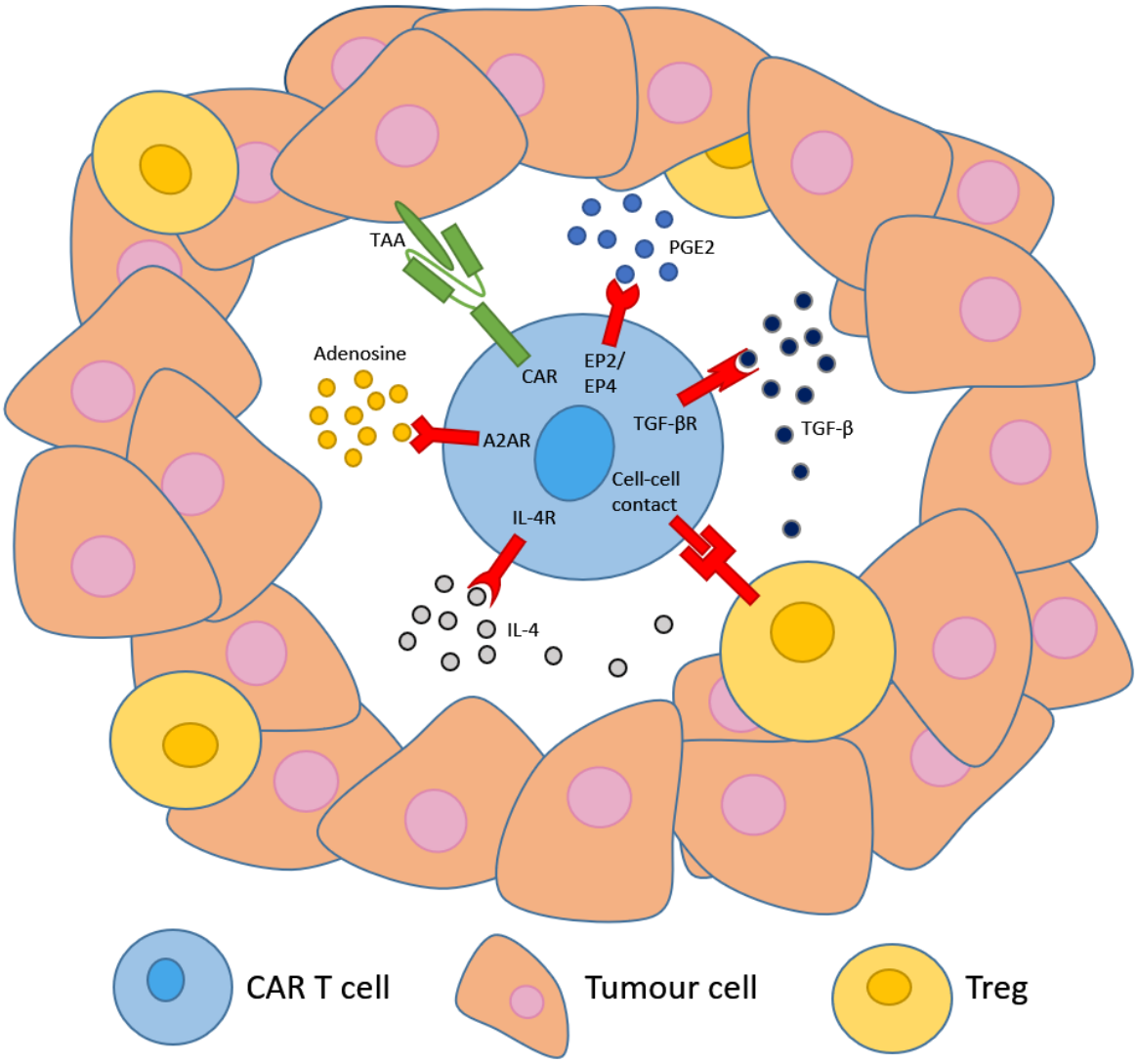
Improving Chimeric Antigen Receptor (CAR) T cell therapy for solid tumours:

Breaking down the suppressive barrier of the tumour microenvironment



Sander Nijhof
Master Biomedical Sciences
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Supervised by prof. dr. J.J. Schuringa and prof. dr. E. Bremer
Experimental Hematology

Abstract

The use of adoptively transferred chimeric antigen receptor (CAR) T cells is a very promising new strategy in the treatment of various cancers, especially haematological cancers. In this strategy, T cells of a patient are isolated, engineered *ex vivo* with a CAR to recognize a specific tumour antigen, and finally re-administered to the patient. These CAR T cells recognize the patient's tumour cells *in vivo*, effectively redirecting CAR T cells to the tumour to provide tumour directed immune responses. Two therapies utilizing such an approach have been approved by the FDA. In these therapies, CAR T cells directed against CD19 are used to effectively treat different types of haematological cancers. Due to the efficacy of these therapies, the use of CAR T cells might be a promising anti-tumour strategy in other types of cancer as well. However, clinical responses with CAR T cell therapy are considerably less frequent in cancers with solid tumours than in haematological cancers. This could partly be explained by the abundancy of different immunosuppressive factors in the tumour microenvironment (TME) of these solid tumours, which can suppress CAR T cells. The abundancy of immunosuppressive factors can be caused by changes in the metabolism and cytokine composition at the tumour site. In this review, a selection of these immunosuppressive factors that inhibit CAR T cells is discussed, including adenosine, PGE2, TGF-β and IL-4. Additionally, an overview of novel approaches to overcome the immunosuppression and to potentially improve CAR T cell therapy in solid tumours is provided in this review. The wide array of immunosuppressive factors in the TME and the multiple approaches to potentially overcome their suppression provide a challenge to determine what is the most effective CAR T cell approach for specific types of cancers, whilst also preventing unwanted toxicity of these new CAR T cell therapies. Therefore, an extensive understanding of the composition of the TME in different types of cancers is needed, and novel CAR T cell approaches should be able to specifically target the tumour site to limit the toxicity of new therapies. With these aspects in mind, the efficacy of CAR T cell therapy for solid tumours can potentially be improved, allowing CAR T cells to become an effective standardized method for treating various types of cancers in humans.

Contents

1. Introduction

The adoptive transfer of chimeric antigen receptor (CAR) T cells is a relatively new and very promising anti-cancer strategy. For this strategy, T cells of a cancer patient are isolated, after which they are engineered to recognize a tumour-associated antigen (TAA). These T cells are engineered by transduction of a new gene in the cell, which leads to the expression of a CAR on the T cell surface. This CAR consists of parts of a B-cell antibody that can recognize a TAA, the single chain antibody fragment, combined with T-cell activating components. After successful transduction of the gene, the newly engineered CAR T cells can be cultured and expanded *in vitro,* after which they are re-administered to the patient. They are then able to recognize the cancer cells of the patient, and can provide effective tumour directed immune responses[1, 2].

CAR T cells generally contain an extracellular single chain antibody fragment that recognizes the TAA, a transmembrane domain, and an intracellular signalling domain that activates the T cell when the TAA is recognized. The first generation of CAR T cells only contains a CD3-ζ signalling domain to activate the CAR T cells, whereas the second generation also contains a co-stimulatory domain to more fully activate the cells. The most commonly used co-stimulatory domains are derived from CD28, CD27, 4-1BB and OX40. The third generation of CAR T cells combines multiple of these co-stimulatory domains, whereas the latest generation of CAR T cells contains a domain that allows the production of cytokines[3, 4]. See Figure 1 for a general overview of the different CAR T cell generations.

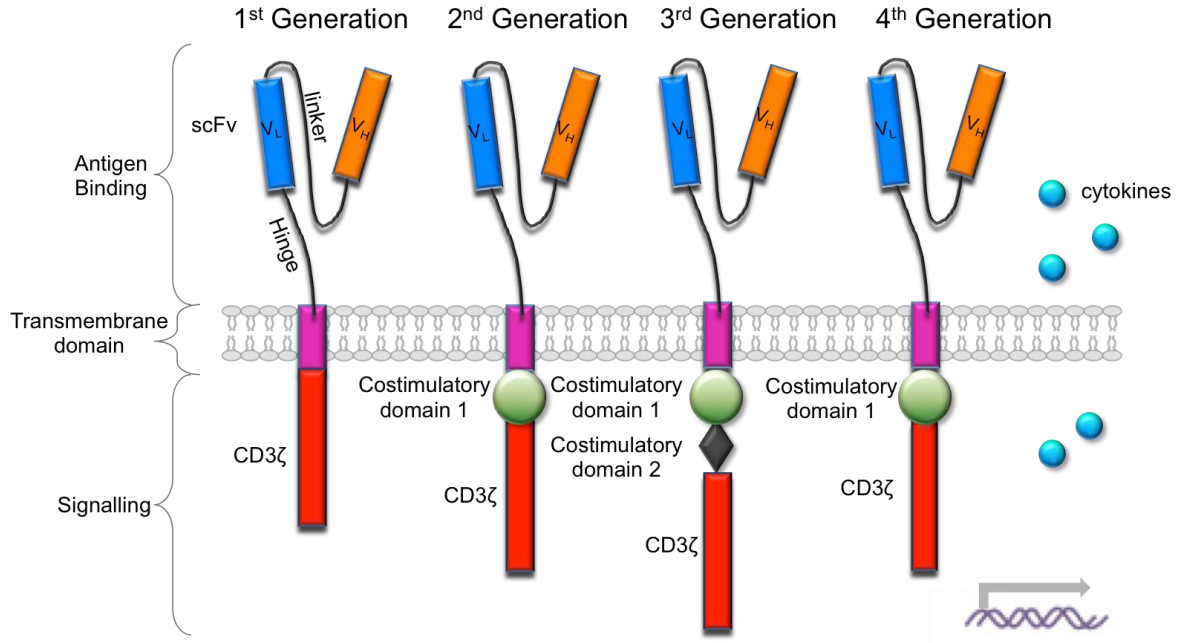

Figure 1: Generations of CAR T cells. VL = variable light chain and VH = variable heavy chain, together with the linker and hinge these make up the extracellular single chain antibody fragment (ScFv) that recognizes a TAA. Adapted from Cell Culture Dish, 2017[5].

CAR T cells been particularly effective in the treatment of haematological cancers, such as leukaemia and lymphoma. Specifically, patients suffering from B cell acute lymphoblastic lymphoma (B-ALL) and diffuse large B cell lymphoma (DLBCL) were shown to greatly benefit from therapies using CAR T cells directed against CD19[6-10], an antigen that is expressed on B cells. Various anti-CD19 CAR T cell therapies in B-ALL remarkably resulted in complete remission in 70% to 94% of patients (reviewed[11]). Two CAR T cell therapies directed against CD19 have also been approved by the FDA; tisagenlecleucel for the treatment of B-ALL and axicabtagene ciloleucel for the treatment of DLBCL[12, 13]. Both these CAR T cell

types contain an anti-CD19 single chain antibody fragment, an intracellular CD3-ζ signalling domain and a co-stimulatory domain. In tisafenlecleucel this co-stimulatory domain is derived from 4-1BB, while in axicabtagene ciloleucel it is derived from CD28[14, 15].

Given the impressive efficacy of these CAR T cell therapies in haematological cancers, CAR T cells have been tested for the treatment of solid tumours as well. CAR T cells directed against disialoganglioside GD2 in neuroblastoma have shown the best results so far, with 3 out of 11 patients achieving complete remission[16]. Most CAR T cells therapies in solid tumours have less favourable responses however, as was the case for CAR T cells directed against EGFR in non-small-cell lung cancer (2 out of 11, only partial remission)[17], and against HER2 in sarcoma (4 out of 17, only stable disease)[18]. This shows that CAR T cell therapy in solid tumours can in some cases lead to a clinical response, but in general its efficacy is a lot lower than in haematological cancers.

The lower efficacy of CAR T cell therapy in solid than in haematological cancers might be partly explained by a decreased infiltration of CAR T cells into the tumour site. This site is more difficult to reach in solid tumours due to the physical barriers presented by epithelial and/or mesenchymal layers[19]. More importantly however, when CAR T cells do reach the solid tumour, they are likely subject to strong suppression by multiple factors in the tumour microenvironment (TME). These can be related to changes in the metabolism or cytokine composition at the tumour site for instance, and can inhibit T cell proliferation, cytokine production and other effector functions of the CAR T cells. Together these effects of the TME can potentially decrease the efficacy of CAR T cell therapy in solid tumours. In this review, we aim to detail a selection of factors in the TME that can suppress CAR T cells, and provide potential approaches to overcome their suppression in CAR T cell therapy. An overview of the suppressive factors to be discussed is shown in Figure 2.

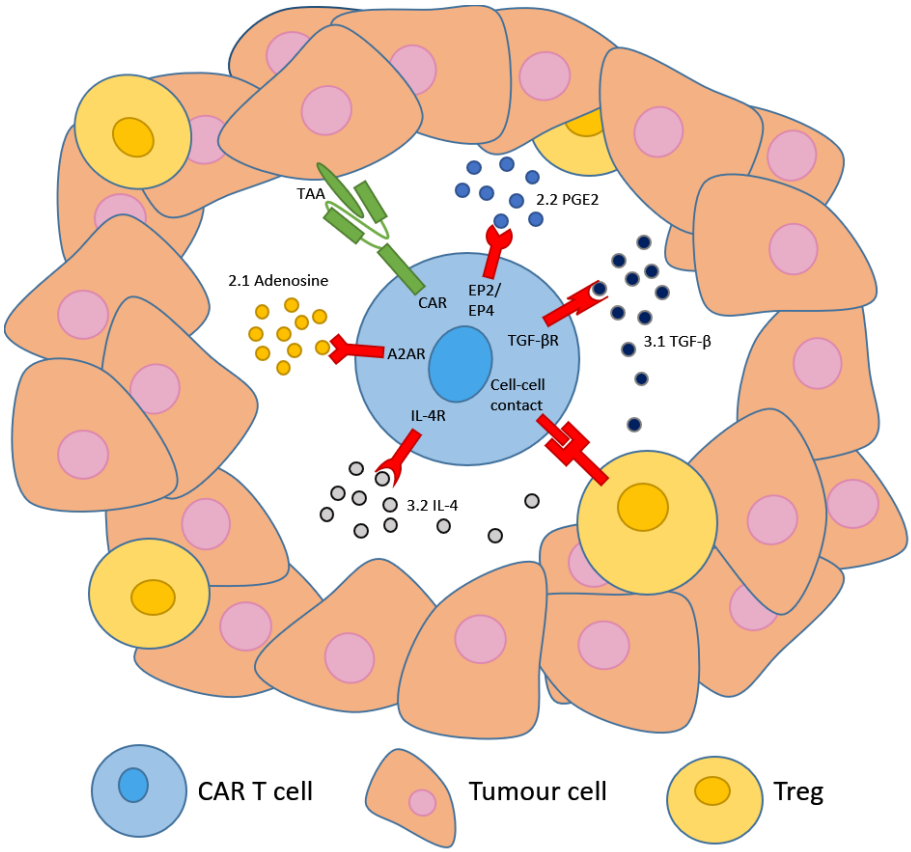

Figure 2: Schematic overview of suppressive factors present in the TME. Receptors marked in red indicate interactions promoting CAR T cell inhibition, while the interaction between the TAA and the CAR shown in green promotes CAR T cell activation. Numbers indicate in which section the suppressive factors are discussed.

2. Metabolic influences

One of the major factors in the TME-induced suppression of CAR T cells is the change in metabolic processes at the site of the tumour. Especially the levels of adenosine and prostaglandin E2 (PGE2) are highly increased in the TME compared to normal tissue. As detailed in the sections below, these molecules can have widespread immunosuppressive effects[20].

2.1 Adenosine

The increased level of the metabolite adenosine in the TME is one of the major contributors to the suppression of CAR T cells in solid tumours. Normally, intracellular adenosine plays a role in metabolic processes such as energy metabolism and nucleic acid metabolism[21]. However, solid tumours are often characterized by high concentrations of extracellular adenosine in the TME, due to hypoxic conditions in the tumour[20]. These hypoxic conditions induce the release of extracellular ATP and ADP into the TME, which are then converted into AMP by CD39 expressed on tumour cells and (tumour infiltrating) lymphocytes. This AMP can be further converted into adenosine by CD73 expressed on tumour cells, which leads to the increased levels of extracellular adenosine[22-24]. Additionally, hypoxia inhibits adenosine kinase, an enzyme that converts adenosine back into AMP. The decreased activity of this enzyme further increases the concentration of extracellular adenosine in the TME[25, 26].

When present in the TME, extracellular adenosine can bind to G protein coupled adenosine receptors expressed on the membrane of different immune cells. There are four known adenosine receptors; A1, A2A, A2B and A3, and of these four, adenosine receptor A2A (A2AR) has been reported to be the most frequently expressed receptor on T cells, at least in mice[26]. Binding of adenosine to this receptor increases intracellular cyclic adenosine monophosphate (cAMP) through activation of adenylyl cyclase, inducing the activation of protein kinase A (PKA) and the downregulation of many pro-inflammatory molecules[26]. For instance, the amount of interferon gamma (IFN-γ) that is produced after stimulation of the T cell receptor (TCR) on T cells is drastically reduced with the activation of A2AR on CD4+ T cells of mice. IFN-γ is an interferon that is vital in the activation of macrophages, and is mainly produced by the inflammatory Th1 subset of CD4+ T cells[27]. Additionally, adenosine inhibits both the effector function of CD8+ T cells and their priming after TCR stimulation[28], and can inhibit proliferation and interleukin-2 (IL-2) production of activated T cells through cAMP independent pathways as well[29]. Furthermore, adenosine increases the number of regulatory T cells (Tregs) and enhances their effector funtion[30]. These Tregs can further suppress CAR T cells through direct cell-cell contact with the CAR T cell, and through the release of soluble factors such as anti-inflammatory cytokines[31].

All these effects indicate how the presence of adenosine in the TME can inhibit tumour directed T cell reactions in general and, more specifically, how this may reduce the efficacy of CAR T cell therapy in solid tumours. The activation of CAR T cells both *in vitro and in vivo* has been shown to increase A2AR expression, making them susceptible to suppression by adenosine[32]. Therefore, it is of great important to find ways to evade or reduce the effects of adenosine in the TME in order to improve CAR T cell therapy in solid tumours.

Targeting the adenosine-induced suppression has already been proven to be an effective method to improve CAR T cell functionality in a mouse model using anti-HER2 CAR T cells[32]. The use of A2AR deficient CAR T cells in this model was shown to increase activation of CD4+ and CD8+ T cells compared to the use of A2AR wild type CAR T cells, and was accompanied by reduced tumour growth. Furthermore, both administration of an A2AR antagonist and knockdown of A2AR in the CAR T cells by short hairpin RNAs effectively rescued the A2AR induced inhibition of IFN-γ production by CAR T cells. However, the effects of these methods in an *in vivo* tumour model are lacking[32]. If proven to be

effective in reducing tumour growth *in vivo*, these methods might be an effective way to evade the suppression of adenosine in the TME. The short hairpin RNAs were transduced in a similar fashion as how the CAR is transduced in T cells, making it a relatively easily applicable strategy to potentially improve CAR T cell efficacy in solid tumours[32].

Only knocking down or out A2AR in CAR T cells will not prevent the increased suppression by Tregs resulting from the presence of adenosine in the TME. Therefore, the administration of antagonists of A2AR or a combinational approach with A2AR knockdown or knockout in CAR T cells might be the more effective strategy, as it can also limit Treg mediated effects. The administration of an A2AR antagonist to humans has previously been examined for safety in a clinical study in Parkinson's disease and was shown to be generally well-tolerated, indicating the possible use of A2AR antagonists in humans[33].

More recently it was shown that A2AR-specific antagonist can also be delivered at the site of the tumour through the use of CAR T cells conjugated with drug-loaded nanoparticles[34]. These nanoparticles contain a small molecule inhibitor of A2AR and are attached to the surface of the CAR T cell by chemical conjugation. Using this method, the antagonist can be more effectively targeted to the tumour, where it can counter the adenosine mediated T cell suppression through the prevention of A2AR activation. The addition of these nanoparticles to anti-CD19 CAR T cells both suppressed tumour growth and increased survival rates in an *in vivo* mouse model, accompanied by a higher percentage of tumour infiltrating lymphocytes and increased IFN-γ production[34]. This method should be more extensively studied for CAR T cells directed at other TAAs and in other tumour models as well, but these results provide an additional promising new method to engineer CAR T cells to overcome one of the burdens of the TME.

2.2 PGE2

Another molecule present in the TME of solid tumours that can suppress CAR T cells is PGE2. This molecule, like adenosine, is an important regulator of inflammatory responses. The levels of one of the enzymes responsible for the production of PGE2, called cyclooxygenase-2 (COX-2), are increased in the TME. This enzyme, in combination with COX-1, catalyses the conversion of arachidonic acid into PGH2, an unstable molecule that is rapidly converted into PGE2 and other prostaglandins[35, 36]. As COX-2 is overexpressed in tumour cells of various types of tumours, this leads to an increase in the levels of PGE2 in the TME as well[35, 37-40].

The PGE2 in the TME can bind to G protein coupled receptors EP1, EP2, EP3 and EP4[35]. Akin to adenosine, the binding of PGE2 to EP2 or EP4 on immune cells such as T cells induces cAMP production by adenylyl cyclase[35], which activates PKA. This activation leads to a decrease in the production of pro-inflammatory cytokines such as IFN-γ, and inhibits cytotoxic CD8+ T cells[41]. Furthermore, PGE2 can decrease the production of IL-2 and reduce the expression of its receptor on T cells[42]. Together, these effects suppress general T cell functionality at the tumour site, and can thus suppress CAR T cells.

PGE2 also plays a role in the induction and effector function of inhibitory immune cells in the TME. First of all, PGE2 increases the number of Tregs through the induction of FOXP3, a transcription factor vital in Treg functionality and development, and enhances their inhibitory capacity *in vitro*[43, 44]. Secondly, the receptors EP1, EP2 and EP4 have been demonstrated to play a role in the induction of myeloid-derived suppressor cells. These cells are present in many cancer types, and have general immunosuppressive effects that aid in further evasion of the immune system by tumours. The induction of these cells has been shown to be counteracted through the use of PGE2 antagonists and COX-2 inhibitors[45]. These effects of PGE2 on Tregs and myeloid-derived suppressor cells further promote general immune suppression and the specific suppression of CAR T cells in solid tumours.

Interestingly, the inhibitory effects of PGE2 and adenosine seem to be increased substantially if both molecules are present, and are very similarly mediated through activation of the cAMP-PKA pathway[41]. This indicates the importance of this general pathway for the immune suppression in solid tumours, which is why this pathway has been extensively studied and targeted to promote anti-tumour responses. One method to more generally target this pathway is aimed at disrupting the interaction between PKA and adenylyl cyclase. Adenylyl cyclase produces the cAMP that is necessary to activate PKA, but for this activation to occur PKA and adenylyl cyclase need to be close together. This is realized by the localization of PKA and adenylyl cyclase in lipid rafts, for which A-kinase anchoring proteins such as ezrin are important[46, 47]. Ezrin can be targeted by a peptide called regulatory subunit I anchoring disruptor (RIAD), which disrupts the localization of PKA in lipid rafts and thereby prevents PKA activation and reduces T cell inhibition[48]. This peptide has been transduced in CAR T cells, as this could to be very effective to combat the suppression of CAR T cells by both PGE2 and adenosine[49]. Indeed, CAR T cells transduced with RIAD showed improved cytotoxic capabilities and increased IFN-γ production *in vitro* compared to regular CAR T cells, and were resistant to inhibition by both PGE2 and adenosine. Furthermore, CAR T cells transduced with RIAD effectively reduced tumour growth in various *in vivo* mice tumour models[49]. This indicates the possible use of RIAD-transduced CAR T cells to overcome the suppression induced by both PGE2 and adenosine in the TME, potentially improving the efficacy of CAR T cell therapy in solid tumours.

An overview of the approaches discussed in this section to evade or overcome the suppression of CAR T cells induced by molecules associated with changes in tumour metabolism is shown in Figure 3.

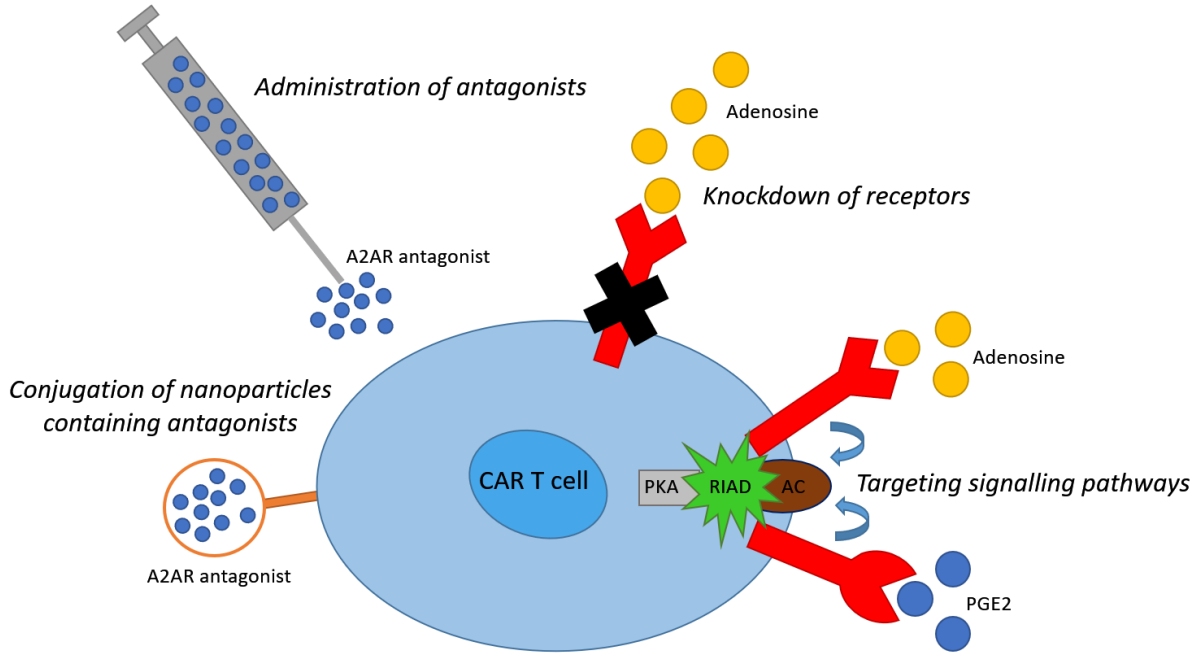

Figure 3: Schematic overview of approaches to evade or counter suppression of CAR T cells induced by molecules associated with changes in tumour metabolism. AC = adenylyl cyclase

3. Cytokines

Solid tumours are not only characterized by increased adenosine and PGE2 which suppress CAR T cells, but often also display many changes in the cytokine composition of the TME. Most notably, the concentrations of many anti-inflammatory cytokines such as transforming growth factor beta (TGF-β) and IL-4 are increased in the TME[50]. These cytokines add to the TME-induced suppression of CAR T

cells, which further limits the efficacy of CAR T cell therapy in solid tumours. Therefore, targeting these cytokines might be an effective method to improve CAR T cell therapy in solid tumours.

3.1 TGF-β

TGF-β is one of the most important immunosuppressive cytokines in the TME and is primarily produced by macrophages at the tumour site[51]. TGF-β can bind to the TGF-β receptor type 1 (TGF-βR1) and TGF-βR2 that are expressed on various immune cells. In the presence of TGF-β, TGF-βR2 can interact with TGF-βR1, which induces phosphorylation and activation of TGF-βR1. This active receptor is then able to phosphorylate intracellular SMAD2 and SMAD3, after which these proteins translocate to the nucleus where they regulate gene transcription[52]. Through this signalling pathway, TGF-β can have widespread immunosuppressive effects, including the inhibition of natural killer cells, B cells and CD8+ cytotoxic T cells. Additionally, TGF-β induces Treg differentiation, further inhibiting tumour-directed immune responses in the TME. Lastly, TFG-beta also inhibits the recruitment of immune cells to the tumour by supressing the expression of chemokines such as CXCL1 and CXCL5, thereby reducing the number of tumour infiltrating immune cells (reviewed[53]).

Due to the extensive immunosuppressive effects of TGF-β in the TME, TGF-β signalling has been an important target to improve immune responses in various types of tumours. For instance, TGF-β signalling has been targeted to potentially improve adoptive T cell transfer in Hodgkin lymphoma, a disease characterized by high concentrations of TGF-β in the TME[54]. Cytotoxic immune cells from patients with relapsed Hodgkin disease were transduced *ex vivo* with a dominant-negative TGF-βR2. This receptor lacks its intracellular domain, preventing phosphorylation of TGF-βR1 and thus removing the TGF-β signalling cascade which normally induces immune suppression. This approach showed promising results, as transduced cytotoxic T cells maintained their effector functions in the presence of TGF-β. However, all experiments were performed *in vitro*, and studies on the effects of this approach on tumour directed immune responses were lacking[54].

More recently, a similar approach was used in CAR T cells directed against prostate-specific membrane antigen for the treatment of prostate cancer[55]. Again, CAR T cells were engineered to express a dominant-negative TGF-βR2 in order to limit TGF-β induced suppression in the TME. These engineered CAR T cells exhibited increased proliferative capacity and increased secretion of cytokines compared to “regular” CAR T cells when co-cultured *in vitro* with prostate cancer cells. Additionally, co-cultures with CAR T cells that expressed the dominant-negative TGF-βR2 contained a reduced number of T cells expressing FoxP3, which is the immunosuppressive Treg subset of T cells. Furthermore, the engineered CAR T cells exhibited increased proliferative capacity and T cell persistence at tumour sites in three different *in vivo* mouse experiments, and were able to effectively eradicate the tumours[55]. These results of TGF-β resistant CAR T cells seem to be very promising to increase the efficacy of CAR T cell in prostate cancer, and this approach is therefore currently undergoing a phase 1 clinical trial using CAR T cells directed against prostate-specific membrane antigen in patients suffering from refractory castration-resistant metastatic prostate cancer[56]. Results of this clinical trial will show whether or not this approach is safe and effective in humans as well. Based on these findings, such an approach might also be tested in the CAR T cell therapy of other types of cancer.

An opposite and seemingly counter-intuitive approach has been studied as well, one where CAR T cells are engineered to actually be specific for TGF-β. However, the CAR T cell is engineered in such a way that its extracellular TGF-β binding domain is coupled to intracellular CD3ζ and CD28 signalling domains. With this approach, TGF-β can be utilized to provide the CAR T cell with a stimulatory signal instead of an inhibitory signal when it binds TGF-β receptors on the CAR T cell. This effectively induces immunostimulatory instead of immunosuppressive pathways in the engineered CAR T cells, promoting

inflammatory cytokine production and enhancing proliferative capacities *in vitro*[57]. However, this approach was limited by the fact that these CAR T cells were not tumour specific, as they only specifically recognized soluble TGF-β and not the tumour cell itself. These CAR T cells directed against TGF-β are however able to enhance the effector functions of surrounding tumour infiltrating T cells as well, as enhanced tumour directed cytotoxicity of CD8+ T cells and a reduction in the number of Tregs was observed in *in vitro* co-cultures that included engineered CAR T cells directed against TGF-β. Likely, this is due to the sequestering of TGF-β from neighbouring immune cells by the CAR T cells, and their production of pro-inflammatory cytokines[58]. This removes the suppression of immune cells induced by TGF-β, while simultaneously stimulating immune cells through the production of pro-inflammatory cytokines.

3.2 IL-4

A comparable approach has been used to evade the immunosuppressive effects of the cytokine IL-4 in the TME. The levels of IL-4 are increased in many types of solid tumours including breast, prostate and bladder cancer[59, 60]. The IL-4 in these tumours is primarily produced by tumour infiltrating lymphocytes[60], and can bind the IL-4 receptor on immune cells such as T cells. This interaction leads to phosphorylation of signal transducer and activator of transcription 6 (STAT6), which increases the production of multiple anti-inflammatory cytokines while decreasing the production of pro-inflammatory cytokines[61, 62]. Through these effects, IL-4 can inhibit tumour directed immune reactions in the TME of solid tumours.

Due to the importance of this cytokine in the inhibition of immune responses in many solid tumours, the IL-4 pathway has been extensively targeted to improve tumour directed immune reactions, also in CAR T cell therapy. For instance, just as with the TGF-β receptor coupled to CD3ζ and CD28 signalling domains, the IL-4 receptor has been engineered to switch the immunosuppressive signal of IL-4 to an immunostimulatory signal. One of the first studies employing this strategy specifically for CAR T cells, coupled the IL-4 receptor to the intracellular signalling domain of IL-2. As IL-2 can induce proliferation and differentiation of T cells, this chimeric receptor is able to provide CAR T cells with immunostimulatory signals in the presence of IL-4. Transduction of this chimeric receptor in CAR T cells improved proliferative capacity and tumour directed immune responses of anti-mucin 1 CAR T cells *in vitro*[63].

Building upon these promising results, similar strategies have been investigated using different signalling domains for CAR T cells in various types of tumours. For instance, CAR T cells directed against prostate stem cell antigen in pancreatic cancer and CAR T cells directed against mucin 1 in breast cancer have been transduced with chimeric IL-4 receptors. In this case, the CAR T cells were transduced with an IL-4 receptor coupled to the signalling domain of IL-7. This approach had already been tested in T cells transduced only with this chimeric IL-4 receptor but not with a CAR that recognizes a TAA. Using this strategy, the proliferative capacity and effector function of cytotoxic T cells was improved in the presence of IL-4, which was accompanied by improved tumour directed responses *in vitro*[61]. Using this exact approach for CAR T cells directed against prostate stem cell antigen and mucin 1 also improved proliferative capacity and tumour directed responses *in vitro,* with similar results in *in vivo* mouse tumour models[64, 65].

3.3 Cytokine administration

A different approach to counter cytokine induced suppression in the TME is to administer large doses of pro-inflammatory cytokines. One of the pro-inflammatory cytokines that has been used is IL-12, a cytokine that among other effects promotes IFN-γ production, induces differentiation of naive T cells

into inflammatory T cells, inhibits the development of IL-4 producing cells and promotes the effector function of cytotoxic T cells[66-68]. The administration of IL-12 has been shown to effectively induce tumour regression and prolong survival in mice tumour models[69-72]. However, administration of IL-12 in human clinical trials has been accompanied by severe toxicity and even death in some cases[73]. In order to prevent such toxicity, it is vital for IL-12 to be administered specifically at the tumour site.

One way to more specifically administer cytokines has been adapted in fourth generation CAR T cells. These fourth generation T cells, also known as armoured CAR T cells, are engineered in such a way that they can release cytokines[3, 4]. In this way, the concentration of IL-12 can be increased locally at the tumour site, utilizing the pro-inflammatory effects of IL-12 whilst simultaneously limiting toxicity. This approach has for instance been used to improve the efficacy of CAR T cell therapy in ovarian carcinoma[74]. Anti-Muc16^{ecto} CAR T cells engineered to release IL-12 increased the concentration of IFN-γ in the TME, and exhibited improved proliferative capacity and tumour directed responses in an *in vivo* mouse tumour model. No IL-12 induced toxicities were observed in these experiments. Based on these promising results, a phase 1 trial is currently being undertaken to assess the safety of these armoured CAR T cells in patients suffering from ovarian carcinoma[74, 75].

CAR T cells engineered to release Il-12 are also able recruit other immune cells such as macrophages to the tumour site through the increased concentration of IL-12 in the TME[76]. This effect is vital for an effective tumour-directed immune response, as tumours are sometimes able to evade tumour directed CAR T cell reactions. This is because mutations in tumour cells can lead to loss of expression of TAAs on these tumour cells[77, 78]. Such mutated tumour cells have an evolutionary advantage against CAR T cell therapy, as they are unrecognizable by the CAR T cells directed against a specific TAA. Therefore, these mutated tumour cells can potentially be a cause of persisting tumours in the patient, even after CAR T cell therapy[77, 78]. Even though these tumour cells are unrecognizable by the CAR T cells they can still be effectively eliminated by macrophages, as was shown in an *in vivo* mouse model using IL-12 producing CAR T cells directed against carcinoembryonic antigen in colorectal cancer[76]. Thus, the effects of IL-12 release on CAR T cells in combination with the recruitment of other immune cells to the tumour are important for effective tumour directed responses in the CAR T cell therapy of solid tumours.

An overview of the approaches discussed in this section to evade or overcome cytokine mediated suppression of CAR T cells in the TME is shown in Figure 4.

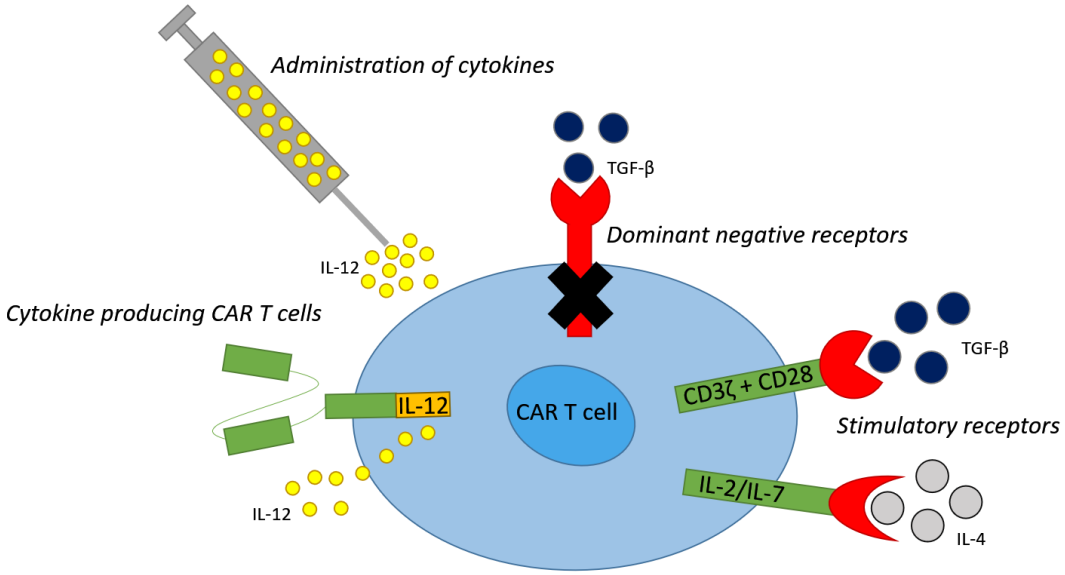

Figure 4: Schematic overview of approaches to evade or counter cytokine induced suppression of CAR T cells.

4. Discussion/perspectives

As this review shows, there are many factors present in the TME that can suppress CAR T cells in solid tumours. These include metabolic factors such as adenosine and PGE2, and cytokines such as TGF-β and IL-4. These bind to receptors on CAR T cells, activating signalling cascades that generally lead to suppression of CAR T cells by inhibition of proliferation and/or their effector function. Furthermore, these factors can activate immunosuppressive cells in the TME, further promoting inhibition of CAR T cells at the tumour site. An overview of the different immunosuppressive factors discussed in this review is shown in Figure 2. Together these factors provide a suppressive barrier to overcome in the CAR T cell therapy of solid tumours.

As this review also shows, many novel approaches are being developed to overcome this suppressive barrier in the CAR T cell therapy of solid tumours. Some approaches to improve CAR T cell therapy discussed in this review employ engineered CAR T cells in which receptors have been knocked down or made dysfunctional in order to prevent inhibition, for instance in the context of adenosine and TGF-β. Other approaches use the increased levels of certain metabolites and cytokines to actually stimulate the CAR T cells in the TME, by coupling the receptor of a certain molecule to an intracellular pro-inflammatory signalling domain. This approach has for instance been tested for the cytokines TGF-β and IL-4. Other more general approaches include the targeting of similar inhibitory signalling pathways, the administration of antagonists against the receptors of immunosuppressive factors, and the administration of pro-inflammatory cytokines to overcome the effects of anti-inflammatory cytokines in the TME. An overview of these different approaches to evade or overcome TME induced suppression of CAR T cells is shown in Figures 3 and 4. Even though most of these approaches have only been studied in *in vivo* mouse models their results have been promising, indicating a possibility of their translation to human applications.

In order for these approaches to be as effective as possible, it is important to take a few considerations into account that relate to CAR T cell efficacy and toxicity. First, it is important to appreciate the presence of other immune cells besides CAR T cells in the TME. For instance, the TME contains immunosuppressive cells such as Tregs and myeloid-derived suppressor cells. A general approach that can limit CAR T cell suppression by these cells is the depletion of the lymphocytes of a patient prior to the administration of CAR T cells. Depleting lymphocytes in patients will ensure the removal of the immunosuppressive immune cells from the TME, decreasing the production of anti-inflammatory cytokines such as IL-4 and TGF-β and also increasing the persistence of the adoptively transferred T cells at the tumour site[78].

Cyclophosphamide is one of the most commonly used drugs for lymphodepletion, as it considerably improves efficacy of adoptive T cell therapy in general. It is often combined with administration of fludarabine prior to adoptive CAR T cell therapy[79, 80], which has been shown to further improve the efficacy of anti-CD19 CAR T cells[81]. The depletion of lymphocytes using cyclophosphamide and fludarabine prior to CAR T cell therapy has already been standardized in anti-CD19 CAR T cell treatments with tisagenlecleucel and axicabtagene ciloleucel[79, 80]. Lymphodepletion by administration of cyclophosphamide and fludarabine has also been tested in the CAR T cell therapy of solid tumours. A phase 1 trial of CAR T cell directed against disialoganglioside GD2 in neuroblastoma showed increased expansion of CAR T cells in patients that were lymphodepleted prior to adoptive CAR T cell transfer[82]. However, depletion of lymphocytes can have many side effects as it is a drastic and nonspecific measure that targets not only immunosuppressive but all other immune cells as well. Lymphodepletion can for instance lead to general leukopenia, lymphopenia, neutropenia and thrombocytopenia, making patients very susceptible to infections[83, 84].

Furthermore, the tumour site also contains immune cells that are beneficial for tumour directed responses, such as pro-inflammatory T cells and macrophages. These cells can for instance target tumour cells that evade CAR T cells by loss of TAA expression[76]. In this context, it might be important to not remove all immune cells from the TME, but to promote the anti-tumour effects of a patient's own immune cells in order to improve tumour directed responses during CAR T cell therapy. The expression of dominant negative receptors on CAR T cells could help with this by sequestering immunosuppressive factors from the TME, thereby reducing inhibition on (surrounding) tumour infiltrating immune cells. Preferably however, CAR T cells engineered with receptors coupled to pro-inflammatory signalling domains can simultaneously sequester immunosuppressive factors and induce activation of the CAR T cell itself. This leads to a greater activation of neighbouring immune cells due to the additional production of pro-inflammatory cytokines by these activated CAR T cells[58].

A second consideration to take into account in the development of novel CAR T cell therapies is that new approaches, such as the ones mentioned above, need to be tumour specific. The approaches should not target surrounding healthy tissue or have widespread systemic effects, as this could lead to severe toxicity such as observed in the administration of IL-12[73]. Therefore, the use of CAR T cells that can release IL-12 locally is a more desirable approach to stimulate immune cells in the TME for instance. The same applies to the use of CAR T cells transduced with dominant negative receptors or receptors coupled to pro-inflammatory signalling domains. These CAR T cells should express both the chimeric receptor that targets an immunosuppressive molecule, and the chimeric receptor directed against the TAA of a specific tumour. Using this approach, these CAR T cells are generally only activated in the tumour, thereby restricting the effects of CAR T cell therapy to the tumour site. This applies to the administration of antagonists as well, where nanoparticles conjugated to the surface of CAR T cells are a promising new approach to locally administer antagonists[34].

Other difficulties arise with the multitude of immunosuppressive factors that can be present in the TME. This review details a selection of the factors that can suppress CAR T cells and there are even more which are beyond the scope of this review, such as the expression of certain inhibitory checkpoints on the surface of tumour cells. Due to this wide array of different factors, it is challenging to develop novel approaches in CAR T cell therapy that are effective in every type of solid tumour. Therefore, it is vital to understand the differences and similarities in the composition of the TME in various tumours to be able to specifically engineer CAR T cells for individual types of cancers. For instance, adenosine has been reported to be increased in the TME of many solid tumours, especially in cancer types with hypoxia such as lung cancer[20]. Perhaps some cancers, such as those with less severe hypoxia like rectal carcinoma[85], do not have highly increased adenosine. In this case, patients suffering from lung cancer would benefit more from CAR T cells that are engineered to be resistant to adenosine than patients suffering from rectal carcinoma. Therefore, to improve the efficacy of new CAR T cell therapies it might be useful to stratify patients for specific CAR T cell therapies based on the type of cancer. This requires an extensive understanding of the presence and importance of different immunosuppressive factors, such as metabolites and cytokines, in different types of cancer.

However, even within specific cancer types there can be variations in the level of metabolites or cytokines. For instance, a group of patients with low levels of TGF-β and a group with high levels of TGF-β can be distinguished in colorectal cancer, where high TGF-β is associated with higher mortality rate[86]. The same is true with levels of IL-4 in clear-cell renal carcinoma, where high levels of IL-4 are associated with increased recurrence and reduced survival[87]. Also, the degree of IL-4 production in patients with cervical intraepithelial neoplasia can differ based on the extensiveness of HPV infection[88]. This indicates that the development and use of newly engineered CAR T cells might not only benefit from the stratification of patients based on cancer type, but also on the identification of individual

differences in the composition of the TME. Therefore, in the future it might be beneficial to base the administration of specific CAR T cell therapies on immunohistochemical stainings of tumour biopsies for instance, in order to identify the most important immunosuppressive factors present in the tumour of a specific patient. In this way, a more patient specific and potentially more effective CAR T cell therapy could be provided, by using CAR T cells engineered to be resistant against the most abundant immunosuppressive factor present in the TME of a specific tumour.

In conclusion, there are many new developments and techniques available to potentially improve CAR T cell therapy in solid tumours. The difficulty lies however in determining the most effective approach, whilst still preventing unwanted toxicity. Important things to consider for this are the composition of the TME in different types of cancers or even in specific tumour biopsies, and the ability of novel CAR T cell approaches to only target the specific tumour site. If these aspects are well examined in humans, CAR T cells can be engineered to be effective and safe in patients suffering from various types of cancers. This can improve the efficacy of CAR T cells in solid tumours, allowing CAR T cell therapy to become an effective standardized method to treat various types of cancers in humans.

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