosine A2a receptor (A2aR) has been identified as a potential target for cancer immunotherapy. Similar to the CTLA-4 and PD-1/PD-L1 pathways, activation of A2aR inhibits the immune response. In this article, I will describe the recent developments in checkpoint inhibitors, including current preclinical and clinical therapies, discussing the mechanism of action, adverse events and treatment limitations. The current data suggest that targeting A2aR has great potential to improve clinical outcomes in cancer patients.

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Introduction

In the past few years, cancer immunotherapy has regained interest within the oncology research field after success with checkpoint inhibitor therapy (Lee, Sun, Sandler & Hoang, 2018). Cancer immunotherapy is directed at manipulating the body's own immune system, reactivating it to enhance the anti-tumour response. The major focus of immunotherapy concentrates on the T cell because of their capacity to induce immune responses. Immune checkpoints consist of inhibitory pathways that regulate the immune system in the event of an active immune response. The duration and extent of the response is regulated with the negative feedback system created by immune checkpoints while maintaining self-tolerance and preventing collateral damage to tissues. (Leone, Lo & Powell, 2015, Pardoll, 2012). Tumour cells abuse these checkpoints to influence the immune system and thereby interfere with the anti-tumour response. Targeting these immune checkpoints composed a potential target for cancer immunotherapy; which has shown promising responses and at the moment continues to be the main focus of cancer research (Leone, Lo & Powell, 2015, Pardoll, 2012, Postow, Callahan & Wolchock, 2015).

For a successful elimination of cancer cells, a series of steps named the cancer immunity cycle, must be completed (Fig. 1). When cancer cells are phagocytosed, Antigen presenting cells (APCs) take up neoantigens, which are then processed and presented on MHCI and MHCII molecules to T cells (Chen & Mellman, 2013, Lee, Sun, Sandler & Hoang, 2018). Priming and activation only occurs when MHC-peptide complex binding to a T-cell receptor (TCR) (signal 1) simultaneously takes place with co-stimulatory CD28 - B7.1/2 signalling (signal 2) (Fig. 2). Absence of both signals leads to anergy or apoptosis of the T cell (Hahn, Gill, Pal & Agarwal, 2017, Sharma & Allison, 2015, Zou & Chen, 2008). In the following step, activated T cells traffic to the tumour and infiltration occurs. Recognition through interaction between the TCR and the MHC-bound antigen results in killing of the cancer cell. Dying cancer cells are eaten by phagocytes, presenting neoantigens once again, and the cancer immunity cycle is initiated (Chen & Mellman, 2013, Lee, Sun, Sandler & Hoang, 2018).

Through multiple mechanisms, cancer cells are able to hijack signals within the cancer immunity cycle. This can be achieved by activating inhibitory checkpoint proteins or dysregulating immune checkpoint expression. For instance, cells within the tumour microenvironment upregulate molecules of the B7 family; molecules which deliver the co-stimulatory signal to activate T-cells or inhibitory signals to maintain homeostasis (Leone, Lo & Powell, 2015, Xu, Jiang, Gao & Zhao, 2014, Zou & Chen, 2008). The reprogramming of immune cells into dysfunctional cells creates an imbalance which allows the tumour to grow, evolve and metastasise (Zou & Chen, 2008). To restore the balance of the immune system, multiple studies have been carried out to research these immune checkpoints as potential targets for treatment. Several immune checkpoints, as well as co-stimulatory proteins are located within different stages of the cancer immunity cycle. CD28, Inducible T-cell Co-stimulator (ICOS), glucocorticoid-induced TNFR-related protein (GITR), OX40, 41BB and CD40L are co-stimulators, while Programmed Cell Death Protein 1 (PD1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), Lymphocyte-activation gene 3 (LAG-3) and T cell immunoglobulin and mucin domain 3 (TIM-3) are inhibitory immune checkpoints. Immune checkpoint pathways mostly consist of ligand-receptor signalling, providing multiple opportunities for interfering with the signals (Hahn, Gill, Pal & Agarwal, 2017, Pardoll, 2012).

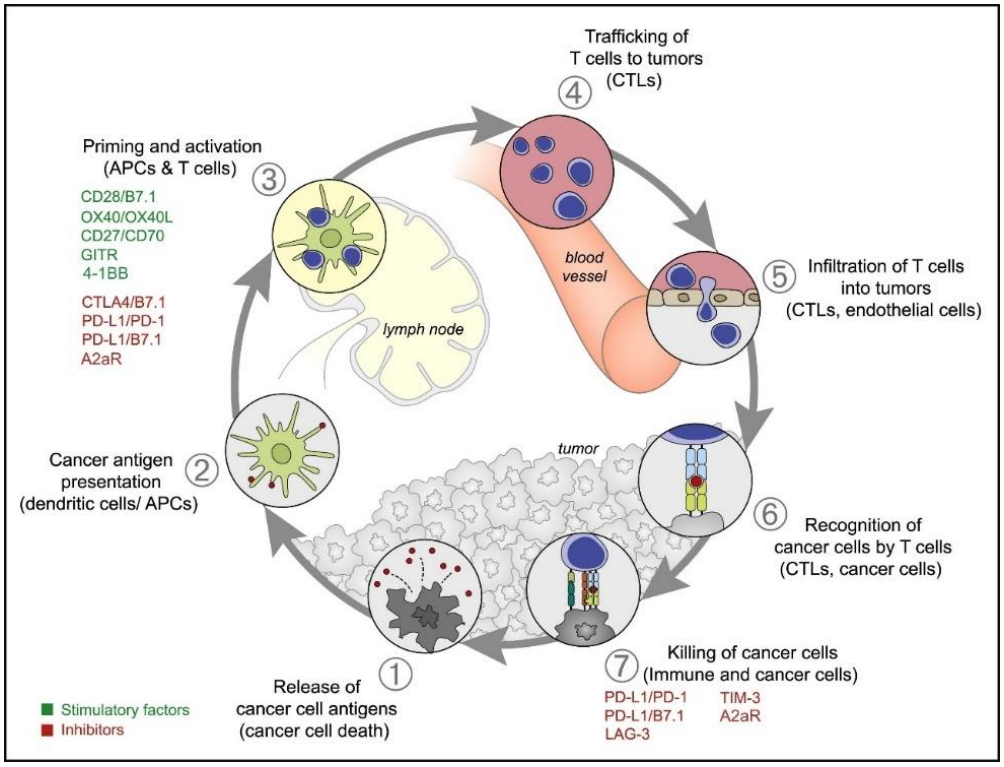

Figure 2 | The cancer immunity cycle. 1) Tumour neoantigens initiate the cancer immunity cycle. 2) APCs present neoantigens to naïve T cells. 3) Priming and activation of T cells after MHC-peptide complex binding to TCR and CD28-B7.1/2 signalling. CLTA-4, PD-1/PD-L1 and A2aR inhibitors exert effect during this stage. 4) Trafficking of T cells to tumours. 5) Infiltration of T cells into tumours. 6) Recognition of tumour cells by T cells. 7) Killing of tumour cells. PD-1/PD-L1 and A2aR inhibitors intervene in this stage of the cancer immunity cycle **(**Chen & Mellman, 2013).

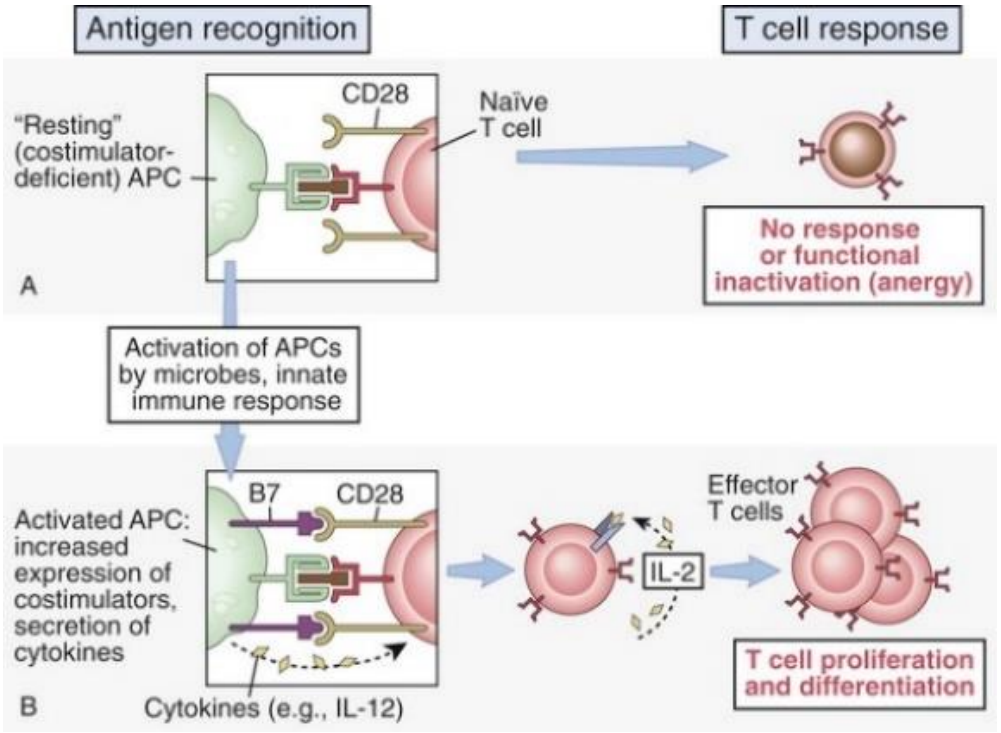

Figure 1 |A) No response in T cells after binding of MHC-peptide complex solely to TCR. B) Activation and priming of T cell after binding of MHC-peptide complex to TCR, simultaneous with binding of CD28 to B7.1/2 (Abbas AK, Lichtman AH, Pillai S: Cellular and molecular immunology, ed 6, Philadelphia, 2010, Saunders-Elsevier).

Ipilimumab is a checkpoint inhibitor that targets CTLA-4 and has been approved by the FDA (Hahn, Gill, Pal & Agarwal, 2017, Lee, Sun, Sandler & Hoang, 2018). Pembrolizumab, nivolumab, avelumab and atezolizumab are monoclonal antibodies targeting either PD-1 or Programmed Death Ligand 1 (PD-L1), which have also been approved by the FDA for treatment of several malignancies such as metastatic melanoma and non-small-cell lung carcinoma (NSCLC). There are multiple ongoing clinical trials targeting different checkpoints such as LAG-3, TIM-3, B7-H3, GITR, OX40 and 41BB (Hahn, Gill, Pal & Agarwal, 2017). A new emerging target in immunotherapy is the Adenosine A2a receptor (A2aR). Activation of the A2aR by extracellular adenosine inhibits the immunological response against pathogens to prevent collateral damage. A2aR agonist have been evaluated for safety in clinical trials for treatment of Parkinson's disease and generally are tolerated well (Fong et al., 2017). Antagonising A2aR in cancer immunotherapy has shown promising preclinical results and are momentarily being tested in phase I/Ib clinical trials.

In this review, I aim to give insight into the recent developments on the subject of checkpoint inhibitors. I will elaborate on the current clinical therapies targeting CTLA-4 or PD1/PD-L1, discussing their mechanism of action, adverse events and treatment limitations. Furthermore, we will look at the mechanism of action, preclinical studies and the active clinical trials for immune checkpoint inhibitors targeting the adenosine A2A Receptor (A2aR). Through evaluating the results concerning A2aR checkpoint inhibitors, I aim to come to a conclusion and propose directions for further studies.

Current clinically approved checkpoint inhibitors

Targeting checkpoints for cancer therapy has been a major focus of oncological research for the past years. In 1988, Cytotoxic T lymphocyte antigen-4 (CTLA-4) was cloned and found to be essential in regulation of T cell homeostasis and tolerance (Weber, 2012). Preclinical studies reported that CTLA-4 can regulate cytotoxic $CD8^+$ cells and that blockade of CTLA-4 increased responsiveness of $CD8^+$ cells and that tumour growth was controlled (McCoy et al., 1999, Shrikant, Khoruts & Mescher, 1999). CTLA-4 was the first immune checkpoint to be targeted for clinical use and its success in clinical trials has raised interest in the field of immunotherapy for cancer treatment (Pardoll, 2012, Postow, Callahan & Wolchok, 2015). Blocking of CTLA-4 re-activates the priming phase of the T-cell response which results in regained anti-tumour immunity (Van Hooren et al., 2016).

CTLA-4 preclinical background

CTLA-4 is an immune checkpoint molecule which downregulates and inhibits T-cell activation. CTLA-4 is expressed on $CD4^+$ cells: regulatory T cells (Tregs) and T helper cells; and is expressed on $CD8^+$ cells: effector T cells (Lee, Sun, Sandler & Hoang, 2018, Pardoll, 2012). CTLA-4 supresses the immune response by downregulating T helper cell activity and enhances Treg immunosuppressive activity (Leone, Lo & Powell, 2015, Pardoll, 2012). After initial activation of effector T cells, through downstream TCR signalling, CTLA-4 is transported to and upregulated on the plasma membrane (Leone, Lo & Powell, 2015, Postow, Callahan & Wolchok, 2015, Weber, 2010). CTLA-4 competes with CD28 for binding

to its ligands B7.1 and B7.2 and as CTLA-4 has a much higher binding affinity for both ligands, it interferes with CD28/B7.1/2 signalling activation (Leone, Lo & Powell, 2015, Rudd, Taylor & Schneider, 2009). CTLA-4 actively removes B7.1 and B7.2 from the APC cell surface via trans-endocytosis to prevent CD28 interaction and sends a direct inhibitory signal to T effector cells (Qureshi et al., 2011). Occurrence of these events results in inhibition of T-cell priming and activation (Leone, Lo & Powell, 2015, Pardoll, 2012, Rudd, Taylor & Schneider, 2009). CTLA-4 binds to PI3K, SHP1/2 and Protein Phosphatase 2 (PP2A) to interfere with various TCR downstream signals. Activation of Akt is interfered by PP2A, whereas recruitment of SHP/1 by CTLA-4 results in TCR signalling protein dephosphorylation (Bour-Jordan et al., 2011, Pardoll, 2012, Rudd, Taylor & Schneider, 2009, Salama & Hodi, 2011). Interfering with the effects of CTLA-4 through targeting with monoclonal antibodies will result in a regained anti-tumour response though T effector cell expansion and depletion of Tregs. The resulting effect being that the ratio of effector T cells to Tregs will shift towards a more favourable tumour microenvironment (Van Hooren et al., 2016, Pardoll, 2012).

Clinical checkpoint inhibitors: CTLA-4

Ipilimumab is a monoclonal human IgG1 κ-isotype antibody (Zorginstituut Nederland, 2018). The therapeutic agent entered clinical trials in the early 2000s and was the first immunity checkpoint inhibitor to be approved by the FDA for treatment of metastatic melanoma (Sharma & Allison, 2015). In two phase III trials, ipilimumab showed improved survival in metastatic melanoma patients, with 20% of patients reaching long-term survival of 3-4 years (Lee, Sun, Sandler & Hoang, 2018, Leone, Lo & Powell, 2015, Postow, Callahan & Wolchok, 2015, Sharma & Allison, 2015). Research into treatment of other types of cancer with ipilimumab is being conducted, however, a drawback of the treatment is that administering ipilimumab comes with a high incidence of immune-related adverse events. (Lee, Sun, Sandler & Hoang, 2018).

PD-1/PD-L1 preclinical background

Because of the success achieved with monoclonal antibodies targeting CTLA-4, more research was carried out to discover more immune checkpoints. In 2000, Programmed cell death Protein 1 (PD-1) was identified as an immune checkpoint and this discovery has henceforth lead to the development and production of multiple new checkpoint inhibitors that target this axis (Sharma & Allison, 2015). Blocking of the PD-1/PD-L1 pathway enhances effector T cell activity in the tumour microenvironment and in the tissues.

PD-1 is an inhibitory immune checkpoint which regulates effector T cells within the tissue and tumour environment (Pardoll, 2012). PD-1 is expressed on the surface of a variety of activated T cells, Tregs, B cells, NK cells, activated monocytes and tumour infiltrating lymphocytes (TIL). As illustrated by Figure 1, PD-1 functions during the effector phase of the immune response in the elimination of the cancer cells (Lee, Sun, Sandler & Hoang, 2018, Pardoll, 2012, Postow, Callahan & Wolchok, 2015, Weber, 2010). Programmed death ligand 1 (PD-L1) and PD-L2 are ligands which interact with PD-1. PD-L1 is expressed on T cells, B cells, APCs, macrophages, tumour infiltrating immune cells and non-immune cells, whereas PD-L2 is mostly expressed on APCs. However, PD-L2 expression can be induced in other

immune cells and also in non-immune cells when subjected to specific stimuli within the microenvironment. Both PD-L1 and PD-L2 are expressed on a variety of tumours. PD-L2 expression has also been detected in the absence of PD-L1 (Lee, Sun, Sandler & Hoang, 2018, Weber, 2010, Yearley et al., 2017). When PD-L1/PD-L2 bind to PD-1 on activated T effector cells, cytokine production, T cell proliferation and effector T cell activity is reduced (Chen & Mellman, 2013, Lee, Sun, Sandler & Hoang, 2018, Postow, Callahan & Wolchok, 2015). Other effects of PD-1 signalling include inhibition of the TCR 'stop signal' and enhancement of Treg proliferation. The TCR stop signal is the signal which is given by the TCR to the T cell. This results in reducing the T cells' motility and consequently prolongs the engagement of the T cell with an APC, an important factor in T cell activation. Inhibition of this signal possibly modifies the duration of contact between T cells and their targets while an increase in Tregs leads to further suppression of effector immune responses (Pardoll, 2012). A rather unexpected discovery revealed that apart from binding to PD-1, PDL-1 also forms a pathway with CD80, in which CD80 rather behaves as a receptor than a ligand and generates an inhibitory signal. When PD-L1 engages with CD80, T cell proliferation decreases, cytokine production declines and cell-surface activation marker expression is reduced (Butte, Keir, Phamduy, Sharpe & Freeman, 2007, Pardoll, 2012). These pathways have been investigated and has lead to the development of multiple PD-1/PD-L1 checkpoint inhibitors.

Clinical checkpoint inhibitors: PD-1/PD-L1

Checkpoint inhibitors that target the PD-1/PD-L1 pathway can be divided into two categories: antibodies that target PD-1 and antibodies that target PDL-1. Nivolumab (Bristol-Myers Squibb bv.) and Pembrolizumab (Merck Sharp & Dohme bv.) target PD-1, whereas Avelumab (Merck Serono Europe Ltd.) and Atezolizumab (Roche Nederland bv.) target PD-L1 (Chen & Han, 2015, Postow, Callahan & Wolchok, 2015, Zorginstituut Nederland, 2018).

Nivolumab

Nivolumab is a monoclonal human IgG4 antibody which is produced in CHO cells. It has a high affinity and specificity for binding to PD-1. A hinge region mutation (S228P) makes the antibody resistant to exchange with serum IgG4 molecules and this stabilises the specificity (Bardhan, Anagnostou & Boussiotis, 2016, Philips & Atkins, 2014, Zorginstituut Nederland, 2018). In a phase I/II clinical trial of 2012, nivolumab was the first PD-1 targeting antibody to demonstrate anti-tumour activity (Bardhan, Anagnostou & Boussiotis, 2016). Treatment with Nivolumab has shown increased response rates and improved overall survival in melanoma compared to treatment with chemotherapy (dacarbazine) and has been approved by the FDA for the treatment of melanoma, Hodgkin lymphoma and squamous NSCLC (Chen & Han, 2015, Bardhan, Anagnostou & Boussiotis, 2016, Postow, Callahan & Wolchok, 2015, Sharma & Allison, 2015). Treatment with nivolumab in combination with ipilimumab has shown that after a year, overall survival rate in patients with advanced melanoma was increased to 94%. However, the combination treatment was accompanied with an increase in toxicity and adverse events (ClinicalTrials.gov: NCT01024231) (Chen & Han, 2015).

Pembrolizumab

Pembrolizumab is a monoclonal recombinant humanised IgG4/ κ-isotype antibody which has been produced in CHO cells. A variable region derived of a high-affinity mouse anti-PD-1 is grafted unto human IgG4 and an Fc region is engineered for improved stability (Bardhan, Anagnostou & Boussiotis, 2016, Philips & Atkins, 2014, Zorginstituut Nederland, 2018). Pembrolizumab has shown promising results regarding tumour responses and has been approved by the FDA for clinical use in treatment of metastatic melanoma and for treatment in NSCLC, Hodgkin lymphoma and Renal Cell Carcinoma (RCC) (Bardhan, Anagnostou & Boussiotis, 2016, Postow, Callahan & Wolchok, 2015, Zorginstituut Nederland, 2018). In a phase III trial, the treatment outcome of pembrolizumab was compared to ipilimumab in patients with advanced melanoma (ClinicalTrials.gov: NCT01866319). One-year estimates of survival were measured at 74.1% for treatment of pembrolizumab administered every two weeks and at 68.4% for treatment administered every three weeks, in comparison to 58.2% in patients receiving ipilimumab. Adverse events as a result of treatment were measured at 13.3% and 10.1% for treatment every two weeks or every three weeks respectively, compared to 19.9% measured in patients receiving treatment with ipilimumab. In this study, pembrolizumab has shown significantly improved results in comparison with ipilimumab, as well as a reduction in high-grade toxic events (Robert et al., 2015). In 2018, four phase III clinical studies have started or will start to continue research about treatment with pembrolizumab (ClinicalTrials.gov: NCT03553836, NCT03517449, NCT03425643 and NCT03515837).

Avelumab

Avelumab is a monoclonal human IgG1 antibody which targets PD-L1. It inhibits the PD-1/PD-L1 pathway and PD-L1/CD80 signalling. It induces NK cell mediated tumour cell lysis through the activation of antibody-dependent cell-mediated cytotoxicity (ADCC) Zorginstituut Nederland, 2018). Avelumab is the first therapeutic agent to be FDA approved for the treatment of metastatic Merkel cell carcinoma (MCC), an aggressive cancer with poor prognosis (Shirley, 2018).

Atezolizumab

Atezolizumab is a monoclonal humanised IgG1 antibody which is produced in CHO cells. The Fc region of the antibody has been modified so that it cannot induce ADCC (Phillips & Atkins, 2014, Zorginstituut Nederland, 2018). Atezolizumab has been approved for the treatment of metastatic urothelial bladder cancer (UBC) and of NSCLC (Chen & Han, 2015, Zorginstituut Nederland, 2018). Atezolizumab is believed to be tolerated better than chemotherapy in patients with UBC and further research regarding atezolizumab for therapy of other diseases is currently being conducted (Chen & Han, 2015, Philips & Atkins, 2014).

Overall, therapeutic agents targeting PD-1 or PD-L1 have shown great improvement compared to traditional treatments such as chemotherapy. Compared to CTLA-4 checkpoint inhibitor ipilimumab, PD-1/PD-L1 checkpoint inhibitors demonstrate fewer high grade toxicities and thus a more favourable approach for treatment (Lee, Sun, Sandler & Hoang, 2018).

Adverse effects and treatment limitations

Although immune checkpoint therapy has induced positive anti-tumour responses and delivered promising clinical results, immune checkpoint therapy is accompanied with treatment-induced toxicity and limitations such as no clinical response or treatment resistance. In the following paragraphs, I will discuss the most common adverse effects and the limitations that come with this type of treatment.

Immune-related adverse events

Immune checkpoint therapy aims to target and increase activity of the immune system, in the hope to provide a body's own effective anti-tumour response. Because enhancing the activity of the immune system is not limited to tumour-specific immune responses, immune checkpoint therapy is often accompanied by occurrence of toxicity, named immune-related adverse events (irAE) (Postow, Callahan & Wolchok, 2015, Postow, Sidlow & Hellmann, 2018). Effects involving the gastrointestinal tract, the skin and endocrine glands are the most common irAEs, being evidenced by diarrhoea/colitis, rash or thyroid insufficiency, respectively. Generalised symptoms, such as fatigue or fever can also manifest themselves as a consequence of irAEs (Marin-Acevedo et al., 2018, Postow, Callahan & Wolchok, 2015, Postow, Sidlow & Hellmann, 2018, Thallinger et al., 2017). Treatment of irAEs mostly involves suspending immune checkpoint therapy and administration of immunosuppressors such as glucocorticoids, TNF-α antagonists or other agents (Marin-Acevedo et al., 2018, Postow, Callahan & Wolchok, 2015, Postow, Sidlow & Hellmann, 2018).

In general, the irAEs that arise with anti-CTLA-4 therapy are of a higher toxicity grade and occur more frequent than irAEs after treatment with anti-PD-1/PD-L1 therapy. 7 out of 10 patients treated with anti-CTLA-4 develop irAEs, while the occurrence with anti-PD-1/PD-L1 therapy is 2-3 out of 10 patients (Thallinger et al., 2017). Furthermore, different types or irAEs arise more often in anti-CTLA-4 therapy, while other irAEs are more common with anti-PD-1/PD-L1 therapy (Postow, Sidlow & Hellmann, 2018, Thallinger et al., 2017). After treatment with ipilimumab (3mg/kg), 3 to 4th grade diarrhoea/ colitis was recorded in 10% of the patients, compared to an incidence of 1-2% 3 to 4th grade diarrhoea/ colitis in anti-PD-1/PD-L1 therapy (Postow, Callahan & Wolchok, 2015). Treatment with a TNF-α antibody (Infliximab), which is used to manage symptoms of Crohn's disease and ulcerative colitis, has shown to be effective in the treatment of irAEs (Postow, Sidlow & Hellmann, 2018). It is not clear by which mechanisms irAEs develop. Further research into these mechanisms may lead to development of more precise treatments for irAE. Moreover, because of the high variety in irAE manifestations, multidisciplinary collaboration between specialisations may lead to improved management of irAEs, compared to standard treatment with immunosuppressors (Postow, Sidlow & Hellmann, 2018, Thallinger et al., 2017). For now, 1st grade irAEs are managed by immunosuppressors while continuing immune checkpoint therapy, 2nd and 3rd grade irAEs ask for delaying treatment, while recurrent 3rd grade and 4th grade toxicities require termination of treatment (Thallinger et al., 2017).

Treatment limitations

Even though immune checkpoint therapy has shown favourable results, in most of the patients that receive checkpoint inhibitors a clinical response does not occur. When patients do not elicit a clinical response at all, it is called primary immune escape, whereas patients with acquired treatment resistance respond initially but subsequently relapse (Lee, Sun, Sandler & Hoang, 2018, Marin-Acevedo et al., 2018). This resistance can be due to three causes: impaired formation of $CD8^+$ T cells, insufficient functioning of $CD8^+$ T cells or reduced formation of memory T cells (Jenkins, Barbie & Flaherty, 2018). These problems can arise through tumour intrinsic factors such as absence of tumour-specific antigens and the presentation thereof, downregulation of MHC or reduced sensitivity to T cells. Extrinsic factors may also contribute to primary immune resistance, such as the absence of T cells, the presence of T_{regs} and presence of other immunosuppressive cells (Fig. 3) (Lee, Sun, Sandler & Hoang, 2018, Marin-Acevedo et al., 2018).

At the moment it is very difficult to predict the response of a patient to treatment. Biomarkers are being identified to be able to predict clinical response and irAEs to select patients for treatment. However, actually predicting the clinical response using biomarkers is very difficult. By observing tumour-infiltrating immune cells, tumours can be classified into two categories: immunologically unresponsive tumours and immunologically responsive tumours. Different monitoring strategies such as whole genome sequencing and flow cytometry can be carried out to discover biomarkers and to further differentiate between types of tumours (Lee, Sun, Sandler & Hoang, 2018, Yuan et al., 2016). Biomarkers that have been identified include mutational load, expression of immune checkpoints: PD-L1, LAG-3, TIM-3, the presence of $CD8^+$ T cells and the T_{eff}/T_{reg} ratio. Immunologically unresponsive tumours are associated with a low mutational burden, a reduced count of CD3 cells, a low T_{eff}/T_{reg} ratio and low PD-L1 levels (Fig. 4) (Marin-Acevedo et al., 2018, Yuan et al., 2016). For different types of cancers, different subsets of biomarkers are important to be able to distinguish between selection or exclusion of therapy (Sharma & Allison, 2015). Further identification of different biomarker combinations between tumour types and patients may lead to administration of personalised therapy. Identifying mutated genes by genome sequencing as well as determining gene expression of certain biomarkers may give insight on whether a certain therapeutic agent could elicit a clinical response. To overcome the limiting factors of immune checkpoint therapy efficacy, abundant research into combinations of different therapies is being carried out. Immune checkpoint therapy can be combined with other checkpoint inhibitors, with radiotherapy, chemotherapy agents and tyrosine kinase inhibitors. Efforts are aimed at improving function of anti-tumour activity, as well as by targeting the tumour micro environment to overcome resistance to treatment (Thallinger et al., 2017). Combined treatments are very promising and some patients that experienced resistance to monotherapy responded to combination treatment. However, these outcomes are accompanied by a high incidence of toxicity and adverse events.

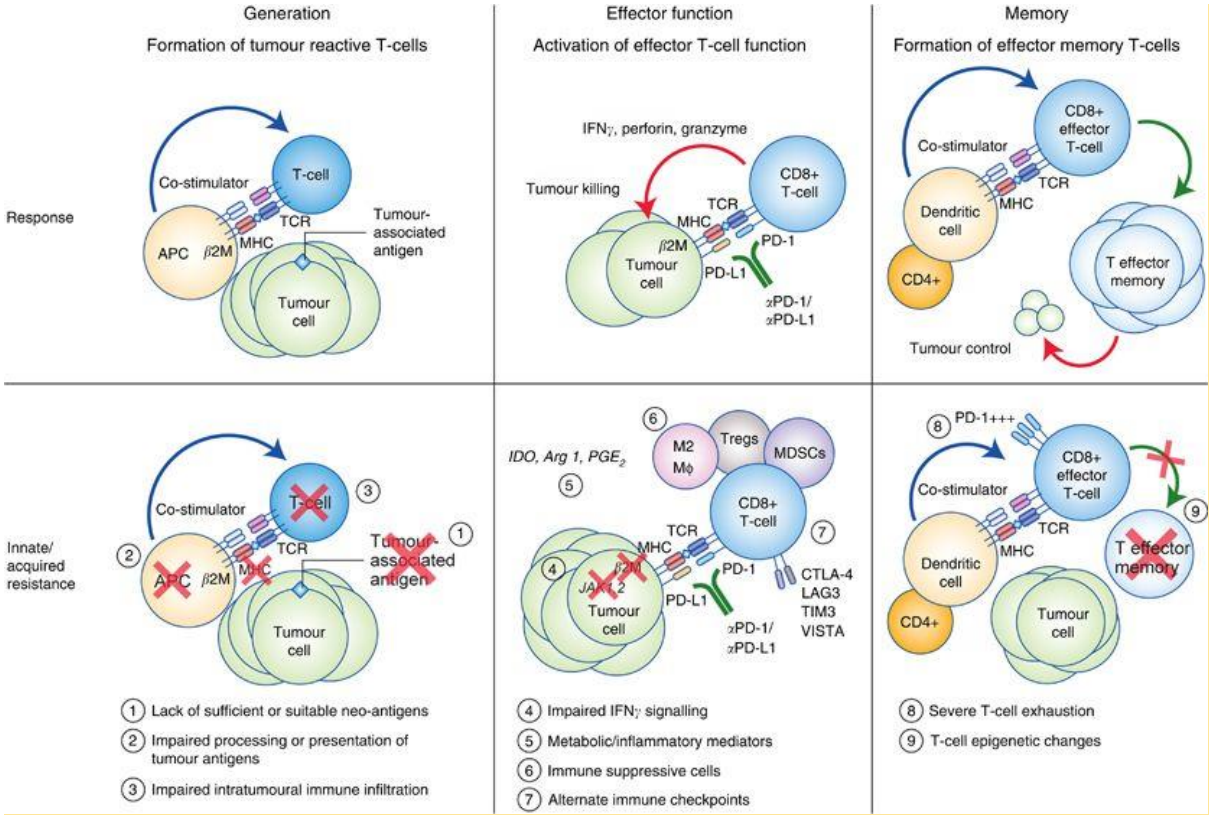

Figure 3 | Mechanisms of response and resistance to immune checkpoint therapy. In the event of a response, APCs present tumour antigens to T cell, resulting in activation and priming. $CD8^+$ T cells bind to rumour cells and secrete IFNγ, perforin and granzyme to kill the tumour cell. After killing the tumour cell, formation of effector memory T cells is initiated. In the event of resistance to therapy, multiple factor can contribute to a disruption in the mechanism (Jenkins, Barbie & Flaherty, 2018).

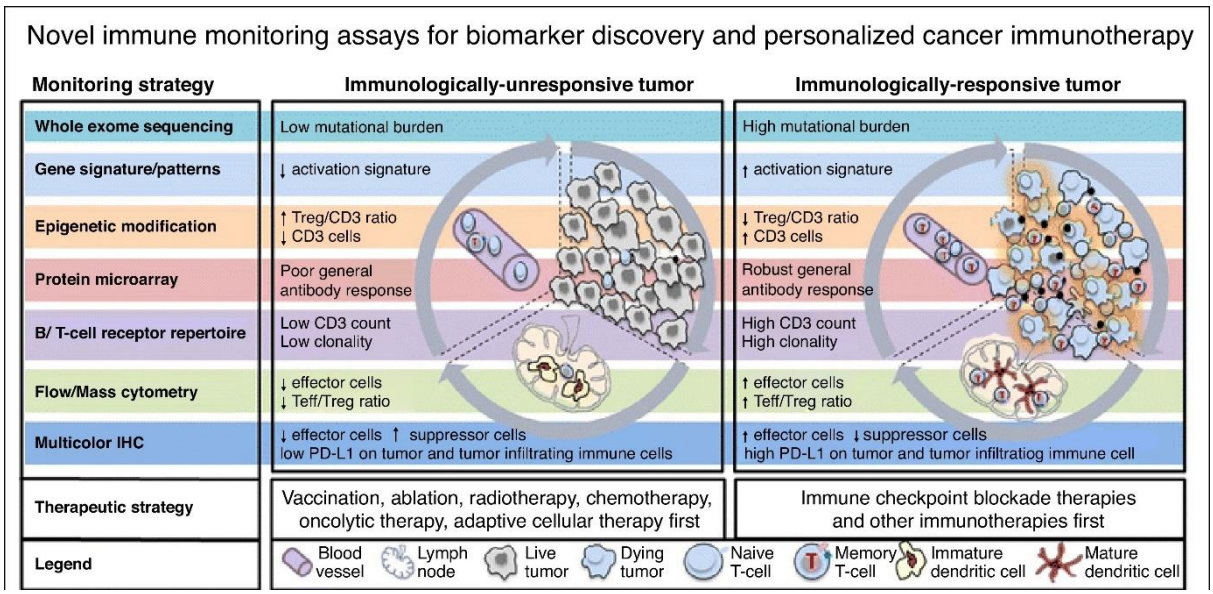

Figure 4 | Novel immune monitoring assays for biomarker discovery and personalised cancer immunotherapy. By determining the presence of immune cells within the tumour microenvironment, tumours can be classified into immunologically unresponsive and responsive tumours. To further distinguish between tumours, different biomarkers can be identified, such as mutational burden and the T_{eff}/T_{reg} ratio. The identification of biomarkers can lead to a personalised clinical treatment strategy (Lee, Sun, Sandler & Hoang, 2018).

Combination therapies

In an effort to enhance and broaden benefits of immune checkpoint therapy, different combinations of therapy strategies are being clinically tested. As mentioned before, immune checkpoint therapy can be combined with other checkpoint inhibitors, with radiotherapy, chemotherapy agents and tyrosine kinase inhibitors. Ipilimumab administered in combination with nivolumab has shown remarkable effects and the combination has been approved by the FDA for treatment of metastatic melanoma in 2015 and for treatment of advanced RCC in 2018 (American Society of Clinical Oncology, 2018, U.S. Food & Drug Administration, 2018). However, combination of these immune checkpoint inhibitors has proven to be very toxic as well. A double-blind phase III study by Larkin et al. (ClinicalTrial.gov NCT01844505) shows that response rates of 57.6% after treatment with nivolumab/ipilimumab combination therapy were higher in comparison with monotherapy of nivolumab and ipilimumab, 43.7% and 19.0% respectively. However, improved overall response rates were accompanied by a higher incidence of 3rd and 4th grade irAEs. With diarrhoea being the most common adverse event, 55.0% of the patients receiving nivolumab/ipilimumab combination therapy experienced irAEs, in comparison to nivolumab and ipilimumab, 16.3% and 27.3% respectively (Larkin et al., 2015). PD-1 and PD-L1 inhibitors in combinations with other immune checkpoint inhibitors have also entered clinical development and the number of combination therapy studies has risen enormously over the last years (Sacher & Gandhi, 2015). A host of combinations is being tested; however, the combination of PD-1 and PD-L1 inhibitors has not been thoroughly investigated. When PD-1 is targeted, interaction between PD-1 and both its ligands (PDL-1 and PD-L2) will be inhibited by the antibody. However, such an antibody is not able to stop the PD-L1/CD80 interaction from occurring. A similar deduction can be made when evaluating PD-L1 checkpoint inhibitors. Antibodies targeting PD-L1 will interfere with PD-L1 and CD80 signalling, as well as with PD-L1 and PD-1 signalling. It will not inhibit the interaction between PD-1 and PD-L2. It is not yet clear whether these interactions are of clinical relevance, however, the combination of PD-1 and PD-L1 inhibitors might lead to a more effective results in patients (Pardoll, 2012, Postow, Callahan & Wolchok, 2015). An ongoing phase 1/2 trial by MedImmune is evaluating the safety and efficacy of a PD-1 inhibitor combined with a PD-L1 inhibitor (ClinicalTrials.gov NCT02118337) (Bardhan, Anagnostou & Boussiotis, 2016).

Another option besides combining different immune checkpoint inhibitors with each other is combining immune checkpoint therapy with other types of treatment. Chemotherapy administered together with PD-1 or PD-L1 inhibitors has shown promising results. Moreover, chemotherapy is thought to modulate immune cells through tumour antigens, which are presented by APCs after tumour cell apoptosis (Thallinger et al., 2017, Lee, Sun, Sandler & Hoang, 2018). In addition, immune checkpoint inhibitors (mostly targeting the PD-1/PD-L1 pathway) are administered together with tyrosine kinase inhibitors (TKI). It is believed that a synergy exists between immune checkpoint inhibitors and TKIs. The identification of oncogenic driver mutations and the clinical activity achieved with TKIs targeting these mutations, combined with the suggestion by preclinical data that abnormal EGFR signalling potentially influences PD-L1 expression, has led to this rationale (Sacher & Gandhi, 2015). Furthermore, disrupting angiogenesis by inhibiting VEGF in combination with immune checkpoint inhibitors is also deemed a potential way to improve clinical outcome. VEGF may have a positive effect on the anti-tumour response, as it has been established that it

can mediate immune suppression. Additionally, in melanoma patients treated with ipilimumab, overall survival was lower in patients with high serum levels of VEGF (Ott et al., 2015). Various studies investigating these combinations are underway and being carried out (Sacher & Gandhi, 2015, Thallinger et al., 2017). Radiation therapy (RT) has the ability to induce an abscopal effect. The abscopal effect is when regression of a tumor is reported outside of the field of radiation. Along with T cells, this effect is mediated by proimmunogenic and proinflammatory factors. RT increases IFN-γ and type 1 IFN production, tumour antigen cross-presentation, expression of PD-1/PD-L1 and diversifies T cell receptor clonality and diversity. As PD-1/PD-L1 inhibitors increase T_{eff} cells and TILs whereas decreasing T_{regs}, it is proposed that the combination of RT combined with PD-1/PD-L1 inhibitors will enhance the anti-tumour response and is therefore a potentially promising therapy combination. Blocking of PD-1/PD-L1 followed with fractionated RT is most optimal and generally has a well tolerable toxicity. Fractionated RT is that administration of radiotherapy therapy is fractionated into lower doses over a longer period of time. Schaue et al. treated B16-OVA murine melanoma mice with radiotherapy in different fractions and compared the results to single doses. They found that 2 fractions of 7.5Gy as well as 3 fractions of 5Gy generated better results than a single dose of 15Gy (Schaue et al., 2012). Dewan et al. have shown that fractionated RT can synergise with an CTLA-4 inhibitor and the combination of these treatment can induce an abscopal effect in secondary tumours, whereas a single dose cannot (Dewan et al., 2009). Timing and dosing are two factors which are of importance in RT and must be considered in following studies. A variety of clinical trials are still investigating the possible effects of RT in combination with immune checkpoint inhibitors and an optimisation of dosing is yet to be determined. Because immune checkpoint therapy has radio sensitisation effects, meaning that it can increase the sensitivity of tumour cells, immune checkpoint inhibitors could also prove to enhance the use of radiotherapy. To be able to determine which of the synergising effects between RT and immune checkpoint therapy is most effective, preclinical and clinical trials must be conducted to investigate these sensitisation properties (Gong, Le, Massarelli, Hendifar & Tuli, 2018, Hahn, Gill, Pal & Agarwal, 2017, Thallinger et al., 2017, Wang et al., 2018). In 2016, He et al. have shown positive effects in mice with metastatic colorectal cancer by combining chemotherapy, a PD-L1 inhibitor and photodynamic therapy. Photodynamic therapy combines photosensitisers, light and oxygen to generate reactive oxygen species (ROS). ROS cause inflammation and induce apoptosis or necrosis. Photodynamic therapy can target the tumour very precisely, is accompanied by minimal toxicity, treatment can be repeated and is less invasive than surgery (Hwang, Shin, Han & Na, 2017). The treatment reduced tumour growth in primary and distant tumours and increased TILs within the tumour microenvironment (He et al., 2016). These different types of combination treatments have shown promising results, but further research is required to select patients for treatment and to assess the most optimal conditions for therapy. Numerous different types of combination therapies are currently being researched to evade primary immune escape, treatment resistance and to enhance overall success rate of the therapies (Hahn, Gill, Pal & Agarwal, 2017). It is difficult to assess which combination is the most effective. Results of similar studies should be compared with each other. For instance, while the combination of ipilimumab and nivolumab has shown enormous potential but is accompanied by a high incidence of 3rd grade irAEs, another immune checkpoint plus immune checkpoint combination may elicit more favourable results. Furthermore, different approaches to combinations must evaluated, such as the comparison of radiotherapy in combination with an immune checkpoint inhibitor compared to photodynamic therapy plus administration of an immune checkpoint inhibitor. After such a

retrospective study is conducted, it may be possible to filter out a few combinations which could provide the best clinical results. It may then be possible to carry out one study which assesses these different approaches. It is also very important to generate different biomarker expression profiles per cancer type, so that patients can be selected or excluded from participation.

A2aR: A novel target for immune checkpoint inhibition

After the successful clinical outcome upon targeting of CTLA-4 and PD-1/PD-L1, research has been focussing on improving these therapies as well as discovering novel immune checkpoint targets. One of these targets has been identified as the A2a adenosine receptor, which is ligated by adenosine. Similar to the CTLA-4 and PD-1/PD-L1 pathways, extracellular adenosine release inhibits the immunological response against pathogens to prevent collateral damage in healthy cells (Mediavilla-Varela et al., 2017, Leone, Lo & Powell, 2015, Ohta et al., 2012). Under normal physiological conditions, low concentrations of ATP and adenosine are present within extracellular fluids (Leone, Lo & Powell, 2015, Leone & Emens, 2018). However, within the tumour microenvironment, adenosine concentrations are high. Hypoxia and cell death lead to an excess of AMP, which is converted to adenosine by ectonucleotidase CD73. Furthermore, high concentrations of extracellular ATP within the tumour microenvironment are dephosphorylated by ectonucleotidase CD39 to form AMP, contributing to the accumulation of adenosine (Hahn, Gill, Pal & Agarwal, 2017, Leone & Emens, 2018, Mediavilla-Varela et al., 2017, Pardoll, 2012). A2aR, the receptor of adenosine, is expressed on a variety of immune cells, including: T cells, monocytes, macrophages, dendritic cells and NK cells (Leone & Emens, 2018, Marin-Acevedo et al., 2018).

When A2aR is ligated by adenosine, it stimulates an increase in intracellular adenylyl cyclase, causing a rise in intracellular cAMP concentration and activating a signalling cascade which results in suppression of a series of immune effects (Leone, Lo & Powell, 2015, Leone & Emens, 2018). A2aR activation increases production of TGF-β, IL-10, upregulates CTLA-4, PD-1 and LAG-3 receptor expression and stimulates $CD4^+$ cells to upregulate FOXP3 expression. Upregulated FOXP3 expression drives the cells to differentiate towards T_{reg} cells. T_{regs} have a high expression of CD73 and CD39, creating an amplifying loop which maintains the generation of excess extracellular adenosine. (Leone, Lo & Powell, 2015, Leone & Emens, 2018, Ohta et al., Pardoll, 2012). Activation of A2aR has multiple other effects which lead to a suppression of immune responses, such as interfering with dendritic cell maturation, inhibiting cell proliferation and effector cytokine production in $CD8^+$ effector T cells, supressing neutrophil chemo attractants and reducing Il-2 secretion to reduce CD28 expression (Hahn, Gill, Pal & Agarwal, 2017, Leone, Lo & Powell, 2015, Leone & Emens, 2018). Because the A2aR pathway induces so many effects, blocking of the A2aR checkpoint has emerged as a potential target and is being investigated (Leone & Emens, 2018).

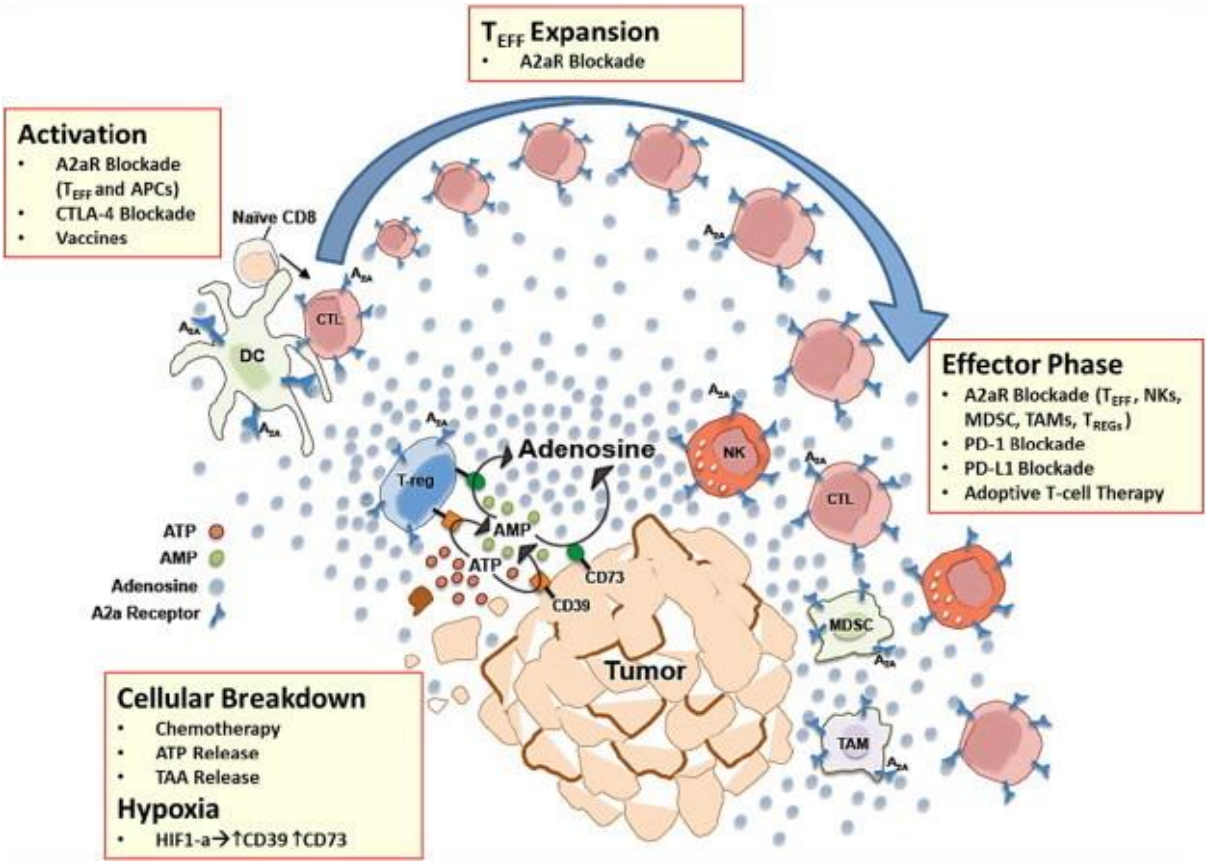

Figure 5 | Blocking of A2aR within the tumour micro environment. Adenosine elicits a variety of effects within the cancer immunity cycle. ATP is dephosphorylated to AMP by CD39. AMP is then catabolised to adenosine by CD73. Blocking of A2aR may lead to an increased activation and proliferation of T_{effs} and can consequently enhance the anti-tumour response (Leone, Lo & Powell, 2015)

Therapeutic agents targeting A2aR have progressed to clinical trials in the treatment against Parkinson's Disease. However, targeting A2aR in the field of cancer immunotherapy has emerged relatively recently and is yet to present results of clinical trials (Young et al., 2016). Preclinical studies targeting A2aR in combination with PD-1 inhibitors have shown promising results. Deletion of the A2aR reduced tumour growth and antagonising the A2aR improved tumour growth inhibition, reduced metastasis and prevented neovascularisation (Ohta et al., 2006, Waickman et al., 2012). Results of various preclinical studies have led to two phase I/Ib clinical trials, which are currently recruiting patients. One is investigating the effects of A2aR antagonist CPI-444 as monotherapy and in combination with atezolizumab in NSCLC, RCC and other advanced cancers (ClinicalTrials.gov NCT02655822), while the other trial investigates the effects of A2aR antagonist PDF-509 as monotherapy and in combination with a PD-1 antibody in NSCLC (ClinicalTrials.gov NCT02403193) (Hahn, Gill, Pal & Agarwal, 2017, Young et al., 2017). According to preliminary results of trial NCT02655822, after administration of CPI-444, patients with RCC and NSCLC had a disease control rate (2 months to > 8 months) of 86% and 50%, respectively. In the RCC patient cohort, all patients that were resistant to anti-PD-1/PD-L1 treatment responded to treatment. In the NSCLC cohort, 42% of patients that were resistant to anti-PD-1/PD-L1 treatment achieved disease control after administration of CPI-444. irAEs were measured mostly at 1st and 2nd grade toxicities with only one occurrence of a 3rd grade irAE. Increased inflammation and >70% $CD8^+$ cells were observed in biopsy after

administration of CPI-444 as single agent. Preliminary conclusions are that treatment with CPI-444 is tolerated fairly well and induces an anti-tumour response in patients after administration of monotherapy or as a combination (Emens et al., 2017, Fong et al., 2017, Marin-Acevedo et al., 2018). Targeting A2aR has gained much attention after various preclinical studies and has sparked interest in combining A2aR with other possible targets. To enhance the anti-tumour response, Young et al. have shown that co-targeting of A2aR and CD73, the ectonucleotidase that converts AMP to adenosine, reduces tumour initiation, growth and metastasis. Fourteen days after administration, the metastatic burden of tumour-bearing lungs was evaluated and the combination therapy reduced the metastatic burden more in the combination than in either monotherapy or the control group. Half of the mice injected with the combination therapy survived after 60 days, whereas after 60 days, the mice injected with either monotherapy did not survive (Young et al., 2016).

While phase I/Ib clinical trials are being carried out with the A2aR antagonist PBF-509, Mediavilla-Varela et al. have published in vivo and ex vivo results on this therapeutic agent. PBF-509 was tested for specificity by testing its effects on a panel of 89 potential targets. PBF-509 did not show relevant effects besides the antagonising of A2aR. PBF-509 was administered orally in two syngeneic mouse models of cancer and significantly reduced tumour burden in both models. Furthermore, tumour infiltrating lymphocyte (TIL) activity was measured in an ex vivo experiment on four resected NSCLC tumours. In two tumours, administration of either anti-PD-L1 or PBF-509 restored TIL activity and the combination had an additive increased TIL activity effect. In the other tumours, administration of either anti-PD-L1 or PBF-509 had no effect on TIL activity. This may be due to the difference in the tumour microenvironment and different biomarkers with each patient. However, the combination did induce an increase in TIL activity in these tumours (Mediavilla-Varela et al., 2017).

Ma et al. have shown that in head and neck squamous cell carcinoma (HNSCC), A2aR expression is increased in TILs. This increased expression of A2aR is correlated with a large tumour size and worse prognosis. Additionally, Ma et al. found that A2aR expression was elevated in recurrent HNSCC and in chemotherapy resistant HNSCC compared to primary HNSCC. This suggests that A2aR activation is associated with therapy resistance and may thus be a potential target for patients that do not respond to anti-CTLA-4 or anti-PD-1/PD-L1 treatment. The efficacy of A2aR antagonist SCH58261 was measured in vivo in mice with HNSCC and tumour growth was significantly reduced after administration. The population of $CD4^+$ T_{regs} with FOXP3 expression declined and the anti-tumour response of $CD8^+$ T effector cells was increased. No additional toxic effects were measured after administration of SCH58261 (Ma et al., 2017).

This past May, Kjaergaard and his colleagues demonstrated that deletion of the A2aR genes leads to enhanced $CD8^+$ T cell immunity. In $A2AR^{-/-}$ mice there was enhanced tumour regression and the mice had significantly prolonged survival compared to $A2AR^{+/+}$ mice. To further analyse these effects, either $CD8^+$ or $CD4^+$ cells were depleted. The depletion of $CD8^+$ cells lead to a loss of therapeutic effect whereas depletion of $CD4^+$ cells demonstrated no difference. Thus, the enhanced tumour regression and prolonged survival was $CD8^+$ dependent. The effect of the A2aR antagonist KW6002 in combination with the transfer of tumour draining lymph node T cells (TDLN cells) can replicate the effects induced by gene deletion of A2aR. Also, KW6002 was developed for the treatment of Parkinson's disease and is therefore capable to pass the blood-brain barrier. Taking this pharmacological ability

into account, they evaluated the effect of KW6002 on intercranial tumours in mice, and complete therapeutic regression was accomplished with administration of $20x10^6$ TDLN cells in combination with KW6002 (20 mg/kg per day) (Kjaergaard, Hatfield, Jones, Ohta & Sitkovsky, 2018).

On June 18th this year, Leone et al. published in vivo results evaluating A2aR antagonist CPI-444, which is currently also being tested in a phase I/Ib clinical trial. As monotherapy, CPI-444 moderately reduced tumour growth in mouse tumour models. As reported in previous studies, blocking of A2aR augments anti-PD-1/PD-L1 treatment. The combination of CPI-444 with anti-PD-1 has shown significant reductions in tumour volume and improved survival compared to monotreatment and mice treated with vehicle. More interestingly, CPI-444 supresses PD-1 and LAG-3 expression on $CD8^+$ T cells within tumour-draining lymph nodes, however, expression is not decreased within tumours and nondraining lymph nodes. This indicates that the tumour-draining lymph node is an important site for A2aR blockage. Because anti-PD-1 therapy requires priming of $CD8^+$ cells within the draining lymph node, the combination hereof might lead to the synergy seen when anti-A2aR and anti-PD-1 therapy is combined. PD-1 expression is decreased on $CD8^+$ T cells in the spleen. PD-1, Lag-3 and FOXP3 expression is also significantly reduced on T_{regs} in mice treated with CPI-444, all of which are important factors in the anti-tumour response. Decreased expression could further improve the anti-tumour response and may improve effects of other immune checkpoint inhibitors.

These studies have shown that anti-A2aR therapies can reduce tumour growth, diminish the population of T_{regs}, reduce metastatic burden, increase TIL activity, supress PD-1 and LAG-3 expression in vitro and in vivo and has various other positive effects on the anti-tumour response. A2aR is known to be generally tolerated well and is accompanied by an incidence of irAEs comparable to placebo (Fong et al., 2017, Hauser et al., 2014, Pourcher et al., 2012). Preliminary results of CPI-444, an A2aR antagonist, show that clinical activity is observed after monotherapy or in combination with atezolizumab. Tumour regression is seen in patients with NSCLC or RCC who had been resistant to PD-1/PD-L1 treatment as well as inflammation and an increase in $CD8^+$ cells. All these results lend significant evidence that the A2aR is highly involved in the regulation of immune responses. Even though clinical trials have yet to release results on their progress, it is obvious that antagonising A2aR is proving to be a valuable target in cancer immunotherapy. Even though the clinical efficacy of A2aR antagonists must still be investigated and a phase III clinical trial has to be conducted, A2aR antagonists have shown less toxicity and responses in primary escape/ treatment resistance patients. Considering that these factors are the greatest limitation of anti-CTLA-4 and anti-PD-1/PD-L1 therapy, either as monotherapy or in combination, these agents may provide the solution. Further research must be conducted to bring suitable therapeutic agents on the market.

Future perspectives

As mentioned before, in therapy with A2aR antagonists, a much better safety profile is observed compared to treatment with CTLA-4 or PD-1/PD-L1 inhibitors. Moreover, patients that initially did not respond to other treatment, or acquired resistance to treatment, did respond after administration of A2aR antagonists. A2aR antagonist have shown synergistic effects when administered in combination with PD-1/PD-L1 inhibitors and may induce similar effects in other treatment combinations. Hypoxia leads to accumulation of adenosine and has a correlation with radiotherapy. Radiotherapy is less effective when there is hypoxia in the tumour microenvironment (Emens et al., 2017). Blocking A2aR might be able to induce radio sensitisation and improve RT and has already shown promising results in combination with anti- PD-1/PD-L1 therapy. Since monotherapy with either anti-CTLA-4 or anti-PD-1/PD/L1 therapy does not induce a response in many patients, and the combination of these inhibitors is paired with extreme toxicities, I believe the focus of cancer immunotherapy should be directed at emerging checkpoint inhibitors. The efficacy of A2aR antagonists has been ascertained in preclinical studies and further research must focus on the evaluating of A2aR antagonists in clinical trials. Research into cancer immunotherapy has been rapidly evolving and will continue to be the main focus in research developments for the next few years. Checkpoint inhibitors have already shown great promise and have revolutionised treatment in various advanced cancers, but there are still limitations to be overcome. A2aR has the potential to overcome these limitations and improve the current treatment of cancer.

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