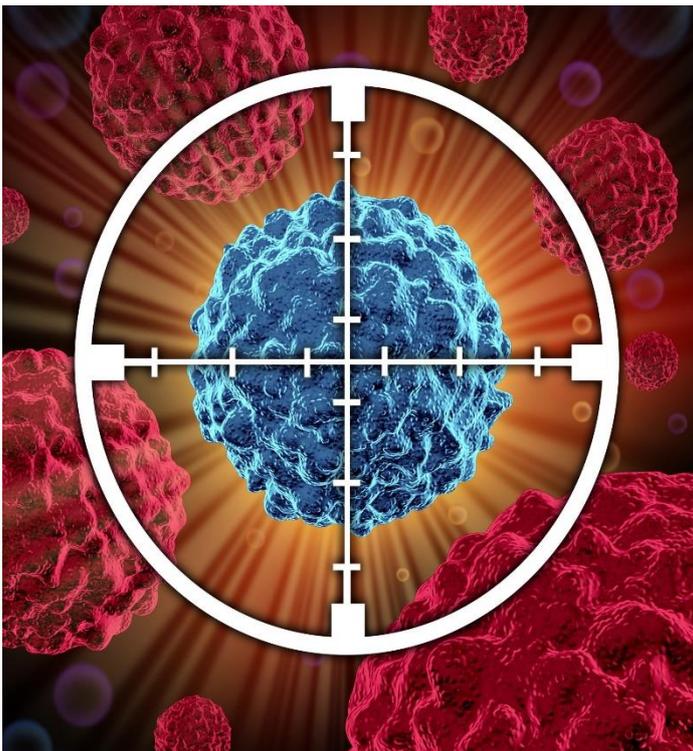


# The ectonucleotidases CD39 and CD73: new targets for cancer immunotherapy



## ABSTRACT

Cancer cells are able to induce immune suppressive pathways, which prevents cancer cells to be recognised by the host's immune response as altered or harmful. Cancer immunotherapy promotes the body's own immune system to fight cancer. Monoclonal antibodies, cancer vaccines, non-specific cancer immunotherapies and immune checkpoint inhibitors are used in cancer immunotherapy. Two prominent immune checkpoints PD1 and CTLA4 are involved in immune suppression by cancer cells. More recently, CD39 and CD73 have been recognised as alternate druggable immune checkpoints. CD39 and CD73 are ectoenzymes present on the surface of different immune cells. CD39 and CD73 cooperate in the conversion of extracellular ATP into adenosine. Normally intracellular ATP serves to provide energy needed for cell processes, whereas extracellular ATP functions as an immune regulator that reduces inflammation by inhibiting the release of pro-inflammatory cytokines. Since CD39 and CD73 are crucial for the conversion of ATP into adenosine, therapeutic blocking of the enzymatic activity of CD39 and CD73 may be of use for cancer immunotherapy. In this bachelor thesis I will discuss the role of ectonucleotidases CD39 and CD73 in cancer and their therapeutic potential as druggable targets in cancer immunotherapy.

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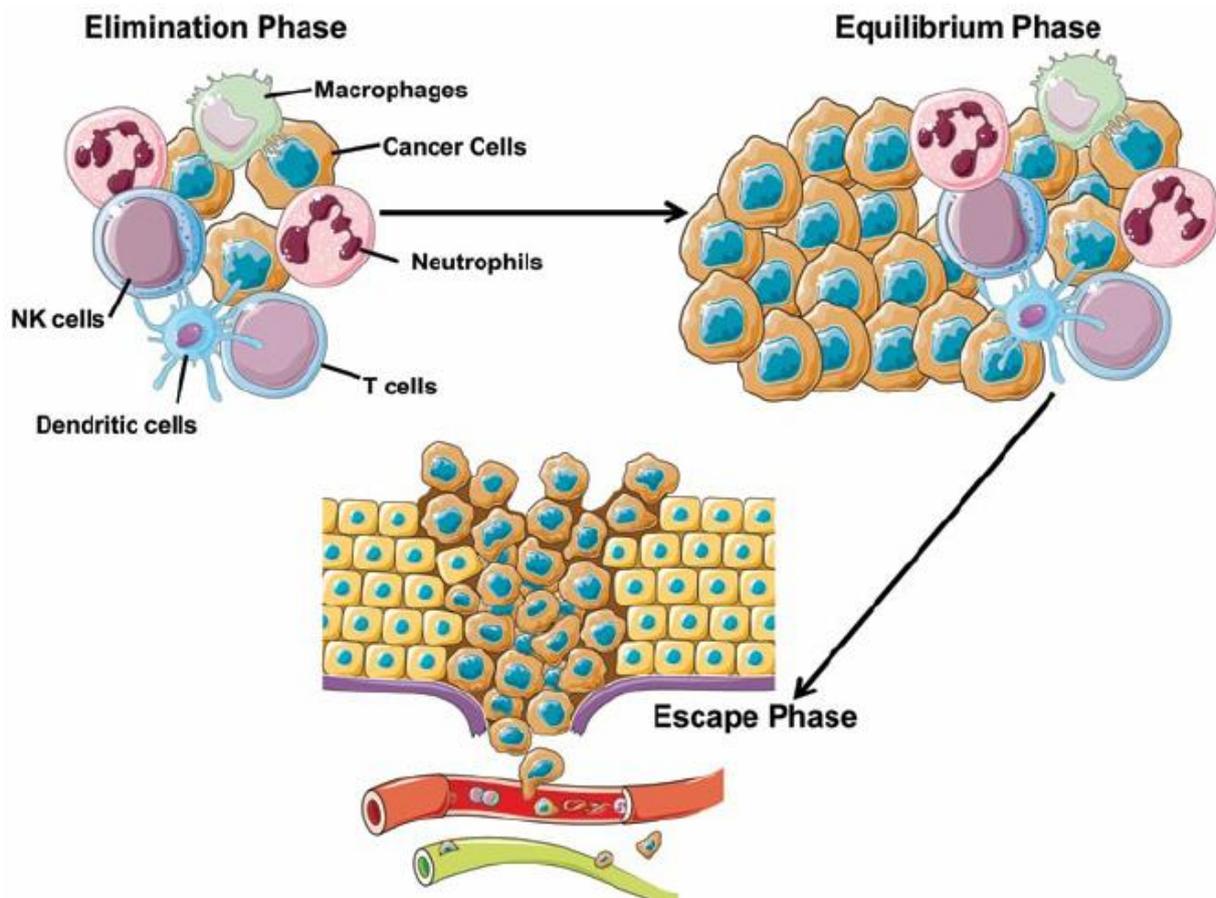
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## Introduction

Cancer caused 8.8 million deaths in 2015, being the second leading cause of death globally. Cancer is characterized by the growth of mutated cells beyond the body tissue from which they have developed. This causes invasion in other parts of the body and spreads to organs, sites or tissues [1]. Cancer cells exhibit several characteristics, also known as the hallmarks of cancer. Cancer cells can induce growth signals, are insensitive to anti-growth signals, invade tissue and metastasis, have unlimited proliferation capacity, sustain angiogenesis and evade apoptosis [2].

Most beginning tumours are detected in a process called immunosurveillance. In this process, immune responses eliminate most of the tumour cells. Subsequently, remaining tumours undergo a process called immuno-editing consisting of three phases; elimination, equilibrium and escape. In the equilibrium phase tumour cells are kept in check by immune cells. However, cancer cells with further reduced immunogenicity evolve in this phase. Finally, some cancer cells escape immune control and develop into full-blown malignant disease. Thus immune-editing produces a selective pressure in the tumour microenvironment that drives tumour progression (Figure 1). [3, 4].



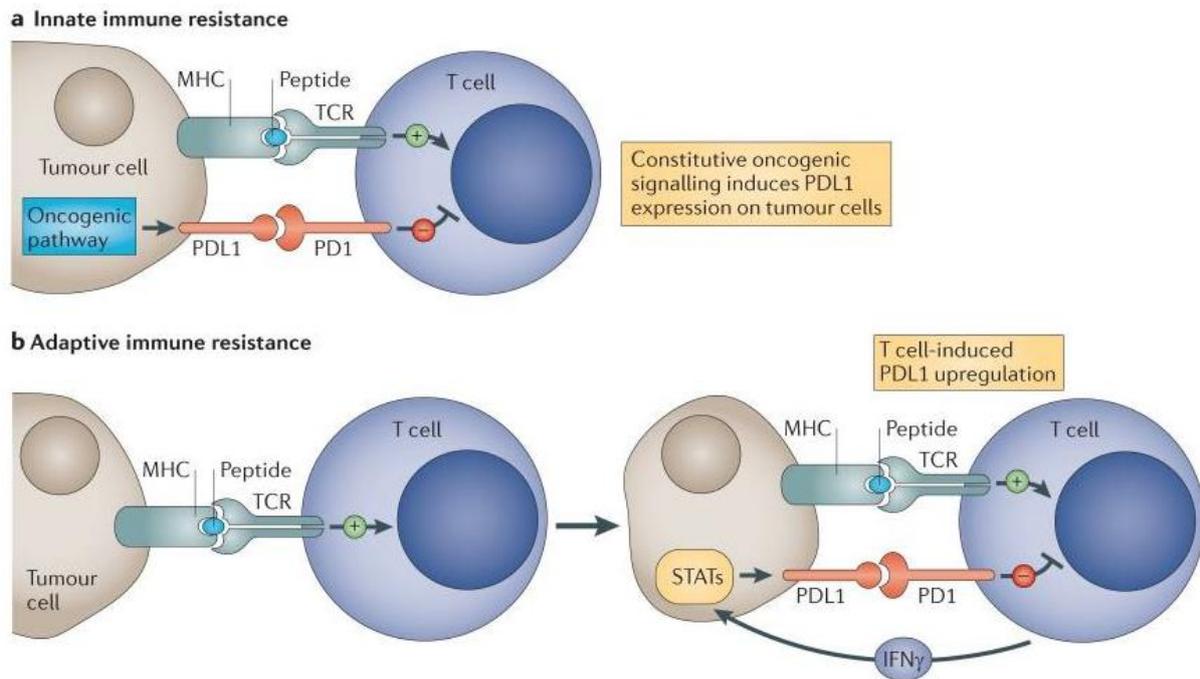
**Figure 1. Overview of cancer immunoediting.** Cancer immuno-editing consists of three phases; elimination, equilibrium and escape. In the elimination phase the initial immune response takes place. Immune cells interact with tumour cells and a strong anti-tumour response occurs. Some tumour cells survive the elimination phase, these enter the equilibrium phase. Selection pressure occurs, immunogenic cancer cells are eliminated and selected cancer cells escape this phase. These go into the escape phase, the cells escape and the can grow to a malignant tumour (figure from [32]).

### **Inhibiting immune checkpoints causes immune suppression**

Cancer cells are able to misuse immune checkpoints to avoid elimination by the host's immune response. The two best-known immune checkpoints are T lymphocyte-associated antigen 4 (CTLA4) and programmed death 1 (PD1). CTLA4 regulates T cell-activation and is in large part dependent on CD4 T cells [5]. CTLA4 counteracts co-stimulatory receptor CD28 on T cells [6]. Normally, CD28 signalling strongly activates T cells when an antigen is recognized. CTLA4 and CD28 both interact with B7, however CTLA4 has a higher affinity for B7 [7]. When CTLA4 is bound to T cells, it downregulates the activity of helper T cells and enhances the immunosuppressive activity of regulatory T cells (Tregs) [8]. Tregs maintain tolerance to self-antigens and prevents auto-immune disease. When Treg activity is enhanced it suppresses immune responses. This creates an immune-suppressive environment.

PD1 limits the activity of T cells during an inflammatory response and limits auto-immunity [8]. PD1 is expressed by various immune cells, including; cytotoxic T cells and tumour infiltrating lymphocytes (TILs) [8, 9]. Programmed death ligand 1 (PD-L1) is expressed by tumour cells in response to IFN $\gamma$  produced by activated T cells. PD1 expression is induced upon T cell activation (Fig. 2) [8]. PD-L1 is highly expressed on tumour cells and this expression has been associated with poor prognosis in patients with cancer [10]. PD-L1 on cancer cells interacts with PD1 on immune cells. When binding occurs, it inhibits kinase signalling pathways involved in T cell activation [8]. This interaction downregulates a CD28 stimulated response, resulting in a strong inhibition of T cell activation [11]. These interactions promote tumour progression.

T helper 1 cells can be converted to Tregs under influence of PD-L1 [12]. This provides more Tregs in the tumour microenvironment, making it more immunosuppressive. PD1/PD-L1 interactions also interfere with T cell receptor signalling. This interference interrupts the interaction of T cells with dendritic cells and stable contact does not form. Stable contact is important for the interaction with and the activation of T cells [13]. Overall cancer misuses immune checkpoints to suppress the immune system by inhibiting T cells. T cells become un-responsive to cancer cells. The amount of Tregs become elevated and contributes to the immune-suppressive environment.



**Figure 2. Binding of PDL-1 expressing tumour cells with PD1 on T cells.** a) Some tumours continuously upregulate PD-L1. T cell receptor and PD1 bind to MHC and PD-L1 on tumour cells. This results in an unresponsive T cell. B) shows the adaptive immune response. T cells become stimulated by tumour cells and induces T-cell-induced PD-L1 upregulation. STATs in tumour cells regulate PD-L1 expression. Upon binding with T cells PD-L1 become upregulated. PD-L1 binds to PD-1 on tumour cells and inhibits T cell activation. T cells produce IFN $\gamma$ . IFN $\gamma$  sustains the upregulation of PD-L1 by STATs, resulting in a positive inhibitory feedback loop (figure from [8]).

### Blocking CTLA4, PD1 and PD-L1 in cancer immunotherapy

Checkpoint inhibitors used in cancer immunotherapy are focused on blocking the immune-suppressive effects of cancer cells on these checkpoints. CTLA4 and PD1 are clinical targets. Blocking CTLA4 prevents the interaction of CTLA4 with B7. This block allows B7 to interact with CD28 on T cells and T cells become activated. Blocking CTLA4 with an antagonistic monoclonal antibody (mAb) elevated the anti-tumour function of CD8<sup>+</sup> T cells [14] and caused depletion of Tregs at tumour sites [15]. The elevation of CD8<sup>+</sup> T cells and depletion of Tregs increased the ratio of CD8<sup>+</sup> T cells to regulatory Foxp3<sup>+</sup> T cells, inhibiting suppressive functions of Tregs. Ipililumab is the first approved monoclonal antibody (mAb) immune-checkpoint inhibitor by the FDA to block CTLA4. Tremelimumab is a fully human mAb against CTLA4 [16]. Both Ipililumab and Tremelimumab are antagonistic anti-CTLA4 mAbs used in clinical trials. Ipililumab improved cancer survival and showed to be an effective antibody in clinical trials to treat melanoma. Melanoma patients treated with Tremelimumab showed sustained anti-tumour responses and an increase in long-term survival [17].

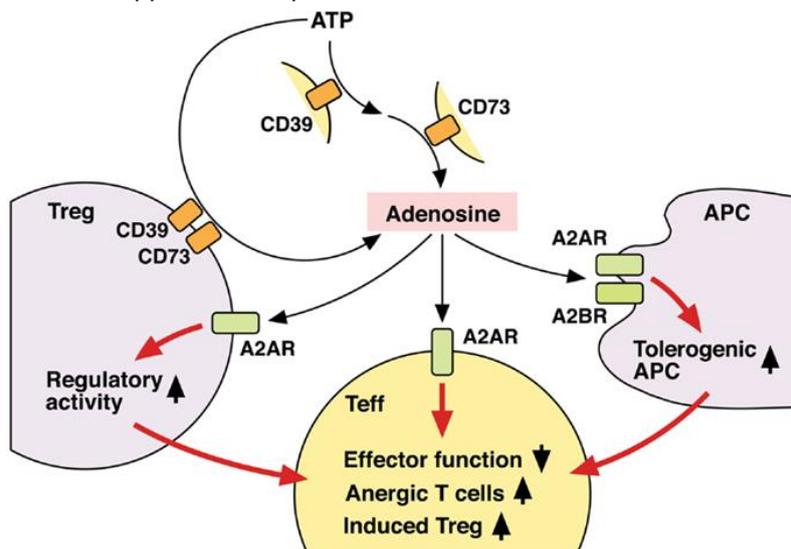
Blocking PD1 on immune cells or blocking PD-L1 on tumour cells with mAbs increased patient survival in clinical trials [18]. Pembrolizumab and Nivolumab are two mAbs that block PD1, both approved by the FDA. Pembrolizumab was the first anti-PD1 antibody to be approved by the FDA and is used to treat different cancers; Hodgkin Lymphoma, melanoma, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) cancer, non-small cell lung cancer, squamous cell carcinoma of the head and neck and urothelial carcinoma [19]. Pembrolizumab has been reported to improve survival in cancer patients compared to Ipililumab. Robert *et al.* showed that treatment with Pembrolizumab improved progression-free and overall survival in patients with advance melanoma. Melanoma

patients treated with Nivolumab did not show similar effects [20]. Nivolumab blocks the interaction of PD1 with PD-L1. This prevents the downregulation of T cell activation. Immune responses remain active [18].

Atezolizumab, Avelumab and Durvalumab are mAbs which block PD-L1. Atezolizumab is the first antagonistic anti-PD-L1 mAb approved for treatment of patients with metastatic non-small cell lung cancer (NSCLC) [21]. Patients treated with Atezolizumab showed ongoing responses to therapy [22]. Atezolizumab increased overall survival in cancer patients compared to chemotherapy. Better responses were associated with high expression of PD-L1 on tumour cells [23]. Avelumab was approved by the FDA in 2017 for treatment of metastatic Merkel Cell Carcinoma (MCC). Clinical trials showed a meaningful and durable overall response rate in MCC patients treated with avelumab [24]. Also Durvalumab was approved by the FDA in 2017. Clinical trials showed meaningful response rates. Response rates correlated with PD-L1 expression. Patients with high PD-L1 expression had better responses than patients with low PD-L1 expression [25, 26].

### Immune checkpoints CD39 and CD73

Blocking CTLA4, PD1 and PD-L1 are promising in cancer immunotherapy. CD39 and CD73 are other checkpoints involved in immune suppression. CD39 and CD73 are responsible for the conversion of ATP to adenosine (Fig. 3). CD39 hydrolyses ATP into ADP [27] and CD73 converts ADP into adenosine [28]. CD39 and CD73 have been considered to be pivotal in the generation of an immuno-suppressive environment via adenosine production. Adenosine is known to have anti-inflammatory activity when released in the extracellular milieu [29]. Adenosine also contributes to suppressing anti-tumour immunity by interacting with specific receptors on T cell [30]. Adenosine also limits the effects of CTLA4 inhibition by mAbs. Blocking of CTLA4 CD73 and both resulted in a decrease in tumour growth [31]. Inhibiting the enzymatic activity of CD39 and CD73 blocks the adenosine generation and inhibits immuno-suppressive responses.



**Figure 3. CD39 and CD73 convert ATP into adenosine.** CD39 and CD73 produce extracellular adenosine by converting ATP. Adenosine binds to adenosine A2A receptors and induces cell responses. The conversion by CD39 and CD73 results in inhibited T cell effector functions, unresponsive T cells and increases Tregs. Antigen presenting cells (APCs) become more tolerogenic, resulting in less antigen presentation. Also the regulatory activity of Tregs are upregulated. These effects are involved in suppressing immune responses. (Figure from [33]).

The aim of this bachelor thesis is to discuss CD39 and CD73, the effects of adenosine in immune suppression and to show that CD39 and CD73 are promising enzymes to be targeted for cancer immunotherapy.

## CD39 and CD73 convert ATP into adenosine

CD39 and CD73 are ectonucleotidases which belong to the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family. CD39 and CD73 hydrolyse extracellular tri- and diphosphates to monophosphate derivatives [34]. All NTPDases have highly conserved sequence domains which are involved in active site formation and extracellular nucleotide catalysis by phosphohydrolysis [35]. CD39 and CD73 are highly expressed on the surface of different immune cells [36, 37]. CD39 hydrolyzes adenosine triphosphate (ATP) and/or adenosine diphosphate (ADP) to adenosine monophosphate (AMP) [37], which is the rate limiting step of the ATP to adenosine conversion. AMP is then dephosphorylated by CD73 to adenosine [38].

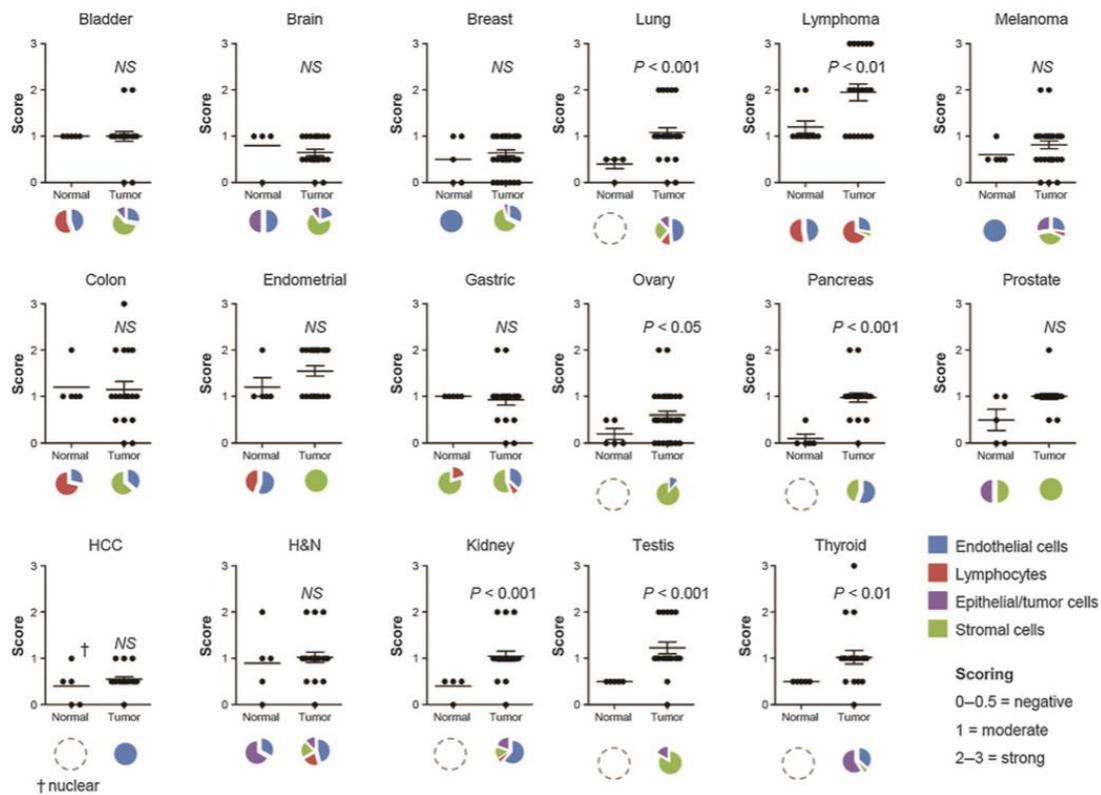
ATP is normally present within cells, where it provides energy needed for cell processes. Compared to other tissues, ATP levels are elevated in the tumour microenvironment. ATP and ADP are important in inflammation and tissue homeostasis. Release of extracellular ATP serves as a mediator for purinergic responses. The activation of purinergic P2 receptors plays an important role in inflammation and immunomodulation. Extracellular ATP acts through 2 purine receptors; P2X and P2Y. P2X are ligand-gated ion channels, P2Y are G-protein coupled receptors (GPCRs). Extracellular ATP inhibited CD4<sup>+</sup> T cell activation via P2Y receptors [40]. Extracellular ATP inhibits proliferation of melanoma cells in a dose dependent manner. When P2X<sub>7</sub> and P2Y<sub>1</sub> receptors are blocked, no inhibition of proliferation of melanoma cells was found. This suggests that P2X<sub>7</sub> and P2Y<sub>1</sub> receptors are involved in cell proliferation. Extracellular ATP also promoted tumour cell death when bound to the P2X<sub>7</sub> receptor [41]. Overall extracellular ATP is a pro-inflammatory biological which suppresses tumour growth and promotes anti-tumour responses. However extracellular ATP is quickly converted to adenosine by CD39 and CD73. The presence of adenosine in the tumour microenvironment results in different responses.

## CD39 and CD73 are expressed on various cells and are crucial in adenosine generation

Extracellular adenosine can act as an immune modulating factor and as such can play an important role in tumour progression. The conversion from ATP to adenosine by cancer cells results in locally elevated levels of adenosine at tumour sites. Alterations in adenosine levels by CD39 and CD73 suppressed cellular responses of immune cells. In 2006 it was found that elevated levels of extracellular adenosine play a role in the evasion of anti-tumour immune responses [42].

## CD39 and CD73 are expressed on cancer cells

CD39 is overexpressed on cell surfaces in various human cancers. CD39 expression was higher in cancer cells than cells in normal tissues (Fig. 4). CD39 and CD73 expression has been studied in different cancers, here I will discuss melanoma, ovarian cancer and endometrial cancer. Melanoma cancer cells expressing CD39 hydrolysed ATP, resulting in anti-tumour responses. Melanoma cells without CD39 expression did not hydrolyse ATP [43]. CD73, also known as ecto-5'-nucleotidase (NT5E), is a glycosylphosphatidylinositol (GPI) -anchored receptor on the cell surface [44]. It was found that melanoma cells also express CD73. Melanoma cancer cells expressing both CD39 and CD73 suppressed CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation through adenosine generation. These melanoma cells also inhibited CD8<sup>+</sup> cytotoxic T cell (CTL) functions, this suggests that the adenosine generation by CD39 and CD73 inhibits the generation of effector CTLs [43].



**Figure 4. The expression of CD39 in normal tissue and tumour tissue.** CD39 is significantly upregulated in some cancers compared to the corresponding normal tissues (figure from [43]).

Ovarian cancer cells express CD73. The medium of epithelial ovarian cancer ID8 cells with CD73 expression slightly inhibited CD4<sup>+</sup> T cell proliferation. CD73 expressing tumour cells suppressed the cytotoxic activity of CTLs, inhibited cancer cell killing and inhibited the survival of tumour-specific T cells [45]. Ovarian cancer cells also express CD39. Cancer cells expressing both CD39 and CD73 produced 30-60 times more biologically active adenosine than Tregs. Adenosine production by these cancer cells inhibited the cytotoxicity of NK cells and T cells. These CD39 and CD73 expressing cells also suppressed CD4<sup>+</sup> T cell proliferation [46].

CD39 and CD73 were detected in endometrial cancer (EC). Expression of CD39 and CD73 in endometrioid (type 1) and serous (type 2) cancer has been studied and had been compared to nontumoural endometrium. Expression of CD39 and CD73 were significantly higher than their nontumoural counterparts [47]. This study adds on to the list of human cancers with CD39 and CD73 overexpression and supports the growing evidence of CD39 and CD73 as potential therapeutic targets for cancer immunotherapy.

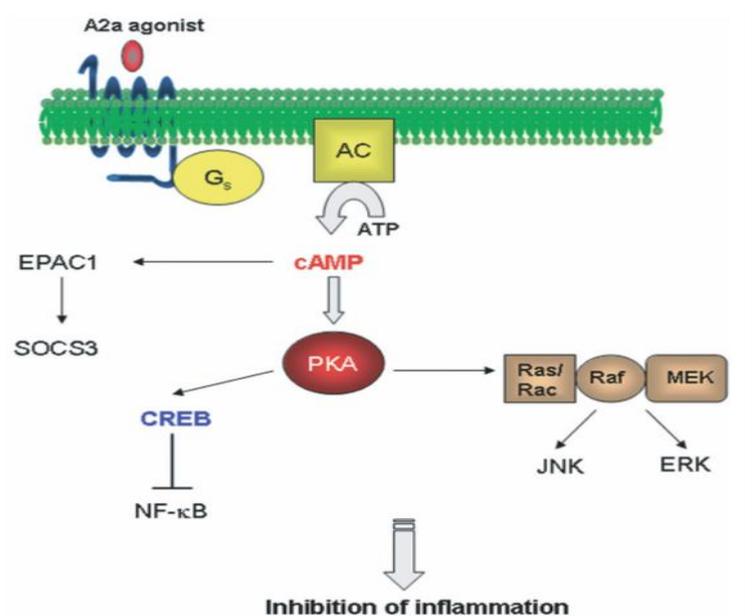
### CD39 and CD73 are expressed regulatory T cells

CD39 and CD73 are also present on various immune cells. CD39 is highly expressed on Foxp3 T cells and suppresses Foxp3 T cell functions. Foxp3 Treg CD39-null mice showed impaired functions of Tregs, suggesting that CD39 is involved in the functioning of Tregs [36]. CD39 expressing Foxp3 Tregs directly suppressed NK cell-controlled tumour expansion, inhibiting the effects of NK cells [48]. CD39 expressed on endothelial cells promoted tumour cell growth by scavenging ATP present in the tumour microenvironment [41]. Also CD73 is highly expressed on Foxp3 Tregs. Together with CD39, CD73 dampens anti-tumour responses. By the generation of adenosine, CD39 and CD73 inhibit the release of cell killing mediators such as TNF and IFN $\gamma$  preventing cell killing [49]. Increased expression of CD73 induced immune-suppressive functions modulating the lymphocyte microenvironment [50]. this adds up to effects CD39 and CD73 have on Tregs. CD39 and CD73 expression on cancer together with CD39 and CD73 on Tregs provides more evidence for CD39 and CD73 as therapeutic targets for cancer immunotherapy.

### Adenosine binds to G protein coupled receptors (GPCRs)

There are four known adenosine receptor subtypes, namely; A1, A2A, A2B and A3 [51]. A2A and A2B are coupled to a G $_s$  protein. When adenosine binds to a GPCR, cAMP production increases. cAMP activates protein kinase A (PKA) and PKA phosphorylates response element binding protein (CREB) (see fig. 5). This signalling cascade results in the inhibition of inflammation. A1 and A3 are coupled to G $_{i/o}$  proteins, decreasing cAMP and hereby turning down this signalling cascade [52]. Stimulation of the adenosine A2A receptor provides an immuno-suppressive signal, inhibiting T cell activities such as cytokine production and cytotoxicity. A2A receptor stimulation by adenosine or the adenosine agonist CGS 21680 inhibited the cytotoxicity of NK cells and inhibited CD4 $^+$  and CD8 $^+$  effector functions [53]. Adenosine A2A receptors are predominantly found on T cells, specifically on activated CD8 $^+$  T cells [54]. When these T cells are active, adenosine binds and inhibits the inflammatory effects of these cells.

**Figure 5. Adenosine signalling cascade.** Adenosine to a G $_s$  protein coupled receptor. cAMP levels increase. cAMP activates PKA and PKA phosphorylates CREB. CREB inhibits NF- $\kappa$ B, a pro-inflammatory biological. This signalling cascade results in inhibition of inflammation (figure from [55]).



## Blocking CD39 and CD73 enzymatic activity

Blocking CTLA4 and blocking PD1 or PD-L1 showed to be effective in reducing anti-tumour responses. Not only CTLA4 and PD1 or PD-L1 are involved in tumour responses. As described above, CD39 and CD73 are just as important in tumour progression as CTLA4 and PD1 or PD-L1. CD39 and CD73 continue to be found in human cancers, indicating CD39 and CD73's importance in tumour progression. This evidence suggests that CD39 and CD73 can be therapeutic targets for immunotherapy.

### Inhibition of CD39 by small molecules and monoclonal antibodies

CD39 inhibition has been studied using small inhibitory molecules and using monoclonal antibodies (mAbs). These inhibitory molecules work as antagonists, blocking the active site of CD39. Blocking the active site prevents binding with ATP, inhibiting ATP to ADP conversion. ARL67156 (ARL) is a chemical inhibitor of CD39. OREG-103/BY40 (OREG) is a mAb against CD39. POM-1 is a non-specific inhibitor of E-NPTDases. IgG<sub>2a</sub> antibody 9-8B is a monoclonal antibody which also inhibits CD39. Blocking CD39 with ARL chemical inhibitor inhibited the conversion of ATP in a dose-dependent manner. Blocking CD39 with OREG mAb and CD39 inhibition with POM-1 also showed inhibited activity of CD39. The inhibition of CD39 activity resulted in restored CTL and NK cell activity and increased the CD8 T cells in the tumour microenvironment [43].

Deleting or blocking CD39 has effects on the anti-tumour response. Blocking CD39 on endothelial cells enhanced anti-tumour effects of ATP [41]. Without CD39 enzymatic activity, extracellular ATP will not be converted to adenosine. ATP accumulates in the microenvironment and induces anti-tumour responses. Inhibition of CD39 on tumour cells showed a decrease of their immunosuppressive characters [54]. Small interfering RNA (siRNA) was used to downmodulate CD39. The downmodulation by siRNA decreased the suppressive activity on CD8<sup>+</sup> T cells. CD8 T cells were no longer suppressed and could execute their normal functions killing infected/abnormal cells [56]. Anti-CD39 gG<sub>2a</sub> antibody 9-8B has been generated and identified in a study by Hayes *et al* (2015). The CD39 enzymatic inhibitory function was first determined using a flow-based platelet aggregation method. Then the Anti-CD39 gG<sub>2a</sub> antibody 9-8B was used to examine the inhibition of CD39 nucleotidase activity. Anti-CD39 gG<sub>2a</sub> antibody 9-8B significantly reduced CD39 activity. It showed a greater inhibition of CD39 than when positive control POM-1 was used to inhibit CD39. This showed that Anti-CD39 gG<sub>2a</sub> antibody 9-8B's ability to inhibit CD39 was greater than the ability of POM-1 to inhibit CD39. Then the effects of 9-8B in patient-derived xenograft in mice were assessed. This showed that the CD39-specific antibody 9-8B significantly improved survival in this metastatic sarcoma model [57].

### Inhibition of CD73 by small molecules and monoclonal antibodies

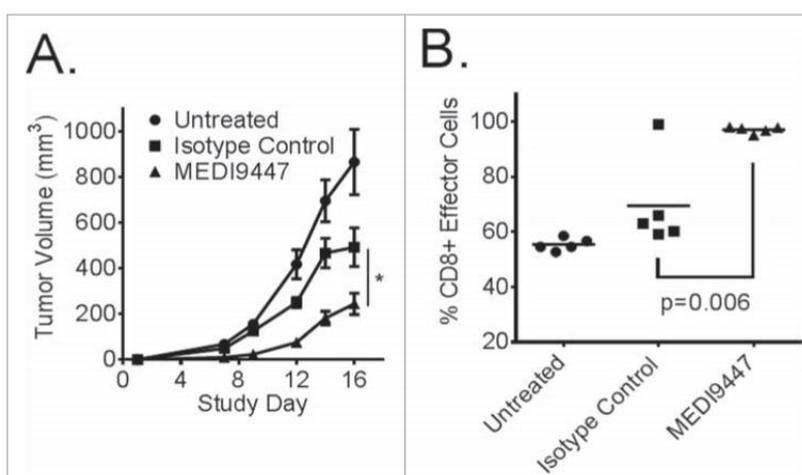
Small molecules, siRNA and antagonistic monoclonal antibodies have been used to study the inhibition of CD73. TY/23 and AD2 are antagonistic CD73 mAbs. APCP is a small molecule which binds to CD73 and blocks enzymatic activity.

APCP was used to study the inhibitory effects on CD73 in human colorectal cancer. APCP reversed the effects of adenosine in colorectal cancer, inhibiting tumour growth [58]. Therapy with the anti-CD73 antagonistic mAb TY/23 downregulated CD37 expression of CD8<sup>+</sup> and CD4<sup>+</sup> tumour infiltrating lymphocytes (TILs) [59] and therapy with the anti-CD73 antagonistic mAb TY/23 significantly delayed

primary tumour growth. Additionally anti-CD73 antagonistic mAb TY/23 decreased spontaneous metastasis [60], the latter being in accordance with the findings of Terp *et al.* (2017). Terp *et al.* (2017) found that the anti-CD73 AD2 mAb inhibits metastasis in a spontaneous metastasis model [61]. Downregulation of CD73 using siRNA showed that T cell proliferation was promoted, even in the presence of AMP. This suggests that CD73 is responsible for the inhibitory effects on T cells [45].

MEDI9447 is a new clinical antagonistic therapeutic and is targeted against CD73. This is a fully human IgG1 $\lambda$  monoclonal antibody. MEDI9447 selectively binds to the ectonucleotidase CD73 and inhibits the activity. MEDI9447 binds to a site in the N-terminal of CD73 and this results in non-competitive inhibition [64]. A study of Hay *et al.* (2016) showed MEDI9447 to be a promising mAb when it comes to targeted therapy. MEDI9447 had the ability to reduce AMP levels in cell culture supernatant, where an irrelevant isotype control antibody could not. Additionally to reducing the enzymatic activity of CD73, MEDI9447 has the ability to internalize CD73 upon binding. This resulted in cytotoxicity of cancer cells when incubated with MEDI9447 and a toxin-conjugated secondary antibody. The isotype control antibody together with a toxin-conjugated secondary antibody did not result in cytotoxicity of cancer cells. Hay *et al.* also examined the effect of CD73 inhibition by MEDI9447 on T cell proliferation. In the presence of 100  $\mu$ M of extracellular AMP, T cell proliferation was suppressed. MEDI9447 could counteract the suppression, the effect of extracellular AMP was relieved. The isotype control antibody did not show this effect.

However this was all done using a purified T lymphocyte system. Hay and colleagues also examined the effects of MEDI9447 in the two-way mixed leukocyte reaction (MLR). The inhibition of CD73 with MEDI9447 enhanced antigen presentation, lymphocyte activation and increased levels of T1 helper cells (a subset of T cells which promote inflammation) in the MLR supernatant. Interferon- $\gamma$ , interleukin 1- $\beta$  and tumour necrosis factor- $\alpha$  were increased when CD73 was blocked with increasing levels of MEDI9447. The isotype control antibody showed a smaller increase when similar levels were used. MEDI9447 also significantly inhibited tumour growth in a murine colon carcinoma model. MEDI9447 inhibited tumour growth by 50% or more when compared to the isotype control antibody. MEDI9447 treated tumours also showed significantly more activated CD8<sup>+</sup> Lymphocytes (see fig. 6). Also CD4<sup>+</sup> TILs were significantly higher in responsive tumours, this was also seen with CD8<sup>+</sup> TILs. Taken this data together, MEDI9447 is a promising target for inhibiting the effects of CD73. It was able to inhibit or decrease the adenosine generation and it could counteract the inhibitory effects of adenosine on T cell activity more than another antibody [37].



**Fig. 6. Effects of MEDI9447 on tumour volume and CD8<sup>+</sup> effector cells.** MEDI9447 shows significant inhibition of tumour volume compare to isotype control and untreated group. Also the CD8<sup>+</sup> cells show an increase when treated with MEDI9447, which is higher than treatment with isotype control. (figure from [56]).

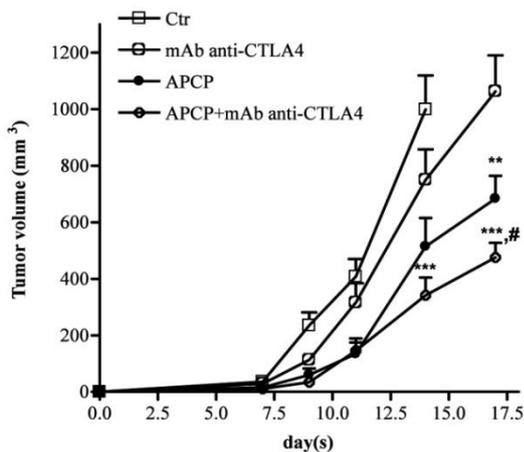
### Combined blockade of CD39 and CD73

Since cancer cells express both CD39 and CD73, blocking both immune checkpoints has been examined in ovarian cancer cell lines SK-OV-3 and OAW-42. Specific antagonistic monoclonal antibody A1 was used to block CD39 and mAb 7G2 was used to block CD73. Blocking CD39 with Anti CD39 antagonistic A1 mAb and blocking CD73 with anti-CD73 antagonistic 7G2 mAb relieved the effects of CD39 and CD73 on NK cells. Blocking CD39 and CD73 with these mAbs significantly improved activity of NK cells.

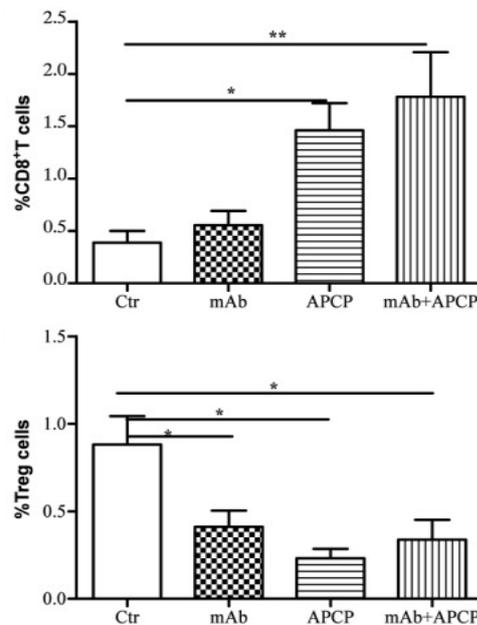
Adenosine receptor A2A overexpressing HEK-293-“sensor cells” were used to examine if anti-CD39 A1 mAb and anti-CD37 7G2 mAb truly interfered with the adenosine generation. Applying both anti-CD39 antagonistic A1 mAb and anti-CD73 antagonistic 7G2 mAb showed a decreased adenosine generation. This confirms that these antibodies can, in addition to increasing NK cell lytic activity, inhibit enzymatic activity for adenosine generation. Anti-CD39 antagonistic A1 mAb and anti-CD73 antagonistic 7G2 mAb also increased CD4<sup>+</sup> proliferation, undoing the inhibitory effects of CD39 and CD73 on CD4<sup>+</sup> proliferation [59].

### Blockade of CD73 combined with CTLA4 or PD1 blockade

Not only CD39 and CD73 could be blocked simultaneously, but also other immune checkpoints could be blocked together. Lannone *et al.* (2014) showed that APCP inhibition of CD73 together with inhibition of CTLA4 with an antagonistic monoclonal antibody enhanced anti-tumour effects. Melanoma-bearing mice showed a significant decrease in tumour growth when the combination of inhibitors was used compared to inhibiting with APCP or an Anti-CTLA4 mAb alone [see fig 7). The combined blockade of CD73 and CTLA4 resulted in increased levels of CD8<sup>+</sup> TILs increased. Blocking CD73 and CTLA4 also resulted in reduced Treg levels (fig. 8) [31].



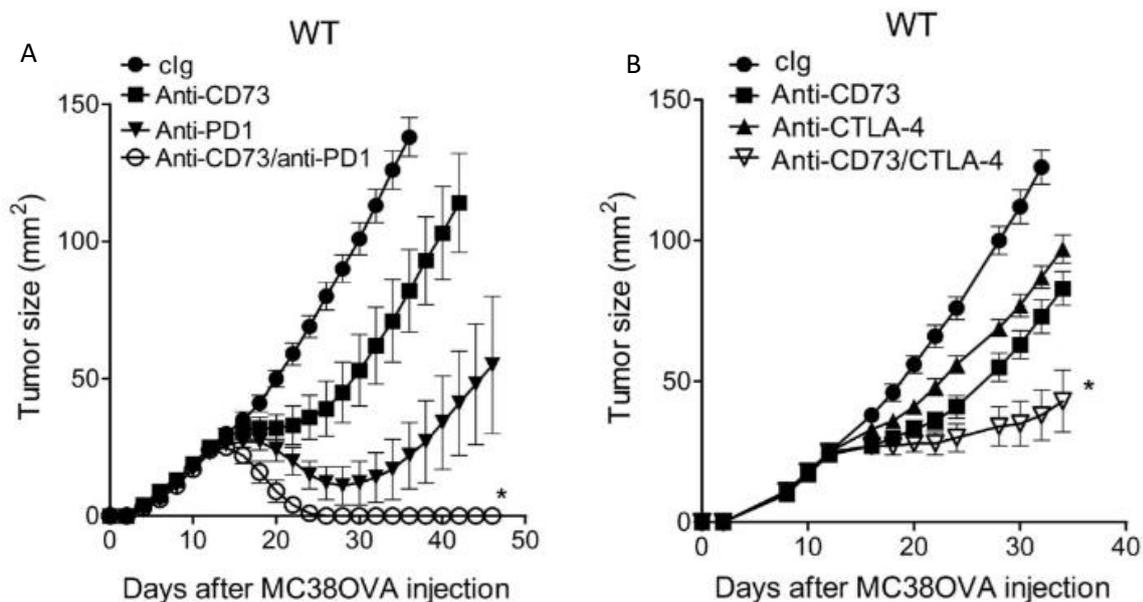
**Figure 7. Effects of different inhibitors on tumour growth.** Anti-CTLA4 mAb and anti-CD73 APCP both show reduction in tumour volume. The combination of anti-CTLA4 and anti-CD73 show an even further reduction which is highly significant (figure from [31]).



**Figure 8. Effects of different inhibitors on CD8<sup>+</sup> T cells and Tregs.** Treatment with mAb or APCP show an increase in CD8<sup>+</sup> T cells and a reduction of Treg cells. The combination of both antagonistic antibodies show an even more significant upregulation of CD8<sup>+</sup> T cells and further reduction of Tregs (figure from [31]).

The effects of inhibiting CD73 together with PD1 inhibition has been examined by Hay *et al.* (2017) examined. TY/23 was used as the CD73 inhibitor, anti-PD1 RMP1-14 mAb was use as the PD1 inhibitor and purified mouse CTLA4 mAbs were used to block CTLA4. Blocking CD73 with MEDI9447 and blocking PD1 with a TY/23 mAb showed increased anti-tumour responses compared to blocking CD73 or PD1 alone [37]. Allard *et al.* also examined the effects of blocking CD73 together with blocking CTLA4 or blocking PD1. Colon cancer bearing-mice showed inhibited tumour growth when treated with either anti-CD73 mAbs or anti-PD1 mAbs.

The combination of blocking CD73 and blocking PD1 showed complete tumour rejection, this was IFN $\gamma$  dependent (see fig 9A). Combined blocking of CTLA4 and CD73 showed that blocking CD73 significantly enhanced the effects of CTLA4 blockade with monoclonal antibodies. However it did not result in complete tumour rejection (see fig 9B). The effects of blocking CD73 and CTLA4 were also IFN $\gamma$  dependent. Both CD73/PD-1 and CD73/CTLA4 blocking combinations were CD8 T cell dependent. Monotherapy blocking CD73, PD1 or CTLA4 independently showed increased CD8 TILs and showed a prolonged survival in metastatic mice. Combination therapy of CD73/PD1 blockade or CD73/CTLA4 blockade further increased CD 8 TIL levels and prolonged survival even more [59]. This study showed that combination therapy increased anti-tumour effects better then monotherapy could. It also showed that the CD73/PD1 blockade combination showed more effective results than combining CD73/CTLA4 blockade. Overall monotherapy seems to be less efficient than combination therapy and combination therapy seems to be more promising.



**Figure 9. A Effects of Anti CD73, anti PD-1 and combination of anti-CD73 and anti-PD1.** Anti-CD73 and anti-PD1 show a decrease in tumour size. The combination of both anti-CD73 and anti-PD1 show complete tumour rejection. **B effects of anti-CD73, anti-CTLA-4 and combination of anti-CD73 and ant-CTLA4.** Both show reducing in tumour size. Combination of both antibodies show a significant decrease in tumour size, however it does not result in complete tumour rejection (figure from [59]).

## Discussion

The current well-known immune checkpoints PD1 and CTLA4 are used by cancer to induce an immune-suppressive environment by inhibiting co-stimulation [6, 11], downregulating T cells and inducing Tregs [8, 12]. Anti-CTLA4, and anti-PD1 and anti-PDL1 antagonistic mAbs are well known to suppress this immune-suppressive environment [14, 18]. However drugs approved by the FDA which inhibit CTLA4 or PD1 show side effects. Ipilimumab causes changes in skin including darker skin and rashes, causes diarrhoea and causes loss of appetite [62]. Tremelimumab, Nivolumab and Pembrolizumab showed similar side effects [63, 64, 65]. According to the review of Jeffrey Weber (2007) inhibition of CTLA4 in patients also showed effects that are called Immune-related adverse events (IRAEs). These IRAEs are inflammatory and may present a decreased tolerance to self-antigens. The most common IRAEs are rash, colitis and hepatitis. These side effects may correlate with the response to Ipilimumab [66]. Atezolizumab, Avelumab and Durvalumab also showed side effects, reaching from nausea to swelling in hands or feet [67, 68, 69]. Since side effects occur, it is important to find other mechanisms to fight cancer.

CD39 and CD73 are immune checkpoints which are also involved in immune suppression. They are pivotal for the conversion of ATP to adenosine. ATP is normally present within cells. In the tumour microenvironment, ATP levels are increased compared to other tissues. Extracellular ATP induces anti-tumour responses [41]. However CD39 and CD73 convert extracellular ATP to adenosine. Adenosine has anti-inflammatory effects in the extracellular milieu and contributes to anti-tumour immunity by interaction with specific receptors on T cells [29, 30].

CD39 and CD73 are overexpressed in various human cancers. The presence of CD39 and CD73 resulted in anti-tumour responses such as suppression of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and inhibition of CD8<sup>+</sup> cytotoxic T cell functions [43]. Presence of CD39 and CD73 enzymatic activity also inhibited cancer cell killing and inhibited the survival of tumour-specific T cells [45]. The continuous finding of CD39 and CD73 in human cancer cells supports the evidence that CD39 and CD73 are potential targets for cancer immunotherapy.

CD39 and CD73 enzymatic activity can be blocked by small molecules, siRNA and mAbs. Inhibition of CD39 and CD73 by small molecules, siRNA and mAbs resulted in the relief of suppressive effects by CD39 and CD73. There are several mAbs produced and identified for blocking CD39 or CD73. All these antibodies are mAbs. Blocking CD39 and CD73 with mAbs have as advantage that antibody drugs typically have long serum half-lives. This characteristic is favourable since mAbs are injected in the blood stream and circulate before mAbs reach its target. CD39 and CD73 mAbs mostly have the same effect, inhibiting enzymatic activity and preventing tumour-favourable responses. MEDI9447 showed more promising results than other existing mAbs. MEDI9447 selectively binds CD73 and inhibits the enzymatic activity. It had the ability to reduce AMP levels where an irrelevant isotope control antibody could not. MEDI9447 causes internalization of CD73 upon binding and together with a toxin-conjugated secondary antibody cancer cells became cytotoxic. An irrelevant isotype control antibody together with the toxin-conjugated secondary antibody did not result in cytotoxicity of cancer cells. MEDI9447 also increased CD8<sup>+</sup> lymphocytes and CD4<sup>+</sup> TILs. These results showed that MEDI9447 has better potential to serve as a therapeutic antibody to treat cancer.

Allard *et al* (2013) inhibited both CD39 and CD73. This resulted in the relief of effects of CD39 and CD73 on NK cells, NK cell activity significantly improved. But the question is; why block both CD39 and CD73 when it has been shown that blocking only CD39 or CD73 is promising? Blocking both CD39 and CD73 do not improve anti-tumour responses compared to blocking CD39 or CD73 alone. Blocking only one already interrupts the adenosine generation, so blocking both might not improve the results significantly compared to blocking only CD39 or CD73. However, blocking CD73 combined with the blockade of PD1 or CTLA4 does improve anti-tumour responses. Blockade of CD73 together with blockade of CTLA4 resulted in increased levels of CD8<sup>+</sup> TILs [31]. Blocking of CD73 with MEDI9447 together with blocking of PD1 increased anti-tumour effects than blocking CD73 or blocking PD1 alone. The combination of blocking CD73 together with PD1 showed even better results than blocking CD73 together with CTLA4 blockade.

As reported, combination therapy seems to be promising for cancer treatment. However as stated earlier, drugs nowadays used to block CTLA4 and PD1 causes side effects. Side effects have not been reported yet for the blockade of CD39 and CD73. Studies should be done to see if CD39 and CD73 show side effects in patients. Also combination therapy has to be studied for possible side effects. CD39 and CD73 are not only present on cancer cells, but also on immune cells. Blocking CD39 and CD73 with mAbs might interfere with other biological processes. To avoid this, tissue specific mAbs have to be generated and identified. When tissue specific mAbs are used, side effects will be minimalised.

## Conclusion

Overall blocking. Blocking CD39 and CD73 inhibited the enzymatic activity and prevented the conversion from ATP to adenosine. Blocking CD39 and CD73 with small molecules, siRNA but mostly antagonistic mAbs have shown to be effective in increasing anti-tumour responses and in limiting tumour progression.

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