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# Extracellular adenosine metabolism in melanoma and pancreatic cancer

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## Summary

Adenosine is a small molecule suppressing the effector arm of the immune system and enhancing the activity of immunosuppressive cells. It is produced through the two-step hydrolysis of extracellular ATP by ectonucleotidases CD39 and CD73. These enzymes have been detected on various populations of immune cells as well as on tumor cells and they can contribute to the immunosuppression developed in the tumor microenvironment. The aim of the current study was to investigate the role of ectonucleotidases CD39 and CD73 in different subsets of immune cells in the regulation of anti-tumor immunity in tumor-bearing hosts. We used the *ret* transgenic mouse model of spontaneous melanoma and the orthotopic Panc02 model of pancreatic ductal adenocarcinoma that resemble the clinical situation. Patterns of ectonucleotidase expression on various immune cell subpopulations were analyzed with regard to their phenotype and function.

We showed that CD39 and CD73 are strongly expressed on murine regulatory T cells (Tregs). Moreover, ectonucleotidase expression was detected on CD4 FoxP3$^-$ conventional T cells (Tcons) and CD8 T cells. Notably, a significant fraction of tumor-infiltrating Tcons and CD8 T cells expressed CD39 or CD73 (especially in the Panc02 pancreatic adenocarcinoma model), thereby suggesting that the expression of ectonucleotidases on T cells might be influenced by the tumor microenvironment. Next, we found that Tcons with the activated phenotype (CD25$^+$) were highly enriched in CD39$^+$ and CD73$^+$ cells. Furthermore, *in vitro* activated Tcons and CD8 T cells also contained increased frequencies of CD39$^+$ cells, as compared to unstimulated cells. The observed upregulation of ectonucleotidases on T cells upon activation suggests a possible mechanism of negative feedback regulation through auto- and/or paracrine adenosine signaling. In memory CD4 T cells, the frequency of CD39$^+$ and CD73$^+$ cells was found to be significantly higher than within naïve CD4 T cells. Memory CD8 T cells displayed an enrichment in CD39$^+$ cells, whereas the numbers of CD73$^+$ cells were similar in memory and naïve cell compartments. In addition, we found that IFN-γ-producing CD4 and CD8 T cells harbored increased frequencies of both CD39$^+$ and CD73$^+$ cells as compared to respective IFN-γ-negative populations. These data implied that CD39 and CD73 might mediate adenosine production by memory cells, thereby providing a mechanism of auto- and/or paracrine regulation to prevent an adverse memory T cell response.

Studying the role of ectonucleotidases in CD11b$^+$Gr1$^+$ myeloid-derived suppressor cells (MDSCs), we demonstrated that the majority of CD11b$^+$Ly6G$^+$Ly6C$^-$ granulocytic MDSCs

coexpressed CD39 and CD73. Importantly, the frequency of $CD73^+$ cells in granulocytic MDSCs was significantly higher than in immature $CD11b^+Gr1^+$ myeloid cells considered as a counterpart of MDSCs in healthy mice. In addition, we observed a substantial frequency of $CD73^+$ cells among monocytic MDSCs in pancreatic tumors. These data indicate that ectonucleotidase expression might contribute to the MDSC suppressive function in tumor-bearing hosts. Using the *ret* transgenic mouse model of melanoma, we showed that the administration of a CD73 inhibitor APCP or an A2a receptor antagonist SCH58261 prolonged the survival of tumor-bearing mice and reduced the MDSC infiltration into tumors, suggesting that this might be a promising strategy for cancer therapy.

Taken together, we found that CD39 and CD73 might be involved in the regulation of T cell activation and memory as well as in the activity of MDSCs, suggesting that agents targeting adenosine production and signaling might be instrumental in the development of novel strategies for tumor immunotherapy.

## Zusammenfassung

Adenosin ist ein kleines Molekül, das die Effektorzellen des Immunsystems supprimiert und die Aktivität von immunsupprimierenden Zellen verbessert. Dies geschieht durch die zweistufige Hydrolyse von ATP durch extrazelluläre CD39 und CD73 Ektonukleotidasen. Diese Enzyme sind in verschiedenen Populationen von Immunzellen sowie auf Tumorzellen exprimiert und sie können zur Immunsuppression beitragen, welche sich in der Tumor-Mikroumgebung entwickelt. Das Ziel der vorliegenden Arbeit war, die Rolle von CD39 und CD73 Ektonukleotidasen in der Regulation der anti-Tumor-Immunität in tumortragenden Organismen zu untersuchen. Hierzu wurden unterschiedliche Populationen von Immunzellen betrachtet. Wir nutzten *ret* transgene Mäuse, welche spontan Melanome entwickeln, und das orthotopen Panc02 Mausmodell duktaler Adenokarzinome des Pankreas. Beide Modelle ähneln stark der klinischen Situation im Patienten. Die Expression von Ektonukleotidasen auf verschiedenen Subpopulationen von Immunzellen wurde hinsichtlich ihres Phänotyps und ihrer Funktion analysiert.

Unsere Untersuchungen zeigten, dass CD39 und CD73 auf der Oberfläche von regulatorischen T-Zellen (Tregs) in Mäusen stark exprimiert sind. Darüber hinaus wurde die Expression von den Ektonukleotidasen auf $CD4^+FoxP3^-$ konventionellen T-Zellen (Tcons) und CD8 T-Zellen beobachtet. Die tumorinfiltrierenden Tcons und CD8 T-Zellen enthalten große Anteile von $CD39^+$ und $CD73^+$-Zellen (insbesondere im Panc02 Pankreaskarzinommodell). Dies lässt vermuten, dass die Expression von den Ektonukleotidasen auf T-Zellen durch die Tumor-Mikroumgebung beeinflusst werden könnte. Des Weiteren fanden wir, dass Tcons mit dem aktivierten Phänotyp ($CD25^+$) in $CD39^+$ und $CD73^+$-Zellen stark angereichert wurden. Außerdem enthielten *in vitro*-aktivierte Tcons und CD8 T-Zellen höhere Anteile von $CD39^+$-Zellen als unstimulierten Zellen. Die beobachtete Hochregulation der Ektonukleotidasen auf den aktivierten T-Zellen könnte einen Mechanismus der negativen Feedback-Regulation durch auto- und/oder parakrine Adenosineffekte darstellen. Der Anteil von den $CD39^+CD73^+$ Zellen in der Population der Gedächtnis-CD4-T-Zellen erwies sich als signifikant höher als in den naiven CD4 T-Zellen. Die Population der Gedächtnis CD8-T-Zellen zeigte eine starke Anreicherung an $CD39^+$-Zellen, obwohl der Anteil der $CD73^+$ Untergruppe ähnlich in naiven und Gedächtniszellen war. Darüber hinaus stellten wir fest, dass sowohl CD4 als auch CD8 IFN-γ-produzierende T-Zellen einen höheren Anteil von $CD39^+$ und $CD73^+$-Zellen aufweisen im Vergleich zu den jeweiligen IFN-γ-negativen Zellpopulationen. Diese Daten deuteten an, dass CD39 und CD73 auf Gedächtniszellen

Adenosinproduktion vermitteln könnten. Damit stellen unsere Daten einen neunen Mechanismus der auto-und / oder parakrinen Regulation dar, der eine mögliche schädliche Gedächtnis-T-Zell-Antwort verhindern könnte.

Unsere Untersuchungen bezüglich der Rolle der Ektonukleotidasen auf $CD11b^{+}Gr1^{+}$ Myeloiden Suppressorzellen (MDSCs) zeigten, dass die Mehrheit der $CD11b^{+}Ly6G^{+}Ly6C^{-}$ granulocytischen MDSCs CD39 und CD73 koexprimieren. Wichtig ist, dass der Anteil an $CD73^{+}$-Zellen unter granulocytischen MDSCs signifikant höher ist als unter unreifen $CD11b^{+}Gr1^{+}$myeloiden Zellen, welche in gesunden Mäusen als Gegenstück zu den MDSCs betrachtet werden. Darüber hinaus fanden wir einen wesentlichen Anteil an $CD73^{+}$-Zellen unter monozytischen MDSCs in den pankreatischen Tumoren. Diese Daten deuten darauf hin, dass die Expression der Ektonukleotidasen auf den MDSCs zur Immunsuppression in einem tumortragenden Organismus beitragen könnten. Mit dem *ret* transgenen Mausmodell des Melanoms gelang es uns zu zeigen, dass die Verabreichung eines CD73-Inhibitors oder eines A2a-Rezeptor-Antagonisten (SCH58261) das Überleben von tumortragenden Mäusen verlängerte und gleichzeitig die Infiltration von MDSCs in den Tumoren reduzierte. Diese Substanzen könnten somit vielversprechend für eine Krebstherapie sein.

Zusammenfassend ist festzuhalten, dass CD39 und CD73 sowohl in der Regulation der T-Zellaktivierung und des T-Zellgedächtnisses als auch in der Aktivität von MDSCs beteiligt sein könnten. Dies deutet darauf hin, dass die Regulation der Adenosinherstellung und -signalkaskade in der Entwicklung neuer Strategien für Tumorimmuntherapien hilfreich werden könnte.

## Abbreviations

**A**

| | |
|---|---|
| A2AR | type-2A Adenosine receptors |
| A2BR | type-2B Adenosine receptors |
| ACT | adoptive cell transfer |
| ADP | adenosine diphosphate |
| AMP | adenosine monophosphate |
| APC | allophycocyanin |
| APC | antigen-presenting cell |
| APCP | adenosine 5'-(α, β-methylene) diphosphate |
| ARG1 | arginase-1 |
| ATP | adenosine diphosphate |
| ATRA | all-trans-retinoic acid |

**B**

| | |
|---|---|
| BM | bone marrow |
| BSA | bovine serum albumine |

**C**

| | |
|---|---|
| cAMP | cyclic adenosine monophosphate |
| CCL | C-C chemokine ligand |
| CCR | C-C chemokine receptor |
| CD | cluster of differentiation |
| CNS1 | conserved non-coding sequence 1 |
| CTLA-4 | cytotoxic T lymphocyte antigen 4 |
| CTX | cytoxan (cyclophosphamide) |
| CXCL | C-X-C chemokine ligand |
| CXCR | C-X-C chemokine receptor |
| Cy | cyanin |

**D**

| | |
|---|---|
| DC | dendritic cell |
| DKFZ | Deutsches Krebsforschungszentrum |
| DNA | desoxyribonucleic acid |

**E**

| | |
|---|---|
| EDTA | ethylenediamine-tetra-acetic acid |

**F**

| | |
|---|---|
| FACS | fluorescence activated cell sorting |
| FBS | fetal bovine serum |
| FCS | fetal calf serum |
| FITC | flourescein-isothiocyanat |
| Foxp3 | forkhead box P3 (transcription factor) |
| FSC | forward scatter |

**G**

| | |
|---|---|
| GITR | glucocorticoid-induced tumor necrosis factor receptor-related protein |
| G-CSF | granulocyte colony-stimulating factor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |

**H**

| | |
|---|---|
| HER2/neu | Human Epidermal Growth Factor Receptor 2) also known as neu |
| HLA | human leukocyte antigen |
| HPV | human papilloma virus |

**I**

| | |
|---|---|
| IDO | indoleamine 2, 3-deoxigenase |
| IL | interleukin |
| IFN | interferon |

| | |
|---|---|
| IPEX | immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome |
| ITIM | immunoreceptor tyrosine-based inhibitory motif |

**K**

| | |
|---|---|
| KLH | keyhole limpet hemocyanin |

**L**

| | |
|---|---|
| LAG-3 | lymphocyte-activation gene 3 |
| LIN | lineage markers (CD3, CD14, CD19, CD20, CD56) |
| LN | lymph nodes |
| LPS | lypopolisaccharide |

**M**

| | |
|---|---|
| MART1 | melanoma antigen recognized by T cells 1 |
| M-CSF | Macrophage colony-stimulating factor |
| MDA | melanocyte differentiation antigen |
| MDSC | myeloid-derived suppressor cell |
| MHC | major histocompatibility complex |

**N**

| | |
|---|---|
| NDP | nucleoside diphosphate |
| NF-κB | nuclear factor kappa B |
| NK | natural killer cell |
| NKT | natural killer T cell |
| NO | nitric oxide |
| iNOS | inducible nitric oxide synthase |

**P**

| | |
|---|---|
| PBS | phosphate buffered saline |

| | |
|---|---|
| PD-1 | programmed death 1 |
| PDAC | pancreatic ductal adenocarcinoma |
| PDE-5 | phosphodiesterase-5 |
| PD-L1 | programmed death ligand 1 |
| PE | phycoerythrin |
| PerCP | peridinin-chlorophyll-protein complex |
| PMA | phorbol 12-myristate 13-acetate |
| PMEL | premelanosome protein |

**R**

| | |
|---|---|
| RBC | red blood cell |
| *ret* | human *ret* proto-oncogene |
| *ret* tg | *ret* transgenic mice without macroscopically visible tumors |
| *ret* tu | *ret* transgenic tumor-bearing mice |
| RNA | ribonucleic acid |
| siRNA | small interfering ribonucleic acid |
| ROS | reactive oxygen species |
| RPMI | Roswell Park Memorial Institute medium |

**S**

| | |
|---|---|
| SCF | stem cell factor |
| STAT | signal transducer and activator of transcription (a family of transcription factors) |
| SSC | side scatter |

**T**

| | |
|---|---|
| Tcon | conventional ($FoxP3^-$) CD4 T cells |
| TCR | T cell receptor |
| Teff | effector T cells (Tcons and CD8 T cells) |
| TGF-β | transforming growth factor beta |
| Th | T-helper cell |
| TIGIT | T cell immunoreceptor with Ig and ITIM domains protein |

| | |
|---|---|
| TIL | tumor infiltrating lymphocyte |
| TIM-3 | T cell immunoglobulin and mucin domain-containing protein 3 |
| Tr1 | type-1 regulatory T cells |
| Treg | regulatory T cell |
| iTreg | induced regulatory T cell |
| nTreg | naturally occurring regulatory T cell |
| TRP | tyrosinase related protein |

**V**

| | |
|---|---|
| VEGF | vascular endothelial growth factor |
| VEGFR1 | vascular endothelial growth factor receptor 1 |

# 1. Introduction

The major role of the immune system in the host protection from malignancies has been progressively recognized over the last decades. Multiple studies in humans and animal models demonstrated a vigorous activation of innate and adaptive immunity in response to tumor growth (1). This is highlighted by the correlation between the tumor infiltration with leukocytes and the prognosis (1-4). However, although tumors often attract immune cells capable of recognizing and destroying tumor cells, establishment of chronic inflammation and immunosuppressive network hinders tumor eradication (5). The outcome of tumorigenesis is thus largely determined by the balance between anti-tumor immunity and numerous mechanisms of immune escape developed by the tumor (1). One of the main factors of tumor-associated immune suppression is the activation and recruitment of inhibitory immune cells, e.g. regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Over the last years, insights into the mechanisms of tumor-associated immune suppression led to the development of novel immunotherapeutic strategies that can significantly extend the survival of cancer patients (6).

## 1.1 T cells in cancer

Multiple genetic and epigenetic alterations in tumor cells provide a broad diversity of antigens that can be recognized by cells of the adaptive immune system (6). In the periphery, naïve T cells continuously sample antigens in the context of Major Histocompatibility Complex protein (MHC) until they encounter their cognate antigen, which, in the presence of costimulation, leads to their activation through T cell receptors (TCRs). In particular, tumor-specific T cells can become activated upon their stimulation with tumor-associated antigens presented by "professional" antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages and B cells. However, antigen-experienced T cells are capable of directly recognizing antigens bound to MHC class I (MHC-I) on tumor cells (7).

Tumor antigen-specific CD8 T cells (which are MHC-I-restricted) can, in principle, directly recognize different types of tumor cells, since MHC-I is ubiquitously expressed by virtually all nucleated cells. However, malignant cells often downregulate MHC-I expression (1, 7, 8).

Professional APCs uptake, process and present tumor antigens to T cells in the context of MHC-II or MHC-I (in the latter case, through a mechanism termed cross-priming) (7). In many cases, CD4 T cells are unable to recognize cancer cells directly due to the lack of MHC-II on most solid

cancers (9). Hematological malignancies represent an exception, since B and T cells can express MHC-II. However, in certain cases, solid cancers (e.g. over 40 % of melanomas) can also constitutively express MHC-II, (10).

Upon migration to the tumor site, T cells can produce cytokines, chemokines and anti-angiogenic factors that affect tumor growth (1, 7). T cells may also exert direct cytotoxicity against tumor cells either through apoptosis-inducing molecules or through the release of cytotoxic granules (7).

Mature CD8 T cells and certain subpopulations of CD4 T cells produce interferon-γ (IFNγ) and tumor necrosis factor (TNF)-α, which can stimulate the immune response by upregulating the expression of MHC class I and II molecules on both tumor cells and APCs (1, 7). CD4 T cells can orchestrate many aspects of innate and adaptive immunity, including the function of cytotoxic CD8 T cells (1, 7). They can also engage and 'license' APCs, which in turn recruit additional T cells and promote the activation of the innate immune system (7, 9).

Thus, one of the greatest challenges in the field of immunotherapy is the identification of tumor antigens that can be used to redirect the immune system against the tumor with minimal side effects in patients (11).

### 1.1.1 Tumor-specific T cells and adoptive T cell therapy

Tumor-specific T cells can recognize differentiation and germline antigens, products of mutated genes, as well as viral antigens (7). In melanoma patients, it has been shown that tumor-infiltrating lymphocytes (TILs) can be isolated from tumors and expanded *ex vivo* in the presence of interleukin-2 (IL-2). Upon reinfusion, the expanded cells can traffic to the tumor and, in some cases, cause tumor regression (7, 12). Such therapy is called adoptive cell transfer (ACT). Usually, it is preceded by so-called „preparative lymphodepletion“(temporary ablation of lymphoid cells). The latter can be achieved either through chemotherapy alone or in combination with total-body irradiation. The combination of preparative lymphodepletion with ACT and administration of IL-2 can lead to tumor eradication in patients with metastatic melanoma (or other tumor entities, including leukemia and synovial cell sarcoma) who failed to benefit from other treatment modalities (7, 9, 12, 13).

Transformed cells often over-express respective tissue-specific differentiation antigens (7). This offers an opportunity to use those antigens as targets for ACT or vaccination (14, 15). However,

such therapies might entail severe adverse effects, since the targeted antigens are expressed in healthy cells and tissues as well (15). For example, patients who have responded to immunotherapies targeting CD19 on B cell lymphomas also experience long-term depletion of normal polyclonal $CD19^+$ B-lineage cells (16).

Melanocyte differentiation antigens (MDAs) - such as PMEL (also known as gp100), melanoma antigen recognized by T cells 1 (MART-1), tyrosinase, tyrosinase-related protein 1 (TRP-1) and TRP-2 - are expressed in most melanoma lesions. Importantly, they are also present in normal melanocytes in the *stratum basale* of the skin, in the retinal pigmented epithelium and in the inner ear, where they are associated with melanin production (15). Early antigen-cloning studies suggested that self-MDAs could be promising targets for T cell-based therapy (17). However, T cells with gene-engineered TCRs specific for PMEL or MART-1 caused a destruction of normal melanocytes in the skin, eyes and ears of treated patients (18). Objective clinical responses were observed in 9 out of 36 (25%) patients treated in these trials, but only one patient showed a complete response to the treatment. In contrast, TILs usually cause no apparent toxicity, although they have TCRs specific for the same self-antigens (19). Recent data on the melanoma exome indicated the presence of a large number of mutational changes (20, 21), which allows targeting non-synonymous mutations that result in the creation of new epitopes. It has been shown that tumor-specific antigens harboring particular point mutations can be recognized by tumor-infiltrating effector T cells from melanoma patients (22).

Certain tumors caused by transforming viruses can express viral products, which represent attractive targets, since they are absent from normal tissues. In particular, it is possible to target oncogenic proteins encoded by cancer-associated viruses such as those derived from Epstein–Barr virus and human papillomavirus (HPV) (7).

In some tumor entities, epigenetic alterations can trigger expression of non-mutated proteins termed cancer germline antigens or cancer-testis antigens (8, 23). These antigens are normally expressed in germline cells in the testes and fetal ovaries. Genes encoding cancer-testis antigens are silent in normal non-germline tissues but can be activated in many types of tumors (23). Importantly, T cells do not recognize these antigens on male germline cells because these cells lack MHC molecules (24). Tumors commonly expressing cancer testis antigens include

melanomas, lung carcinomas as well as head and neck, oesophagus and bladder cancers (25). Therefore, cancer testis antigens are among the most attractive targets for cancer immunotherapy.

### 1.1.2 Role of T cell subsets in anti-tumor immunity

Recent studies focusing on the clinical impact of TILs in solid tumors (the frequencies of CD3, CD4, CD8, FoxP3 and ratios between them) showed that the presence of CD3, CD8, as well as a high CD8/FoxP3 ratio are indicative of good prognosis (9, 26). In addition, the density and distribution of TILs were shown to independently predict the sentinel lymph node status and survival in patients with melanoma (27). Studies by Fridman et al. (reviewed in (28)) showed that both $CD8^+CD45RO^+$ memory cells and IFN-γ-producing T helper type 1 (Th1) cells represent positive prognostic factors in the vast majority of tumor entities. Moreover, Galon et al. (29) have shown that $CD8^+CD45RO^+$ T cells in the core of the tumor represent a superior prognostic factor compared to the conventional clinical staging in colorectal cancer. Strikingly, low frequencies of T cells in the tumor core indicated a very poor prognosis even in patients with localized disease, similar to that of patients with concomitant distant metastasis. This suggested a particular clinical importance of CD8 memory T cells at the tumor site (9).

On the other hand, suppressor CD8 T cells detected at quite high frequencies in cancer patients display strong suppressive properties (30). The lack of surface markers allowing immunohistochemical detection of these cells in biopsies complicates the interpretation of clinical data on CD8 T cell infiltration in tumors (9).

The data on CD4 T cells are more ambiguous, which could be due to the higher plasticity and diversity of CD4 T cell subpopulations, which can sometimes play opposing roles (9). Moreover, tumor-specific effector CD4 T cells can be converted into immunosuppressive $CD25^+FoxP3^+$ Tregs (31). In addition, certain organs harbor large numbers of Tregs, which underscores the role of the originating tissue in the immune response against cancer (9). Recently, multiple studies showed that a high CD8/Treg ratio can serve as a positive prognostic factor in many malignancies (9). Thus, despite the fact that FoxP3 is not solely expressed by Tregs, these data suggest a predominant expression of FoxP3 in cells with a pro-tumor activity.

### 1.1.3 Phenotypes of tumor infiltrating lymphocytes and the manipulation of immune checkpoints

Tumor-infiltrating T cells show a remarkable phenotipic diversity (32). It has been demonstrated that tumor-infiltrating CD8 T cells display a higher expression of exhaustion markers than CD8 T cells in the peripheral blood or surrounding normal tissue (9). In melanomas, tumor-infiltrating CD8 T cells express the Programmed Death-1 (PD-1) and Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) (often simultaneously) at high levels (7). These two molecules represent major immune checkpoints, and the activation of respective signaling pathways leads to a profound inhibition of T cell activation, proliferation and function (6).

CTLA-4 competes primarily with CD28 (a T cell co-stimulatory receptor) for their common ligands CD80 (also known as B7.1) and CD86 (also known as B7.2) (6). Upon antigen recognition, CD28 engagement by CD80 and CD86 provides the essential second signal for naive T cell activation (6). Since CTLA-4 has a much higher overall affinity for both ligands, it has been proposed that its expression on the surface of T cells dampens the activation of T cells by outcompeting CD28 in binding CD80 and CD86, as well as by actively delivering inhibitory signals to the T cell (6). Normally, CTLA-4 expression is induced in T cells upon their initial exposure to antigen. The level of CTLA-4 upregulation is largely determined by the intensity of the initial TCR-mediated signaling (6). High-affinity ligands induce higher levels of CTLA-4, which restricts the amplitude of the initial response. Naive and memory T cells express high levels of cell surface CD28 (6). On the contrary, CTLA-4 remains sequestered in intracellular vesicles. Once the TCR is triggered by antigen encounter, CTLA-4 is transported to the cell surface, in amounts proportional to the strength of TCR (and CD28) stimulation (6). Therefore, CTLA-4 provides a negative feedback loop to regulate T cell activation after the TCR stimulation with ligands of varying concentrations and affinities.

In contrast to CTLA-4, the major function of PD-1 is to restrain the activity of T cells in peripheral tissues during an inflammatory response to infection and to prevent autoimmunity (6). This turns into a major immunosuppressive pathway in cancer. Activated T cells upregulate PD-1 and continue to express it in tissues (6). Chronic antigen exposure, which is common in chronic viral infections and cancer, often entails high expression of PD-1 (6). This causes a state of exhaustion or anergy among antigen-specific T cells (6). Similarly to CTLA-4, PD-1 can shift the

balance from T cell activation to tolerance at the early stages of T cell responses to antigens within secondary lymphoid tissues (6).

Tumor-infiltrating CD4 T cells also show high expression of PD-1 and CTLA-4 (9). The PD-$1^+$ fraction of TILs displayed impaired effector functions (6). The immune regulatory checkpoints PD-1 and CTLA-4 play a major role in the exhausted phenotype of TILs, and the blockade of these molecules has been shown to increase not only T cell effector functions but also their infiltration into tumor lesions (6). PD-L1 expression has been correlated with a decreased CD8 infiltration, but the correlation with the overall survival or disease progression remains controversial and may differ with regard to different malignancies (33, 34). However, there is clear association between the blocking of these immune-blockade pathways, increased T-cell functionality, increased tumor infiltration and the clinical effect of these novel immunotherapies (9).

### 1.2 Regulatory T cells

Under physiological conditions, regulatory CD4 T cells are characterized by the expression of the forkhead box P3 (Foxp3) transcription factor and interleukin-2 (IL-2) receptor α-chain (CD25) and serve to prevent adverse immune reactions against self, food and environmental antigens (35). Mice with a loss-of-function mutation in Foxp3 lack functional Tregs and die of multi-organ autoimmune and lymphoproliferative reactions early in life (35). In humans, hypomorphic mutations in FOXP3 causes similar pathology termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (36). Numerous studies have linked the Treg accumulation in tumors and peripheral blood of cancer patients to poor prognosis, making Tregs an attractive target for cancer immunotherapy (28, 37). Studies in animal models showed that Treg depletion or inhibition of Treg suppressive mechanisms can restore anti-tumor immunity, eventually leading to a decreased tumor growth or even tumor regression (4, 38).

However, many recent publications challenged this simplistic view of Tregs as a major pro-tumorigenic cell population in the tumor microenvironment. First, a variety of other cells has been implicated in the negative regulation of anti-tumor immune responses (39, 40). Second, it has been established that in certain malignancies, Treg accumulation can diminish tumorigenic inflammation and serve as a positive prognostic factor (41-43). Third, Tregs exhibit a significant phenotypical and functional heterogeneity with different subsets showing a distinct suppressive

efficacy and exploiting distinct suppressive mechanisms (44). Finally, Treg phenotype and function strongly differ in various tumor entities, underlining the necessity to investigate the specific role of Tregs in particular malignancies (45).

### 1.2.1. Treg development: naturally occurring and induced Tregs

During thymic differentiation, T cell receptor (TCR) signals of intermediate strength (together with IL-2 signaling) enable a number of CD4 single-positive thymocytes to escape deletion (35). This population is enriched for cells instructed to differentiate into naturally occurring $Foxp3^+$ Treg cells (nTregs) (35). While the development of nTreg cells in the thymus is promoted by increased affinity interactions with self-peptide-MHC complexes, the conversion of conventional $CD4^+CD25^-FoxP3^-$ T cells (Tcons) into iTregs occurs in the periphery as a result of high-affinity TCR signaling together with suboptimal costimulation in the presence of transforming growth factor (TGF)-β and IL-2 (35, 46). These cells can arise in the response to stimulation with foreign as well as tumor-associated self- or altered self-antigens under tolerogenic conditions (35, 46). The TCR repertoire of iTregs largely differs from that of nTregs (35). In particular, it has been suggested that iTreg often descend from Tcons recognizing antigens from the environment or commensal microbiota (47-50). This implies that iTregs play an important role in the post-thymic education of the immune system and adaptive tolerance (50).

The TGF-β signaling is essential for the induction of Foxp3 in peripheral CD4 T cells (35). IL-2 is required for the TGF-β-mediated induction of Foxp3 in peripheral T cells *in vitro* (35, 46, 51). IL-2 activates the Foxp3 locus through STAT5 and enhances the viability and proliferation rate of target cells in the presence of TGFβ (52). IL-2 also inhibits the development of T helper 17 (Th17) cells (53). Moreover, IL-2 is indispensable for the homeostasis of fully differentiated Treg cells (54).

Type 1 regulatory T cells (Tr1), characterized by IL-10 production, can be induced by the suboptimal activation and TGF-β signaling (55, 56). However, the Tr1 differentiation requires high IL-10 levels and they might be $Foxp3^-$ (55, 57). These data indicate that iTregs and Tr1 may represent competing and alternative cell lineages. It has been proposed that tumor antigen-specific nTregs and iTregs contribute to the pool of tumor-infiltrating Tregs (58). Moreover, the Treg stimulation with tumor antigens may trigger vigorous proliferation of both nTregs and iTregs, significantly increasing their numbers in the tumor (59). Recently, Rudensky's group

generated mice with a deleted CNS1-element, an intronic Foxp3 enhancer, which is essential for the differentiation of iTreg but not nTreg cells (60). This model has already been applied to determine the role of iTregs in mucosal Th2 inflammation (61) and maternal-foetal conflict (62).

### 1.2.2 Treg phenotype and identification

In mice, Foxp3 appears to be a necessary and sufficient marker for the identification of *bona fide* Tregs (35), although it has been detected in a minor fraction of recently activated Tcons (63). However, in humans, FOXP3 is expressed in a significant proportion of activated Tcons (36), which requires the use of additional markers for an accurate analysis of human Tregs. Tregs are routinely defined and isolated based on the surface expression of CD25, although it can also be expressed on activated Tcon (55). Therefore, much attention has been focused on finding further markers that would allow a robust Treg detection and sorting. For instance, several studies suggested that IL-7-receptor, CD127 is expressed on conventional but not on regulatory T cells (64). However, CD127 is not an ideal biomarker for the identification of Tregs. First, some early activated Tcon also downregulate the CD127 expression (65). Second, activated Tregs can be $CD127^{+}$ (66). Another study showed that the integrin CD49d is present on most pro-inflammatory $CD25^{high}$ cells but virtually absent in the Treg compartment (67); it can be used in conjunction with CD127 to improve the purity of sorted Treg preparations. Indeed, sorting of $CD25^{high}CD127^{low/-}CD49d^{-}$ Tregs, with subsequent analysis of FoxP3 expression is now becoming a standard method for human Treg isolation (68).

### 1.2.3 Mechanisms of suppression

Tregs inhibit the activity of various immune cell types, e.g. CD4 and CD8 T cells, B cells, DCs), natural killer (NK) and NKT cells both *in vitro* and *in vivo* (31, 35). *In vitro*, Tregs show hyporesponsiveness, i.e. low proliferation rate in the response to stimulation (36, 57). However, a significant proportion of Tregs exhibit a vigorous homeostatic proliferation *in vivo*, probably reflecting a high turnover rate or representing a consequence of recurrent interactions with cognate antigens (45). Tregs inhibit immune responses in an antigen-unspecific manner but their suppressive function is activated upon antigen-specific stimulation (69).

Treg suppressive effects are mediated by a wide variety of mechanisms. These include surface expression of inhibitory or cell-death-inducing molecules, secretion of immunosuppressive cytokines, production of inhibitory molecules and direct cytolysis. Tregs express high levels of

CD25 (IL-2 receptor α-chain) and thus can compete with effector T cells (Teff) for IL-2 and hamper their proliferation (70). The inhibitory co-receptor CTLA-4 is constitutively expressed on Tregs at high levels and plays an important role in the Treg suppressive function (71). CTLA-4 interaction with co-stimulatory molecules B7-1 and B7-2 on DC leads to the upregulation of the suppressive enzyme indoleamine 2, 3-dioxygenase (IDO) in antigen-presenting cells (APC), and the subsequent suppression of T cell activation and function (72). Moreover, IDO itself is a strong inducer of Treg differentiation (73).

There are several other surface molecules that Tregs use to inhibit the APC activity. For instance, LAG-3, a CD4 homolog and a high-affinity ligand of MHC class II, contributes to the suppressive activity of both nTregs and iTregs (74). Ligation of MHC on DCs through LAG-3 leads to the inhibition of their maturation and function (75). A recently discovered protein TIGIT is highly expressed on Tregs and activated Tcons, representing another example of Treg-mediated control of the DC activity. During Treg-DC interactions, TIGIT induces IL-10 and TGF-β production by DC (76). Besides contact-dependent immune suppression, Tregs can also elaborate a variety of suppressive cytokines, most notably TGFβ and IL-10, which proved to be essential for the Treg homeostasis and function *in vivo* (77-79).

Several mechanisms of the Treg-mediated immune suppression involve production of molecules disrupting metabolic pathways and signaling in target cells. For instance, Tregs can transfer the second messenger cyclic AMP (cAMP) into target cells through gap junctions, which dampens the proliferation and activity of target cells (80). Another mechanism leading to the cAMP elevation in effector cells is the Treg-mediated adenosine production through the enzymatic activity of cell-surface enzymes CD39 and CD73 (81). These proteins can convert pro-inflammatory ATP into immunosuppressive adenosine in a two-step process: CD39 hydrolyses ATP or ADP into AMP, and CD73, in turn, cleaves AMP yielding adenosine (82). However, extracellular enzymes adenylate kinase and nucleoside diphosphate (NDP) kinase can phosphorylate AMP and ADP respectively, thus antagonizing CD39 activity (83). In contrast, adenosine can only be phosphorylated inside the cell by adenosine kinase, although it can also be inactivated in the extracellular space by adenosine deaminase. Remarkably, despite the necessity of CD73 for adenosine synthesis under physiological conditions, CD73-deficiency is usually complemented by compensatory overproduction of alkaline phosphatases (83).

Adenosine activates four distinct G-protein-coupled receptors: A1, A2A, A2B, and A3 (82). The A1 and A3 adenosine receptors are coupled to the Gi/o subunit, and their activation leads to the inhibition of adenylate cyclase, cAMP production, and protein kinase A activation. An engagement of these receptors also activates the phosphatidylinositol 3-kinase pathway (82). Activation of A2A and A2B largely accounts for immunosuppressive effects of adenosine. These receptors are coupled to the Gs subunit that activates adenylate cyclase, thus leading to enhanced cAMP synthesis and subsequent inhibitory effects on target cells (81). However, in contrast to other adenosine receptors, A2B is a low-affinity receptor and can only be activated at high levels of adenosine generated in response to pathological conditions (82).

A2a receptors (A2AR) are expressed on a variety of immune cells (monocytes/macrophages, granulocytes, lymphocytes, DCs and NK cells) (82, 84). The A2AR-triggered cAMP elevation inhibits effector functions of all these cell populations. In particular, adenosine impedes the phagocytic capacity of macrophages, degranulation of neutrophils and IFN-γ production by NK-cells (82). It also drives DC differentiation towards a population producing immune suppressive factors, such as TGF-β, prostaglandin E2, IDO etc. (85). An adenosine-mediated cAMP accumulation and inhibition of the NF-κB pathway (86) in T cells hinders their activation, proliferation, survival and effector functions (87, 88).

Adenosine production strongly contributes to the Treg immunosuppressive activity (83, 89). Tregs from CD39-deficient mice are constantly activated but fail to suppress effector T cell proliferation (88). Furthermore, in CD73-deficient mice, activated CD4 T cells show an enhanced production of pro-inflammatory cytokines (IFN-γ, IL-2 and TNF-α), and Tregs completely lack their suppressive activity (90). Therefore, it was hypothesized that CD73-derived adenosine ensures a tonic inhibition of NF-κB in CD4 T-cells, thereby restricting the development of effector T cell responses (90). CD39 and CD73 on Tregs play an important role in regulating the balance between the pro-inflammatory ATP and immunosuppressive adenosine. ATP can accumulate outside the cell both under normal and pathological conditions. In the context of inflammation and cancer, ATP is released by activated leukocytes, dying, injured or stressed cells (83).

The effects of ATP-mediated signaling are concentration-dependent. High ATP concentrations activate NLRP3 inflammasome through P2X7 receptors, which leads to the activation of caspases

and cell death (91). Low ATP concentrations can trigger the influx of calcium into the cytoplasm or inhibit the synthesis of immunosuppressive cAMP through various P2Y receptors (92). Thus, the regulation of the ratio between extracellular ATP and adenosine by CD39 and CD73 on Tregs provides a means of tipping the balance between an immune activation and suppression. However, the role of CD73 in the human immune system appears to differ significantly from that in animal models. Importantly, while murine Tregs stably express CD73 on the cell surface, in human Tregs, CD73 can only be detected inside the cell (93). This challenges the notion that human Tregs can mediate the whole cascade of ATP conversion into adenosine, thereby suggesting that other cell populations might be involved in catalyzing AMP dephosphorylation.

Another means of Treg-mediated immune suppression is direct cytotoxic activity against effector immune cells (57). Tregs can release cytolytic molecules such as granzyme A/B, perforin or activate the tumor necrosis factor related apoptosis inducing ligand-death receptor-5 pathway.

Thus, Tregs deploy a wide variety of mechanisms to control the effector arm of immunity. This paves the way for the development of numerous strategies to alleviate Treg suppressor function in cancer patients aiming to improve the anti-tumor immune responses.

### 1.2.4 Therapeutic targeting of Tregs

Current strategies to target Tregs in cancer include Treg depletion as well as a blockade of the Treg immunosuppressive activity and migration. Treg depletion has been widely used to restore the anti-tumor immunity and to improve the efficacy of vaccination and adoptive cellular immunotherapy in cancer patients and tumor-bearing mice (38). Depletion agents used in clinical and preclinical studies include low-dose chemotherapeutics, anti-CD25 antibody (daclizumab), IL-2-difteria toxin fusion protein (denileukin diftitox, ONTAK) and CD25-targeting immunotoxins (45, 94). However, current depletion strategies do not allow a specific Treg targeting since the latter share CD25 expression with activated Tcons, and their high proliferative activity makes them sensitive to chemotherapy (45). However, these approaches proved instrumental when Tregs significantly outnumber activated Tcons in the tumor microenvironment. Therefore, Treg depletion might restore the activation, migration, proliferation and activity of remaining Tcons (38).

#### 1.2.4.1 Low-dose chemotherapy

The high proliferation rate of Tregs makes them more sensitive to chemotherapy than effector T cells (45). Therefore, certain chemotherapeutic regimens might lead to a selective Treg depletion. This was confirmed by studies, showing that anti-tumor effects of certain chemotherapeutic agents have distinct mechanisms, depending on the dose regimen (95). Metronomic (low-dose) cyclophosphamide (CTX) administration have been shown to improve an anti-tumor immunity and vaccination efficacy through Treg depletion, whereas high-dose regimens exerted direct cytolytic effects on tumor cells, causing an immunosuppression (95).

The results of an early study by Polak and Turk (96) indicated that CTX might reverse the immune tolerance by targeting a yet unidentified suppressive T-cell population. Further investigations confirmed this hypothesis. Machiels et al. (97) showed that immune-modulating doses of CTX, doxorubicin, and paclitaxel can boost anti-tumor effects of HER-2/neu-expressing whole cell vaccines and reduce mammary tumor growth in *neu* transgenic mice. The tolerance to the *neu* transgene observed in these mice recapitulates irresponsiveness to tumor antigens in cancer patients. The administration of CTX at 50-150 mg/kg one day before vaccination was superior to CTX or vaccination alone. The reversed order of treatment proved to be ineffective. Moreover, higher doses of CTX were rather detrimental for the vaccination efficacy. Metronomic CTX treatment resulted in an augmented Th1 HER-2/neu-specific T cell response, indicating the reversal of tolerance.

In a rat model of colon carcinoma, Ghiringhelli et al. (98) demonstrated that CTX reduces Treg numbers, leading to a restoration of peripheral T cell proliferation and innate killing activities. A single administration of CTX at 25 to 30 mg/kg depleted $CD4^+CD25^+$ T cells while sparing other lymphocytes, delayed tumor growth, and led to a complete tumor regression when followed by immunotherapy that had no curative effect when administered alone. Later, it was shown that metronomic CTX could also selectively deplete $CD4^+CD25^+FOXP3^+$ Tregs in advanced cancer patients. In a study reported by Ge et al. (99), daily oral administration of 50 mg cyclophosphamide in patients with treatment-refractory metastatic breast cancer initially caused a depletion of more than 40% of Treg in the peripheral blood. However, during the treatment, Treg numbers gradually recovered. Nevertheless, the transient Treg depletion allowed an expansion of tumor antigen-specific T cells that persisted during the whole period of treatment. Notably,

numbers of tumor-reactive T effectors but not Tregs correlated with the disease stabilization and increased overall survival.

Preclinical studies showed that low-dose CTX also decreases the functional activity of Treg cells. Lutsiak et al. (100) compared Tregs from cyclophosphamide-treated and control mice 2 and 10 days after the treatment. Tregs from CTX-treated animals had a significantly diminished suppressive capacity, which was restored by day 10 upon the treatment.

In a recent phase II randomized trial (101), Treg numbers, phenotype and function were rigorously monitored in the lymph nodes and peripheral blood of stage II-III melanoma patients vaccinated with HLA-A*0201-modified tumor peptides in conjunction with the low-dose CTX and low-dose IL-2 administration. CTX slightly decreased the number of circulating Tregs in the vaccinated patients followed by a complete recovery of Treg numbers despite the administration of additional CTX doses. However, the treatment resulted in a substantial reduction in Treg numbers as well as IL-10 and TGFβ production in regional lymph nodes. Interestingly, the frequency of activated Tregs was lower in the LNs of patients treated with CTX compared with that in the LN of control patients. This observation is in agreement with the preclinical data indicating that CTX selectively depletes cycling and effector/memory Tregs with enhanced tumor-homing activity (102, 103).

#### 1.2.4.2 Treg depletion by CD25-targeting agents

Several groups showed that prophylactic Treg depletion with anti-CD25 mAb in mice triggered the rejection of syngeneic tumors (104, 105), although such treatment after the tumor inoculation was less effective. However, tumor rejection was substantially facilitated when the anti-CD25 mAb administration was combined with the DC vaccination (106, 107), CTLA-4 blockade (108) or GITR stimulation (57, 109). Since CD25 is expressed not only on Tregs but also on activated CD4 and CD8 T cells, anti-CD25 therapy may entail concomitant deletion of effector T cells. Accordingly, in B16 melanoma model, it was observed that although $CD25^+$ T cell depletion before vaccination enhanced the treatment efficacy, the administration of anti-CD25 mAbs before a rechallenge in pre-vaccinated mice significantly diminished the protective effect of vaccination (106, 108).

In a clinical study, Jacobs et al. (110) evaluated the impact of anti-CD25 mAb treatment on the vaccination efficacy. Thirty HLA-$A2.1^+$ metastatic melanoma patients were vaccinated with

mature DC loaded with the tumor peptide and keyhole limpet hemocyanin (KLH). Half of the patients were pretreated with daclizumab, a humanized blocking antibody against CD25. Daclizumab efficiently depleted all circulating $CD25^{high}$ leukocytes (including $CD4^{+}FoxP3^{+}CD25^{high}$ cells) within four days after daclizumab injection. The presence of daclizumab during DC vaccinations prevented the induction of specific antibodies but not the presence of antigen-specific T cells. However, these $CD25^{+}$ T cells did not acquire effector functions. Daclizumab pretreatment had no significant effect on the progression-free survival as compared to that in the control group. A more recent study by Rech et al. (111) showed that daclizumab did not act through antibody-dependent or complement-mediated cytotoxicity but rather repressed FoxP3 expression selectively in $CD25^{high}CD45RA^{-}$ Tregs. Morevover, daclizumab-treated $CD45RA^{-}$ Tregs lost suppressive function and acquired the ability to produce interferon-γ, indicating their reprogramming towards Th1 lineage. In a clinical trial monitored by the same group (111), daclizumab was administered in combination with an experimental cancer vaccine in patients with metastatic breast cancer. The treatment led to a substantial and prolonged decrease in Tregs. An effective CD8 and CD4 T cell priming and responsiveness to all vaccine antigens were observed in the absence of autoimmune reactions.

Another CD25-targeting agent, denileukin diftitox (ONTAK) was initially approved as an anti-neoplastic agent for the treatment of cutaneous T cell lymphoma (94). Denileukin diftitox consists of a fragment of diphtheria toxin fused to IL-2. Upon internalization, diphtheria toxin irreversibly inhibits protein synthesis, ultimately triggering cell death. In patients with renal cell carcinoma, ONTAK significantly reduced circulating Treg numbers and promoted the expansion of CD4 and CD8 T cells producing IFN-γ after the DC vaccination (112). However, clinical studies of ONTAK in patients with melanoma showed a variable efficacy in the Treg depletion and contradictory data on the clinical outcome. For instance, the ONTAK administration altered neither FoxP3 mRNA levels in CD4 T cells nor the suppressive activity of $CD4^{+}CD25^{+}$ T cells (113). None of the patients showed an objective clinical response. In contrast, Telang et al. (114) showed that ONTAK caused a transient depletion of Tregs, thereby promoting melanoma antigen-specific CD8 T cells. Moreover, in a phase II clinical trial, ONTAK treatment resulted in a partial response in 16.7% of totally 60 patients and markedly increased the one-year survival in the cohort of partial responders as compared to patients with the progressive disease (114).

Two clinical trials explored the administration of immunotoxins linked to CD25 antibodies: RFT5-SMPT-dgIgA, CD25-specific murine antibody linked to a deglycosylated ricin A chain (dgA) (115), and LMB-2, a fusion of a single-chain Fγ fragment of the CD25-specific, anti-Tac monoclonal antibody to a truncated form of the bacterial pseudomonas exotoxin A (116). The administration of RFT5-SMPT-dgA to six patients with metastatic melanoma led to a transient but consistent reduction in the number of $CD25^{high}$ CD4 T cells *in vivo* (115). However, the decrease in total $FoxP3^+$ CD4 T cell numbers was not so drastic since the $CD25^{low/-}FoxP3^+$ subpopulation of CD4 T cells escaped selectively the depletion and persisted at stable numbers. No objective clinical responses were observed. In another study, eight patients with metastatic melanoma received LMB-2 followed by the MART-1 and gp100-specific peptide vaccination (116). The LMB-2 administration resulted in a preferential, transient reduction (up to 79.1%) in circulating $CD25^+$ CD4 T cell numbers. $FoxP3^+$ CD4 Treg cells that escaped LMB-2-mediated depletion were $CD25^{low/-}$. However, despite the decrease in Treg numbers, LMB-2 therapy failed to improve the immune and clinical responses.

Thus, above mentioned Treg-depleting strategies transiently reduce the Treg numbers and function in the peripheral blood of some cancer patients. However, Tregs come back and the blocking of their activity becomes again necessary.

#### 1.2.4.3 FoxP3 vaccination

Since FoxP3 is a "master regulator" transcription factor of Treg development and function, the vaccination against FoxP3 might lead to efficient Treg depletion, representing an interesting approach to neutralize the tumor-associated immune suppression. In the B16 melanoma model, it was shown that the vaccination with FoxP3 mRNA leads to a local intratumoral but not systemic depletion of $FoxP3^+$ cells (106). Nevertheless, the paucity of tumor Treg was associated with the improved anti-tumor reactivity of TRP-2-specific cytotoxic T lymphocytes after an additional vaccination with TRP-2-loaded DC. The mechanisms of this preferential depletion of tumor Treg remain obscure.

#### 1.2.4.4 CTLA-4

Similar to many other markers, CTLA-4 expression is shared by regulatory and activated T cells (57). There have been some conflicting data on the specific role of CTLA-4 expression in either subset. Mice with Treg-specific CTLA-4 knockout succumb to lymphoproliferation and

autoimmune diseases similar to Foxp3 deficiency (117). Nude mice reconstituted with splenocytes with CTLA-4-deficient Tregs showed an enhanced survival and decreased tumor growth as compared to the recipients of control splenocytes (117). On the other hand, it has been demonstrated (118) that although antibody-mediated CTLA-4 blockade on T effector cells significantly improved the survival of mice with B16 melanoma, a specific blockade of CTLA-4 expressed on Tregs alone had no antitumor effects. The maximal prolongation of survival was achieved by concomitant CTLA-4 blockade in both regulatory and effector T cells. In stage IV melanoma patients, the therapy with the anti-CTLA-4 antibody ipilimumab reduced Treg numbers and restored TCR-dependent proliferation of effector T cells (98). Importantly, a randomised trial in a large cohort of patients with previously treated metastatic melanoma (119) showed a significantly increased overall survival upon the ipilimumab treatment.

#### 1.2.4.5 GITR

Glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) is a co-stimulatory molecule constitutively expressed on Tregs (45). It is also expressed at lower levels on resting effector T cells and is upregulated upon the T cell activation (38). GITR stimulation by agonistic antibodies or GITR ligands leads to the suppression of Treg activity and augmented proliferation of effector T cells (45). Administration of anti-GITR mAbs elicited a concomitant immunity in B16 melanoma-bearing mice (105). Moreover, GITR activation triggered the tumor regression in mice with methylcholanthrene-induced fibrosarcoma (120). In contrast to most depletion strategies, anti-GITR mAb therapy showed higher efficacy in mice with established tumors than in prophylactic settings (120). In addition, combination of GITR activation with the tumor antigen stimulation promotes tumor antigen-specific effector T cells (121). Thus, GITR represents an attractive target for cancer immunotherapy.

#### 1.2.4.6 OX40

OX40 (CD134) is a costimulatory receptor expressed on Treg and activated Teff (45). OX40 ligation promotes the T cell proliferation, cytokine production and survival (122). Furthermore, it can also inhibit the Treg differentiation and suppressive activity (123, 124). Studies in preclinical models demonstrated that the OX40 activation provides a strong protection against immunogenic tumors, although poorly immunogenic tumors are more refractory to the therapy (125). Interestingly, a recent study showed that, in the context of chemotherapy-induced lymphopenia,

the OX40 activation induced a novel CD4 T cell population, expressing the transcription factor eomesodermin and producing Th1 and Th2 cytokines (126). This subpopulation promoted eradication of advanced B16 tumors in mice, supporting the use of immune modulation in redirecting the polarization of CD4 T cells. These promising results suggest the necessity of further research to gain a better understanding of the role of OX40 in antitumor immunity.

#### 1.2.4.7 Extracellular adenosine metabolism and signaling

In mice, Tregs coexpress ectoenzymes CD39 and CD73 producing immunosuppressive adenosine (81). Over the last years, multiple studies implicated the adenosine production by Treg in their inhibitory activity, and showed that targeting adenosine synthesis *in vivo* was a promising strategy to diminish tumor-mediated immunosuppression (87, 88, 93, 127-130).

It has been well documented that the deficiency or inhibition of adenosine production and signaling significantly reduces the tumor growth by improving the anti-tumor immunity (87, 127-131). One of the seminal studies showed that A2AR-deficiency promoted the rejection of established immunogenic tumors, and that the administration of A2AR antagonists reduced the tumor growth and metastasis (87). The anti-tumor effects of A2AR targeting were CD8 T cell-dependent. Furthermore, in a transplantable breast cancer model, Stagg et al. (128) demonstrated the efficacy of anti-CD73 mAb therapy in reducing tumorigenesis and metastasis. This study, together with several others (87, 130), showed an importance of CD73 expressed on tumor cells in hindering the anti-tumor immune reactivity.

A more recent research has been focused on the role of the host CD73 in tumor-mediated immunosuppression and on the specific contribution of various cell lineages to adenosine production. Using four different transplantable tumor models, Stagg et al. (127) found that CD73-deficiency enhanced anti-tumor immune responses and resistance to experimental metastasis. The anti-tumor effect of the CD73 deletion was dependent on CD8 T cells and associated with an expansion of antigen-specific IFN-γ-producing CD8 T cells in the peripheral blood and tumor microenvironment. Experiments with the bone marrow chimeras indicated that both hematopoietic and non-hematopoietic expression of CD73 non-redundantly contribute to the tumor immune escape. Furthermore, CD73 expression at least partially accounted for the tumor-promoting effects of Tregs. The pro-metastatic effect of host-derived CD73 was dependent on CD73 expression on non-hematopoietic cells. Complementary data of Wang et al. (129)

suggested that the enzymatic activity of CD73 on non-hematopoietic cells hindered leukocyte migration into the tumor site, whereas CD73 on hematopoietic cells suppressed a systemic anti-tumor T cell expansion and their effector functions. Both groups demonstrated the anti-tumor and anti-metastatic effects of the CD73 targeting with a selective inhibitor α,β-methylene adenosine diphosphate (APCP) or respective monoclonal antibodies. Moreover, Wang et al. (129) showed that anti-CD73 mAbs could completely restore the efficacy of adoptive T cell therapy in tumor-bearing mice. The translational significance of these findings is underscored by the fact that various adenosine receptor antagonists are already used in clinical settings for other indications (45).

Thus, targeting adenosine production and signaling represents a promising strategy to restore spontaneous T cell-mediated anti-tumor reactivity and to improve the efficacy of adoptive cellular immunotherapy in cancer patients. Further studies are necessary to elucidate the role of adenosine in the human immune cells and their interactions with tumor cells in the tumor microenvironment and in the periphery.

## 1.3 Myeloid-derived suppressor cells

### 1.3.1 Subsets, phenotypes and development

MDSCs represent a heterogeneous population of highly suppressive immature myeloid cells (IMCs). In mice, MDSCs are identified by the co-expression of the myeloid-cell lineage differentiation antigen Gr-1 and CD11b (also known as αM-integrin) (132). MDSCs contain subpopulations of ($Ly6G^{+}Ly6C^{low/-}$) and monocytic ($Ly6G^{-}Ly6C^{high}$) lineages (132). In cancer patients, various sets of markers have been use to analyze or isolate MDSCs. In particular, $Lin^{-}$ $HLA\text{-}DR^{-}$ $CD33^{+}CD11b^{+}$ MDSCs have been isolated from the peripheral blood of patients with glioblastoma, breast cancer, colon cancer, lung cancer or kidney cancer (40). These cells shared morphological and functional properties with granulocyte precursors. High frequencies of these cells correlated with a poor prognosis and radiographic progression in patients with breast or colorectal cancer (133, 134). Furthermore, the frequency of each MDSC subset appears to be influenced by the type of cancer. Polymorphonuclear MDSCs ($CD11b^{+}CD14^{-}$ $CD15^{+}CD66b^{+}VEGFR1^{+}$) were detected in patients with renal cancer, whereas $CD14^{+}CD11b^{+}HLA\text{-}DR^{low/-}$ monocytic MDSCs were observed in the peripheral blood of patients

with melanoma, multiple myeloma, prostate cancer, hepatocellular carcinoma or head and neck cancer (40, 135-138).

In the bone marrow of healthy mice, the $CD11b^{+}Gr\text{-}1^{+}$ population comprises 20–30% of all cells. However, $CD11b^{+}Gr\text{-}1^{+}$ cells make up only a small proportion (2–4%) of spleen cells and are absent from the lymph nodes (132). MDSCs accumulate in the bone marrow, spleen and peripheral blood, within primary and metastatic solid tumors, and to a lesser extent in lymph nodes. Tumors release multiple soluble factors, causing substantial alterations in the differentiation and function of myeloid cells. Cytokines such as GM‑CSF, G‑CSF, M‑CSF, stem cell factor (SCF), vascular endothelial growth factor (VEGF) and IL‑3 not only promote myelopoiesis but also block myeloid cell maturation (132, 139). Tumor-derived pro-inflammatory factors (e.g. IL-1β, IL-6, S100A8 and S100A9), as well as cytokines released by activated T cells (such as IFNγ, IL-4, IL-10 and IL-13) convert IMCs into MDSCs (140). The tumor-derived factors CCL2, CCL12, CXC-chemokine ligand 5, prokineticin 2, S100A8 and S100A9 attract MDSCs to the tumor bed (40, 141). Interestingly, MDSCs can also be recruited by nitrated or nitrosylated CCL2, whereas effector CD8 T cells cannot, which may account for the selective accumulation of myelomonocytic cells within murine and human tumors (142).

Lypopolisaccharide (LPS), in combination with IFN-γ, promotes MDSC expansion, probably through the inhibition of DC differentiation (143). Tumor-derived TGF-β also regulates MDSC accumulation and neutrophil polarization (144, 145). Tumors can also promote MDSC expansion through the release of small membrane vesicles – exosomes that contain signaling peptides, mRNAs, microRNAs and lipids (146).

### 1.3.2 Mechanisms of immunosuppression

In tumor-bearing mice, the granulocytic MDSC subset is the prevalent population of MDSCs. Polymorphonuclear MDSCs suppress antigen-specific CD8 T cells predominantly through production of reactive oxygen species (ROS) (40). Although granulocytic MDSCs represent the major subset of circulating MDSCs, they are less immunosuppressive than the monocytic counterparts when assessed on a per cell basis. Monocytic MDSCs express variable levels of usual monocytes markers, such as F4/80, CD115, Ly6C and CCR2 (40). This subset of MDSCs may also include progenitors that give rise to a subset of $CD11b^{hi}Gr\text{-}1^{low}Ly6G\text{–}F4/80^{hi}MHC\text{-}II^{+}$ macrophages with potent immunosuppressive properties. Monocytic MDSCs suppress CD8 T

cells predominantly via the expression of arginase-1 (ARG1) and inducible nitric oxide synthase (iNOS) and through the production of reactive nitrogen species. The increased activity of ARG1 in MDSCs leads to the depletion of L-arginine from the microenvironment. The shortage of L-arginine inhibits T-cell proliferation through several different mechanisms, including downregulation of TCR ζ-chain expression and blockade of the cell cycle. NO and its derivatives cause T cell apoptosis, the nitration of chemokines and T cell receptors (TCR), blockade of T cell migration, cytokine production and cytotoxic activity.

Another type of MDSC suppressive activity perturbs lymphocyte migration and viability (40). For instance, MDSCs express the membrane receptor ADAM17 (disintegrin and metalloproteinase domain-containing protein 17) that decreases CD62L levels on the surface of naive CD4 and CD8 T cells, thereby hindering T cell homing to secondary lymphoid organs (147). Furthermore, MDSCs express galectin-9, which binds to the T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) on lymphocytes and triggers T cell apoptosis (148). MDSCs also decrease the numbers and inhibit the function of mouse and human NK cells, mostly through membrane contact-dependent mechanisms, i.e. through membrane-bound TGF-β (in the case of mouse MDSCs) and through interaction with the NK cell receptor NKp30 (also known as NCR3) (40, 149).

MDSCs have also been shown to activate and expand Tregs, which underscores the interdependence of these two major immunosuppressive populations (137, 150). MDSCs boost the clonal expansion of antigen-specific nTregs and induce the conversion of naive CD4 T cells into iTregs. Human $CD14^{+}HLA^{-}DR^{low/-}$ MDSCs promote the transdifferentiation of Th17 cells into $FOXP3^{+}$ iTregs through TGFβ and retinoic acid (40, 150).

A recent study by Ryzhov et al. (151) suggests that the numbers and immunosuppressive activity of MDSC can be modulated by extracellular adenosine through A2BR. Furthermore, the authors detected high levels of CD73 on the surface of granulocytic MDSC and showed that the MDSC suppressive activity is enhanced in the presence of AMP, thereby suggesting that ectonucleotidase-mediated adenosine production might contribute to the MDSC function.

### 1.3.3 Therapeutic targeting of MDSCs

A large body of evidence indicates that the reduction of MDSC numbers and/or activity significantly improves anti-tumor immunity and improves the survival of tumor-bearing hosts

(39). Several strategies for targeting MDSC showed promising results in preclinical models (39, 40). The first approach is to block MDSC generation from bone marrow progenitors and to promote the maturation of MDSCs into proficient APCs. The inhibition of the migration of MDSCs to the tumor and tumor-draining lymph nodes also showed therapeutic efficacy. Finally, it is important to inhibit the molecular pathways used by MDSCs to suppress T cell function.

It has been shown that MDSC development can be blocked by targeting stem cell factor (SCF) (152, 153). Several studies showed that siRNA-mediated knockdown of SCF or inhibition of SCF signaling by anti-c-Kit antibodies or tyrosine kinase inhibitors (such as sunitinib, pazopanib and sorafenib) hinder MDSC development from bone marrow precursors in humans (*in vitro*) and mice (colon and Lewis lung carcinomas) (152, 153). A decrease in MDSC numbers was associated with improved tumor-specific immune responses, tumor regression and significantly prolonged survival. In addition, sunitinib has been demonstrated to reverse MDSC accumulation in patients with renal cell carcinoma, resulting in an expansion of Th1 cells and reduction in Treg numbers (154). In a mouse model of RCC, sunitinib was also found to neutralize the immunosuppressive functions of tumor-infiltrating MDSCs (154).

Administration of all-trans-retinoic acid (ATRA) or ultra-low non-cytotoxic doses of paclitaxel has been shown to promote the differentiation of MDSCs into mature macrophages, DCs or granulocytes (155, 156). The combination of ATRA with G-CSF enhanced granulocyte differentiation, whereas ATRA together with vitamin D induced the development of monocytes (155). In a recent study, Sevko et al. (157) showed that the administration of the chemotherapeutic drug paclitaxel at ultra-low non-cytotoxic doses (previously described as chemoimmunomodulation (158)) in healthy C57BL/6 mice significantly reduced the frequency of $CD11b^{+}Gr1^{+}$ immature myeloid cells that are known as the MDSC counterparts in healthy mice (132). This was associated with an increase in NK cell frequencies in the bone marrow and their ability to produce IFN-γ. Moreover, paclitaxel-mediated chemoimmunomodulation enhanced the efficacy of vaccination with a peptide derived from melanoma-associated antigen tyrosinase related protein-2. Ultra-low dose paclitaxel administration in the *ret* transgenic (*ret* tg) mouse melanoma model led to a drastic reduction in MDSC frequencies as well as their ability to produce NO and to suppress T cell proliferation *in vitro* (159). Moreover, the concentrations of numerous chronic inflammatory factors (e.g. IL-1β, IL-6, TNF-α, IFN-γ, GM-CSF, and IL-10) in

melanoma lesions were significantly diminished. These changes were associated with a partial recovery of tumor-specific T cell responses that resulted in a delayed tumor growth and prolonged survival (159).

Furthermore, MDSCs can also be sensitive to conventional chemotherapy (39, 40). A selective depletion of MDSCs has been achieved by the administration of gemcitabine (160) or 5‘-fluorouracil (161). These agents depleted MDSCs with no apparent toxicity against other leukocyte subpopulations, which led to a markedly enhanced anti-tumor effect in several transplantable tumor models.

Targeting of tumor-derived chemokines has also been applied to inhibit MDSC migration towards tumor lesions. Thus, prostate, breast and Lewis lung carcinomas, melanoma and colorectal cancer have been shown to produce various ligands for CCR2 (including CCL2) that attracted MDSCs and supported their suppressive activity (162, 163). For instance, direct CCL2 binding by bindarit (164) or the blocking of production of these chemokine in tumors (162) has been shown to diminish MDSC frequencies in tumor microenvironment, to inhibit metastasis and neoangiogenesis and suppress the development of transplantable tumors.

Finally, MDSCs could be neutralized through inhibition of their immunosuppressive activity. It has been reported that phosphodiesterase (PDE)-5 inhibitors (e.g. sildenafil or tadalafil) block MDSC immunosuppressive functions in various transplantable tumor models, which restores antitumor immunity and delays tumor growth (165-167). These effects were due to elevated intracellular concentrations of cyclic guanosine monophosphate leading to the down-regulation of iNOS and ARG-1 activities and decreased NO synthesis in MDSCs.

In the *ret* transgenic mouse melanoma model, chronic administration of sildenafil caused a significant reduction in NO production and ARG-1 expression, which led to an improved anti-tumor CD8 T cell response (168). Moreover, sildenafil significantly diminished chronic inflammation, which was manifested by a reduction in IL-1β, IL-6, VEGF, GM-CSF, CCL2, CCL3 and S100A9 production. All these changes resulted in a significantly prolonged survival of tumor-bearing mice. In addition to PDE-5 inhibitors, the activity of iNOS and ARG-1 can be directly blocked by corresponding inhibitors (165, 169) or by nitroaspirin (170), leading to a reactivation of T cells and strong anti-tumor effects.

Interestingly, some agents preventing the migration of MDSCs towards tumor lesions can also inhibit MDSC immunosuppressive function (39). In particular, COX-2 inhibitors that reduced MDSC trafficking mediated by chemokines CXCR4/CXCL12 and CXCR1-CXCR2/CXCL8, also impaired MDSC-mediated immunosuppression through the down-regulation of ROS and NO production or ARG-1 expression in these cells (171-173).

### 1.4 Tumor models

In the current study, we used two distinct tumor models – the *ret* tg mouse model of melanoma and the orthotopic Panc02 model of pancreatic ductal adenocarcinoma (PDAC).

The *ret* tg model largely recapitulates human melanoma with respect to etiology, tumor genetics, histopathology and clinical development (174, 175). Tumor lesions show typical melanoma morphology and express melanoma-associated antigens, such as S100, tyrosinase, tyrosinase related protein (TRP)-1, TRP-2 and gp100. In these mice, the human *ret* transgene expression is controlled by the mouse metallothionein I promoter/enhancer, which allows its selective expression in melanin-producing cells (39, 174, 176). Ret belongs to the family of receptor tyrosine kinases and its activation promotes malignant transformation of melanocytes and tumor development in *ret* transgenic mice (177). Overexpression of the Ret kinase is associated with the activation of other kinases (such as mitogen-activated protein kinase and c-Jun) and matrix metalloproteinases acting downstream of the Ret kinase. After a short latency period (20–70 days; mean, 40 days), transgenic animals develop skin tumors on the head (nose, ears, eyes), neck, back, or tail with metastases in lymph nodes, lungs, liver, brain, kidney, and bone marrow, similar to the metastatic pattern observed in melanoma patients. It was previously shown that skin tumors of these mice contain high frequencies of TRP-2-specific CD8 T cells, mostly exhibiting the effector memory phenotype (176, 178). Notably, these cells could be detected even at earlier stages of melanoma progression. Upon a co-incubation with DCs pulsed with melanoma lysates, bone marrow-derived T cells produced IFN-γ *in vitro* and exerted an anti-tumor activity after their adoptive transfer into melanoma-bearing *ret* tg mice. However, this anti-tumor effect was merely transient and failed to trigger tumor regression, probably subdued by the complex immunosuppressive network in the tumor microenvironment represented, for instance, by CD4 Tregs and MDSCs (39, 92, 175, 176, 178). Indeed, a strong Treg accumulation in skin tumors and metastatic lymph nodes was observed at the early stage of melanoma development (179). In

addition, increasingly high frequencies of MDSCs were detected in skin melanomas and lymphoid organs during the course of tumor progression. Inhibition of MDSC immunosuppressive activity was shown to improve anti-tumor immunity and survival in tumor-bearing *ret* tg mice (159, 168).

Another model used in our work was an orthotopic model of pancreas adenocarcinoma (180, 181). Panc02 tumor cells were injected directly into the pancreata of immunocompetent mice. The development of pancreatic tumors in their natural microenvironment constitutes an important advantage of that model. It has been found that Panc02 tumors are strongly infiltrated with Tregs (182). The vast majority of tumor-infiltrating Tregs, Tcons and CD8 T cells exhibited the effector/effector memory phenotype. In Tregs, this phenotype had been linked to an enhanced suppressive activity and proliferation capacity.

Although a steady increase in TGF-β levels was observed in pancreatic tumors, treatment with a specific inhibitor of the TGF-β receptor I kinase failed to abrogate Treg accumulation (182). Targeting a major pathway of Treg migration with a CCR4 antagonist also did not affect Treg frequencies in the tumor. However, Panc02 tumors harbor high frequencies of cycling (Ki-67-expressing) Tregs, which suggested that local proliferation might be the driving force of Treg accumulation in the tumor. Accordingly, Treg frequencies in the tumor were strongly reduced upon the administration of low-dose gemcitabine. This resulted in a modestly increased survival of tumor-bearing mice, thereby underscoring the contribution of Tregs to tumorigenesis in the Panc02 model.

## 2. Aims of the study

Recent studies have shown that extracellular adenosine plays an important role in the regulation of various arms of the immune response. Several lines of evidence indicate that extracellular adenosine might be produced by a variety of cell types. In particular, it has been shown that adenosine can be elaborated by ectonucleotidases expressed on Tregs, MDSCs, endothelial cells, mesenchimal cells, cancer cells etc. However, recent studies suggested that ectonucleotidase-mediated adenosine production might also be performed by certain types of effector T cells, in particular, IL-17-producing Th17 cells. Moreover, the patterns of ectonucleotidase expression in Tregs, Tcons and MDSCs, as regards to their subsets and anatomical location, remain largely unknown. Therefore, the goal of this work was to investigate the expression of CD39 and CD73 on various subsets of T cells and MDSCs from tumors and lymphoid organs, in order to understand the role of extracellular adenosine in the regulation of immunity under homeostatic conditions and in cancer. In the current study, following issues were addressed:

- determination of the patterns of ectonucleotidase expression on Tregs in tumor-bearing mice and cancer patients;

- elucidation of the role of CD39 and CD73 in the regulation of T cell activation and function.

- investigation of the function of ectonucleotidases in the myeloid compartment, particularly in MDSCs.

- evaluation of the therapeutic efficacy and immunological effects of CD73 inhibitors *in vivo*.

# 3. Materials and Methods

## 3.1 Reagents

### 3.1.1 Chemicals, buffers and media

| Product | Company | Catalogue number |
|---|---|---|
| Bovine serum albumin (BSA) | Sigma | 7030-50G |
| 0,5M EDTA (pH 8.0) | GIBCO | 15575-098 |
| Fetal bovine serum (FBS) | PAN Biotech, GmbH | 3702-P260718 |
| Sodium azide ($NaN_3$) | Roth | K305 |
| 0.4% Trypan blue solution | Sigma | T8154 |
| Phosphate buffer saline (PBS) | PAA | H15-002 |
| RPMI-1640, with L-glutamine | PAA | E15-840 |
| Red Blood Cell (RBC) Lysis Buffer | Biolegend | 420301 |
| Penicillin-Streptomycin (P/S) | PAA | P11-010 |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma | P1585 |
| Ionomycin calcium salt | Sigma | I3909 |
| Foxp3/Transcription Factor Staining Buffer Set | eBioscience | 00-5523-00 |
| Permeabilization Buffer (10X) | eBioscience | 00-8333-56 |
| Protein Transport Inhibitor (Brefeldin A) | BD Biosciences | 555029 |
| Adenosine 5′-(α,β-methylene)diphosphate | Sigma | M3763 |

### 3.1.2 Buffers and media

| Buffer / medium | Components |
|---|---|
| FACS buffer: | 1 x PBS |
| 2% FBS, | |
| 0.2% NaN3 | |
| 2 mM EDTA | |
| Complete RPMI: | RPMI-1640 w/L-glutamine |
| 10% FCS | |
| 1% P/S | |

### 3.1.3 Antibodies

| Antibody | Conjugate | Isotype | Company | Catalogue No. |
|---|---|---|---|---|
| **anti-human CD3e** | V500 | mouse IgG1, λ | BD | 560770 |
| **anti-mouse CD3e** | PerCP-Cy5.5 | rat IgG2a, κ | BD | 555276 |
| | APC | rat IgG2a, κ | BD | 553066 |
| | V500 | syrian hamster IgG2, κ | BD | 560771 |
| | V450 | syrian hamster IgG2, κ | BD | 560352 |
| | purified | armenian Hamster IgG | eBioscience | 16-0031 |
| **anti-human CD4** | APC-H7 | mouse IgG1, κ | BD | 560158 |
| **anti-mouse CD4** | APC | rat IgG2a, κ | BD | 561091 |
| | APC-Cy7 | rat IgG2b, κ | BD | 552051 |
| | APC-H7 | rat IgG2b, κ | BD | 560181 |
| **anti-human CD8** | APC-H7 | mouse IgG1, κ | BD | 560179 |

| | | | | |
|---|---|---|---|---|
| **anti-mouse CD8a** | APC-Cy7 | rat IgG2a, κ | BD | 557654 |
| | APC-H7 | rat IgG2a, κ | BD | 560182 |
| **anti-mouse CD11b** | PerCP-Cy5.5 | rat IgG2b, κ | BD | 550993 |
| | FITC | rat IgG2b, κ | BD | 553310 |
| | APC | rat IgG2b, κ | BD | 553312 |
| **anti-human CD25** | PE | mouse IgG1, κ | BD | 560355 |
| **anti-mouse CD25** | APC | rat IgG2b, κ | BD | 558643 |
| | V450 | rat IgG1, λ | BD | 561257 |
| **Anti-Mouse CD28** | purified | Golden Syrian Hamster IgG | eBioscience | 16-0281 |
| **anti-human CD39** | PE | mouse IgG2b, κ | BD | 555464 |
| **anti-mouse CD39** | PE-Cy7 | rat IgG2b, κ | eBioscience | 25-0391 |
| **anti-mouse CD44** | PE | rat IgG2b, κ | BD | 553134 |
| **anti-mouse CD45** | V500 | rat IgG2b, κ | BD | 561487 |
| **anti-mouse CD45.2** | PerCP-Cy5.5 | rat IgG2a, κ | BD | BD 552950 |
| **anti-mouse CD45RB** | PE | rat IgG2a, κ | BD | 553101 |
| **anti-mouse CD62L** | APC | rat IgG2a, κ | BD | 553152 |
| **anti-human CD73** | APC | mouse IgG1, κ | BD | 560847 |
| **anti-mouse CD73** | eFluor® 450 | rat IgG1, λ | eBioscience | 48-0731 |
| | PE | rat IgG1, λ | eBioscience | 12-0731 |
| **anti-human CD127** | PerCP-Cy5.5 | mouse IgG1, κ | BD | 560551 |

| | | | | |
|---|---|---|---|---|
| **anti-mouse Gr1** | PE-Cy7 | rat IgG2b, κ | BD | 552985 |
| | APC-Cy7 | rat IgG2b, κ | BD | 557661 |
| **anti-human FOXP3** | FITC | rat IgG2a, κ | eBioscience | 11-4776 |
| **anti-mouse Foxp3** | FITC | rat IgG2a, κ | eBioscience | 11-5773 |
| **anti-mouse Ly6C** | APC | rat IgG2b, κ | BD | 553129 |

## 3.2 Cell line

Highly tumorigenic Panc02 cells were maintained in RPMI-1640 cell culture medium (PAA, Germany) supplemented with 10% FCS (PAA) at 37°C under 5% CO2. Cells were passaged by a brief detachment with 0.025% trypsin. The cells were routinely tested for contamination including mycoplasms. Cell cultivation was done by Mr. Markus Herbst (department of General Surgery, University Heidelberg).

## 3.3 Routine laboratory materials and equipment

### 3.3.1 Routine laboratory materials

| **Material / instrument** | **Type / model** | **Company** |
|---|---|---|
| Needles | 0,4x19 mm Mikrolance | BD |
| | 0,3x13 mm Mikrolance | BD |
| Pipets | 2-20 µl, 20-200 µl, 200-1000µl | Rainin |
| Object carrier | 76x26 mm SuperFrostPlus | Menzel-Gläser |
| Cover glass | 24 x 24 mm | Roth |
| Test tubes | 5 ml / 15 ml / 50 ml<br>0.5 ml / 1.5 ml / 2 ml | BD Falcon<br>Eppendorf |

| | | |
|---|---|---|
| Syringes | 1 ml | BD |
| Cell culture plates | 96-well flat bottom Platte | Greiner |
| Cell strainer | 40 µm; 100 µm | BD Falcon |
| Sterile filter | 0,45 µm, sterile | Roth |

### 3.3.2 Devices and equipment

| **Equipment** | **Model** | **Company** |
|---|---|---|
| Flow cytometer | FACS Canto II, 3 lasers | BD |
| Refrigerator (-80 °C) | HeraFreeze | Heraeus |
| CO2 incubator | HeraCell | Heraeus |
| Refrigerator (-20 °C) | Premium | Liebherr |
| Microscopes | DMIL | Leica |
| pH meter | 766 | Calimatic |
| Laminar flow hood | Hera Safe | Thermo Electron Cooperation |
| Vortex | Vortex Genie 2 | Scientific Industries |
| Balance | BP 3100P | Sartorius |
| Water bath | DC3, HAAKE | GFL |
| Centrifuges | Labofuge 400R | Heraeus |
| | Biofuge primo R | Heraeus |
| | Varifuge K | Heraeus |

## 3.4 Mice

All mice were crossed and kept under specific pathogen-free conditions in the animal facility of the German Cancer Research Center (Heidelberg) or Heidelberg University. Experiments were performed in accordance with governmental and institutional guidelines and regulations.

### 3.4.1 Ret transgenic spontaneous melanoma model

*Ret* tg mice (C57BL/6 background) kindly provided by Dr. I. Nakashima (Japan) overexpress the human *Ret* proto-oncogene in melanocytes under the control of mouse metallothionein-I promoter-enhancer (177). Non-transgenic littermates and transgenic mice without macroscopically visible tumors were used as controls. The mice were kept and crossed under the guidelines of the animal facility of the German Cancer Research Center.

### 3.4.2 Orthotopic model of pancreatic ductal adenocarcinoma

Highly tumorigenic syngeneic murine Panc02 pancreatic ductal adenocarcinoma cells were injected into the head of the pancreas, producing rapidly growing tumors starting from day 5 after transplantation. Experiments were performed under the guidelines of the animal facility of Heidelberg University. Orthotopic transplantation was performed by Dr. Sabine Soltek or by Prof. Dr. Alexandr Bazhin (Department of General Surgery, University Heidelberg). C57BL/6 mice were purchased from CharlesRiver (Germany).

## 3.5 Software

| | |
|---|---|
| FlowJo (Version 7.6.1) | Tree Star, Inc., Ashland, USA |
| GraphPad PRISM (Version 5) | GraphPad Software, Inc., San Diego, USA |

## 3.6 Single cell suspensions

### 3.6.1 Spleen

Mouse spleens were collected into 2 ml Eppendorf tubes with 2 ml of ice-cold PBS (PAA). Single cell suspensions were prepared by mashing spleens with a plunger of a 5 ml syringe through a 40 µm cell strainer. Next, cell suspensions were centrifuged at 300g and the supernatant was discarded. Red blood cells were depleted by resuspending cell pellets in 1 ml of RBC lysis buffer followed by 3 min of incubation on ice. The reaction was stopped by adding 9

ml of PBS. Cells were subsequently centrifuged (300 g, 10 min), and the pellet was resuspended in FACS buffer at the concentration of $10^7$ cells/ml.

### 3.6.2 Lymph nodes

Isolated mouse lymph nodes were mashed on a petri dish using plunger of a 5 ml syringe and passed through a 40 µm cell strainer. Cells were washed with PBS (300 g, 5 min), and the pellet was resuspended in FACS buffer at $10^7$ cells/ml.

### 3.6.3 Bone marrow

Freshly isolated murine femurs and tibiae were cut at both ends and BM was flushed out with ice cold PBS using a 10 ml syringe with a 23G needle. BM was collected into a 10 cm Petri dish and subsequently transferred onto a 40 µm cell strainer. After the BM was mashed through the cell strainer, the resulting cell suspension was centrifuged, and red blood cells were depleted as described in 3.1.1. After washing with cold PBS (300 g, 5 min), the pellet was resuspended in FACS buffer at $10^7$ cells/ml.

### 3.6.4 Tumor

Freshly isolated tumor mass was mashed through a 40 µm cell strainer with a 5 ml syringe plunger. After the depletion of red blood cells, the cell pellet was resuspended in FACS buffer at$10^7$ cells/ml.

### 3.6.5 Cell numbers

Cell numbers were determined using a Neubauer counting chamber. A sample of 10 µl was diluted with trypan blue at 1:100. The following formula was used:

n x $10^4$ x dilution factor = cell number / ml, where n= mean number of cells in a quadrant.

## 3.7 Flow cytometry

Single cell suspensions were dispensed into 5 ml polystyrene tubes at $10^6$ cells per tube and incubated for 15 min with the Fc-blocking reagent. Next, fluorescently labeled antibodies against surface antigens were added, followed by 15 min of incubation in the dark (at 4°C or on ice). Then, cells were washed twice with an excess of FACS buffer. When no intracellular staining was required, the cells were resuspended in 100 µl of FACS buffer and analyzed by flow cytometry with the FACS Canto II cytometer (BD) using the BD Diva Software V.6.1.1. At least

10,000 events were acquired for each population of interest. When necessary, intracellular staining for cytokines and transcription factors was performed. For this, cells were incubated in 1 ml of Fixation/Permeabilization solution (eBioscience) for 30 min and subsequently washed twice with an excess of Permeabilization buffer (eBioscience). Next, cells were resuspended in 50 µl of Permeabilization buffer and stained with fluorescently labeled antibodies against intracellular antigens. After 30 min of incubation, cells were washed twice with the FACS buffer, resuspended in 100 µl of the same buffer and analyzed by flow cytometry.

FlowJo software 7.6.1 (Tree Star) was used to analyze at least 10,000 events per population of interest. Data were expressed as pseudocolor dot plots.

### 3.8 *In vitro* splenocyte activation

Microwells of the assay plate were coated with anti-CD3 antibodies. To this end, a 10 µg/ml solution of anti-CD3 antibodies (eBioscience) in sterile PBS was prepared and 50 µL of the antibody solution were dispensed to each well of the assay plate. As a control, unstimulated cells, 50 µl of sterile PBS were added. The plate was tightly covered with Parafilm™ to avoid sample evaporation and incubated at 37°C for 2 hours. Immediately before adding cells, the antibody solution was removed with a multichannel pipettor, and the wells were rinsed with 200 µl of sterile PBS three times.

Single cell suspensions of splenocytes were centrifuged and resuspended in the complete RPMI-1640 medium at $10^6$ cells/ml. Then 200 µL of the cell suspension were added to each well, and the plate was placed in a humidified 37°C, 5% $CO_2$ incubator. Then soluble anti-CD28 (eBioscience) antibody was added to the cells at 2 µg/ml. The kinetics of ectonucleotidase expression on splenocytes was analyzed by flow cytometry (as described in 3.2) at different time points (5, 24 and 48 hours) after the beginning of activation.

### 3.9 Analysis of the IFN-γ production by T cells

Single cell suspension of splenocytes was centrifuged, and the pellet was resuspended in complete RPMI at $10^6$ cells/ml. Next, 100 µl of the resulting cell suspension were dispensed to each well of a 96-well flat bottom plate followed by the addition of 100 µl of master mix containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), 1 mM ionomycin (Sigma), and GolgiStop (BD Biosciences) (1.2 µl/ml). To the control wells, only GolgiStop was added.

After 5 hours of incubation, cells were stained with antibodies against surface markers, followed by the fixation/permeabilization and intracellular staining for IFN-γ according to the protocol described in 3.2.

### 3.10 Survival analyses

*Ret* transgenic tumor-bearing mice were injected intraperitoneally (i.p.) with APCP at 20 mg/kg or with SCH58261 at 5 mg/kg three times a week for 60 days. Mice were monitored daily for the tumor progression. The control group of mice with tumors of similar size received PBS. Each group comprised 15 animals. Two independent survival experiments have been performed.

### 3.11 Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc.). The paired t-test using was applied to analyze the statistical significance where appropriate. Survival of tumor bearing mice was analyzed with Kaplan-Meier curves with subsequent Log-rank test. A p-value $< 0.05$ was considered significant. Values were expressed as mean ± SEM.

# 4. Results

## 4.1. Expression of ectonucleotidases CD39 and CD73 on Tregs

It was previously shown that the vast majority of murine Tregs express on their surface a high level CD39 and CD73, and adenosine production strongly contributes to Treg immunosuppressive properties (82, 83). However, patterns of the ectonucleotidase expression on Tregs from tumors and lymphoid organs remain poorly understood and deserve further investigation. To this end, we analyzed Tregs from tumors, bone marrow, spleens and lymph nodes of healthy mice and animals bearing melanoma or pancreatic adenocarcinoma (Fig. 1).

Consistently with previously published findings, we found that Treg populations in the spleen contained 90-95% $CD39^{+}CD73^{+}$ cells (Fig. 2A), whereas only a minor proportion of CD4 Tcon and CD8 T cells coexpressed CD39 and CD73. Moreover, the levels of CD73 on Tregs in spleens of healthy mice were far above those on Tcons and CD8 T cells, although CD39 expression was comparable (Fig. 2B, C). These observations confirmed that Tregs possess the whole machinery of adenosine synthesis and can perform it more efficiently than other major T cell subsets.

Remarkably, the patterns of ectonucleotidase expression on tumor-infiltrating T cells were distinctly different from what was observed in splenic T cells (Fig. 2D). In particular, only 60% of Tregs from melanoma lesions in *ret* transgenic mice expressed both CD39 and CD73. On the other hand, in both tumor models, we observed increased frequencies of $CD39^{+}CD73^{+}$ subsets among tumor-infiltrating Tcons and CD8 T cells as compared to these subsets in the spleen. This was especially pronounced in the Panc02 model, where approximately 50% of Tcons and more than 60% of CD8 T cells expressed both enzymes. Moreover, Tcons and Tregs from pancreatic tumors showed nearly the same expression level of both ectonucleotidases (Fig. 2E, F). These profound alterations might be due to the influence of the tumor microenvironment and/or to certain phenotypical and functional differences between T cell populations in the spleen and tumor.

Next, we analyzed the expression of CD39 and CD73 on regulatory ($FOXP3^{+}CD25^{high}CD127^{low/-}$) and conventional ($FOXP3^{-}CD25^{low/-}CD127^{high}$) T cells from the peripheral blood of patients with melanoma or pancreatic adenocarcinoma and healthy donors (Fig. 3A). In contrast to mouse

Tregs, human regulatory T cells contained only a small population of $CD39^+CD73^+$ cells, which suggested that human Tregs have a very limited capacity to produce adenosine through enzymatic hydrolysis of extracellular nucleotides (Fig. 3B, C). However, we observed that approximately a half of the Treg subset showed CD39 expression, which indicates the ability of these cells to hydrolyze ATP. Only a small proportion of Tregs was positive for CD73 expression. Thus, human Tregs can contribute only to the first step of adenosine production outside the cell. Moreover, we detected a two-fold increase in the percentage of $CD73^+$ cells in Tcons from melanoma patients, suggesting, in turn, an increased capacity to produce adenosine from AMP (Fig. 3D).Furthermore, we found that the $CD73^+$ subset comprised only around 10% of human Tcons, and the $CD39^+$ population is also relatively small (Fig. 3D, E). Similarly to our findings in mouse models, we observed increased frequencies of $CD39^+$ and/or $CD73^+$ cells in human Tcon and Treg from the peripheral blood of cancer patients as compared to healthy donors. In particular, Tregs and Tcons from pancreatic cancer patients contained increased frequencies of $CD39^+$ cells, indicating their enhanced capacity to hydrolyze ATP (Fig. 3E).

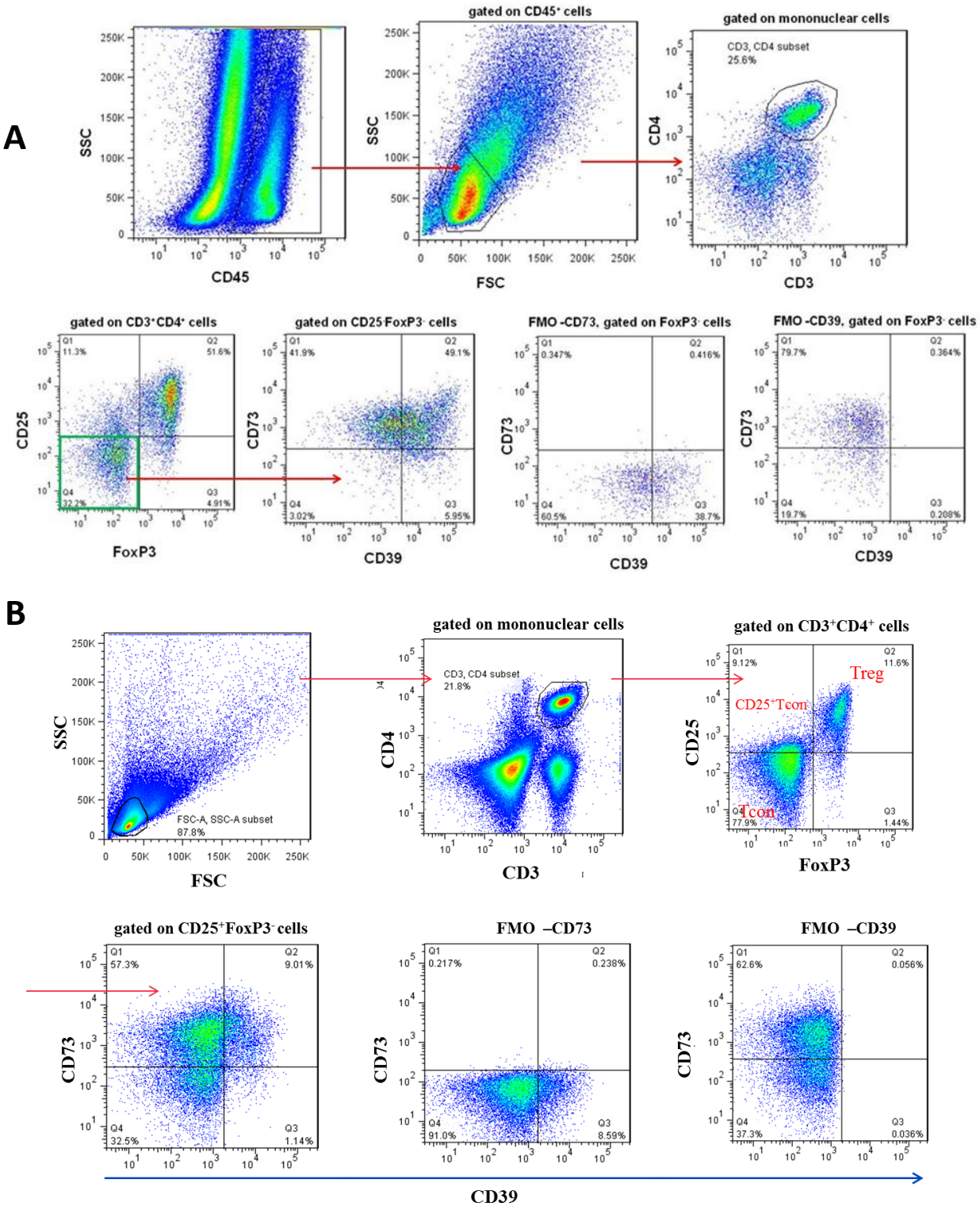


**Figure 1.** Representative dot plots of murine pancreatic tumors **(A)** and spleens from tumor-bearing mice **(B)** showing gating strategy for the analysis of CD73 and CD39 expression on regulatory and conventional CD4 T cells (measured by flow cytometry).

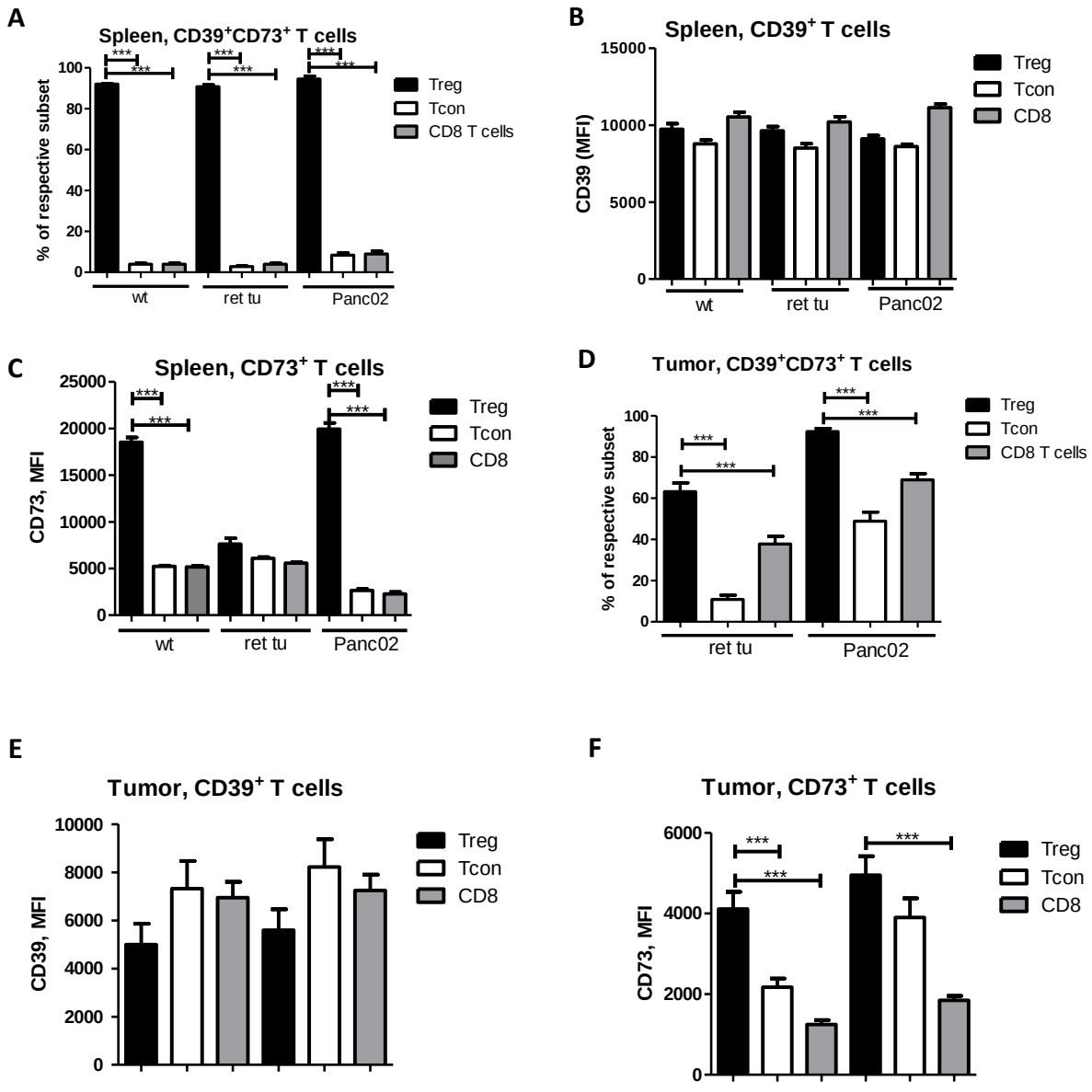


**Figure 2.** Analysis of CD39 and CD73 expression on Tregs, CD4 Tcons and CD8 T cells in spleens **(A-C)** and tumors **(D-F)** of *ret* tg melanoma-bearing mice (ret tu) and animals with pancreatic adenocarcinoma (Panc02) as well as healthy wild type C57BL/6 (wt) mice (measured by flow cytometry; n=20; *p<0.001).

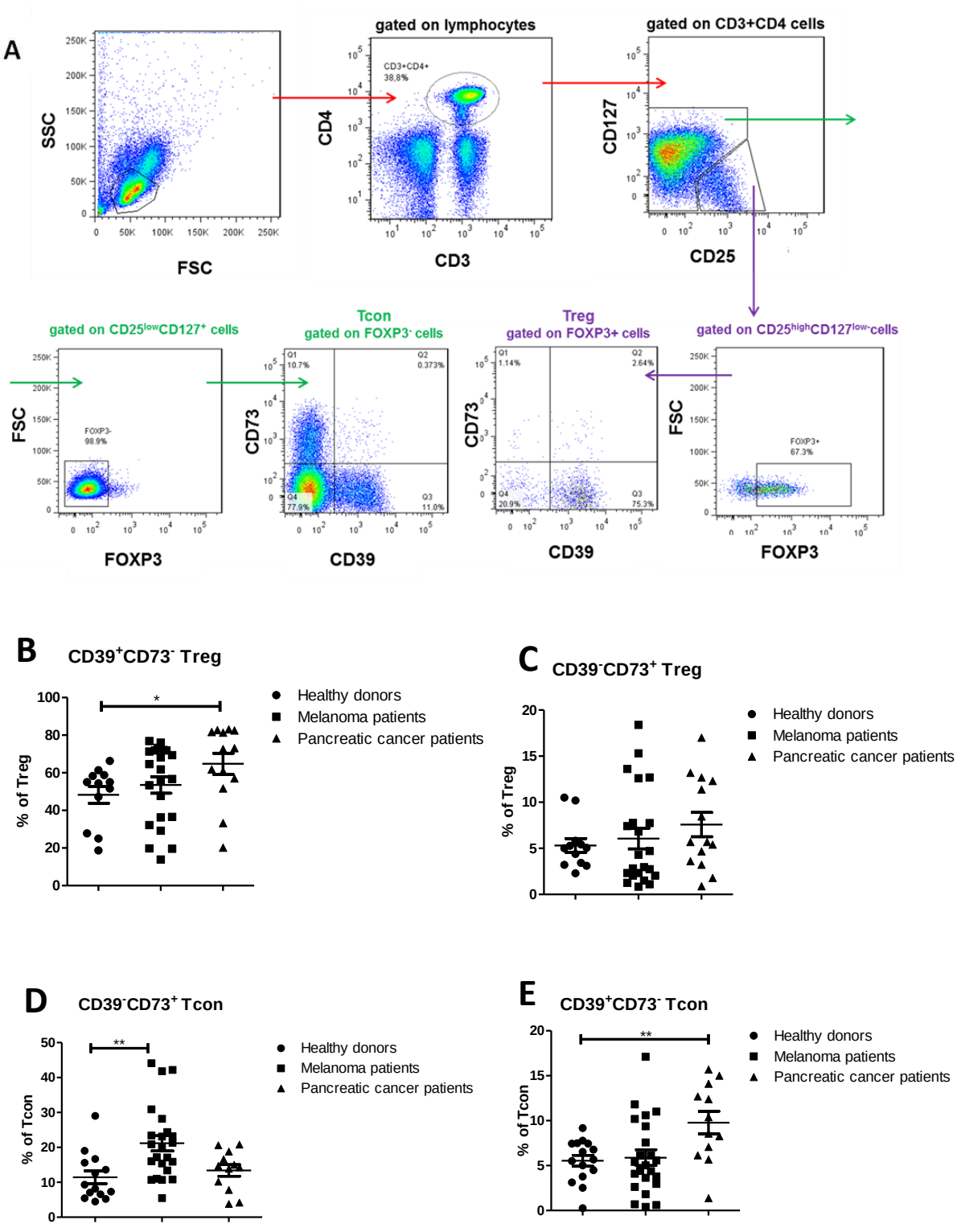


**Figure 3.** Representative dot plots of human peripheral blood showing gating strategy **(A)** for the analysis of ectonucleotidase expression on Tregs **(B, C)** and Tcons **(D, E)** in patients with melanoma or pancreatic cancer and healthy donors (measured by flow cytometry; *p<0.05; **p<0.01).

### 4.2. Analysis of ectonucleotidase expression on CD4 Tcons and CD8 T cells

Since we found a relatively high expression of both ectonucleotidases also in non-regulatory T cells in melanoma and pancreatic cancer, we reasoned that it might be due to the alterations in the milieu and/or activation status of T cells. To address this suggestion, we compared the expression of CD39 and CD73 in $CD25^{-}FoxP3^{-}$ and $CD25^{+}FoxP3^{-}$ T cells (Fig. 4A-H). The latter subset presumably consists of activated T cells, since CD25 (IL-2 receptor α-chain) is induced on T cells upon activation (35). Importantly, $CD25^{+}FoxP3^{-}$ T cells from lymphoid organs and tumors contained significantly higher frequencies of $CD73^{+}$ cells than their $CD25^{-}$ counterparts. Accordingly, the $CD25^{+}$ T cell subpopulation was highly enriched in $CD39^{+}$ cells. This indicated that T cells may upregulate ectonucleotidases upon activation, which can possibly provide a negative feedback mechanism of autocrine regulation.

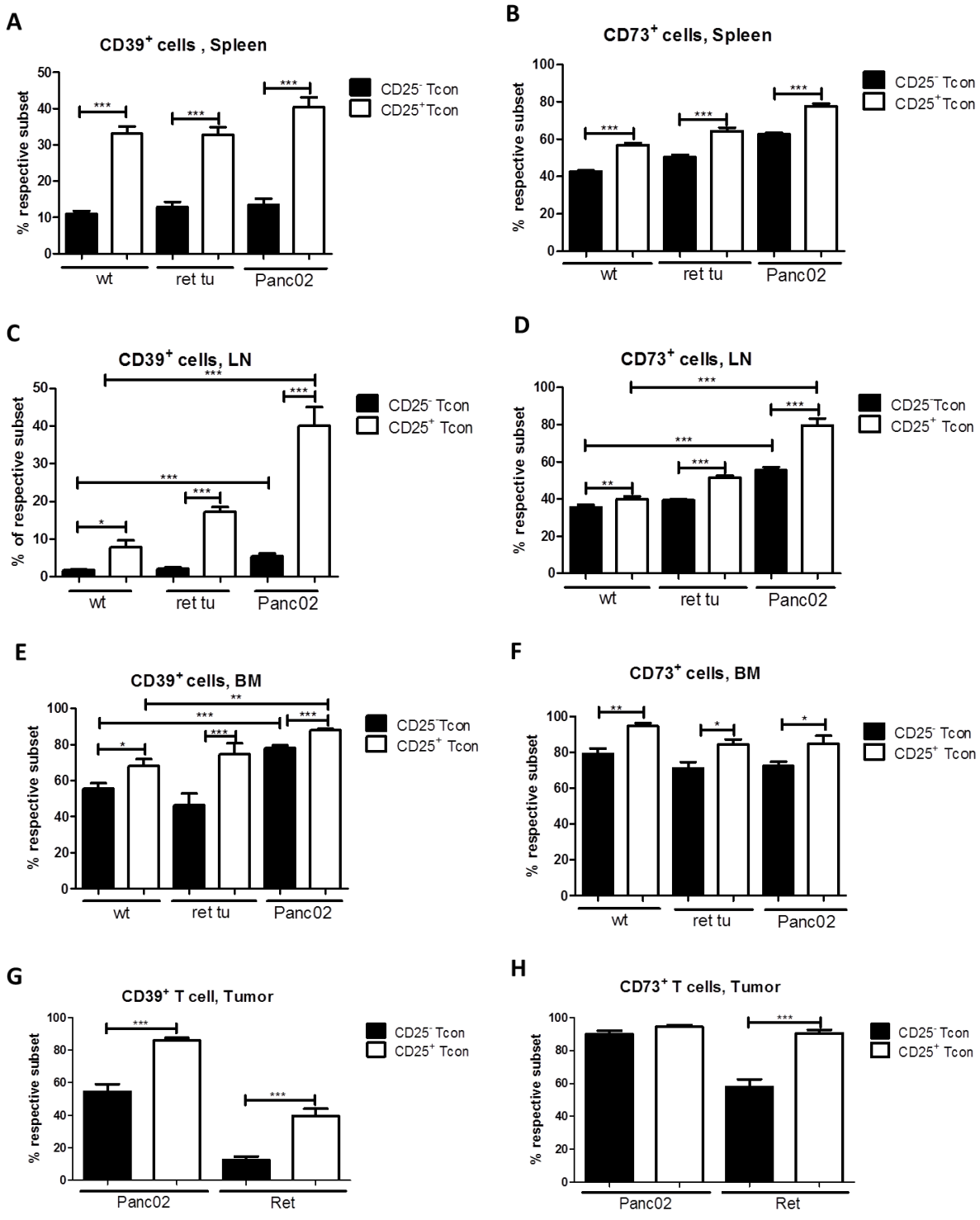


**Figure 4.** Analysis of CD39 and CD73 expression on Tregs, CD4 Tcons and CD8 T cells in spleens **(A, B)** lymph nodes **(C, D)**, bone marrow **(E, F)** and tumors **(G, H)** of *ret* tg melanoma-bearing (ret tu) mice and animals with pancreatic adenocarcinoma (Panc02) as well as healthy wild type C57BL/6 (wt) (measured by flow cytometry; n=20; *p<0.05; **p<0.01; ***p<0.001).

To test this hypothesis, we activated normal murine splenocytes *in vitro* and analyzed the dynamics of CD39 and CD73 expression (Fig. 5A, B). Similarly to our *ex vivo* data, we observed a substantially higher percentage of $CD39^+$ cells among activated CD4 T cells, compared to the non-activated control. However, we did not detect any significant changes in CD73 expression, which may reflect the differences in conditions of *in vitro* and *in vivo* T cell activation.

Studying memory T cells, we found that both central ($CD44^{high}CD62L^+$) and effector memory ($CD44^{high}CD62L^-$) $CD4^+FoxP3^-$ subsets are highly enriched in $CD39^+$ cells (Fig. 6A) as compared to naïve ($CD44^{low}CD62L^+$) CD4 T cells, which contained only a very low percentage of CD39-expressing cells. Similarly, the memory compartments of conventional CD4 T cells showed significantly higher frequencies of $CD73^+$ cells than the naïve subset (Fig. 6B).

Memory CD4 T cells are known to produce rapidly effector cytokines upon restimulation (183). Therefore, to determine if the association of ectonucleotidase expression with the memory phenotype has any functional implication, we analyzed CD39 and CD73 expression in the context of IFN-γ production by CD4 T cells from healthy wild type mice 5h after stimulation. Indeed, IFN-γ-producing cells contained a significantly higher percentage of $CD39^+$ and $CD73^+$ cells than IFN-γ-negative cells (Fig. 7A, B).

Next, we asked if CD8 T cells also might exploit ectonucleotidases as a means of self-regulation. First, we found that virtually all CD8 T cells from lymphoid organs and tumors constitutively express CD73, suggesting that they might produce extracellular adenosine when AMP is available in their environment (Fig. 8A-D). However, CD39 expression appeared to be highly heterogeneous and varying between different lymphoid organs and the tumor site. In the spleen and lymph nodes, only a minor fraction of CD8 T cells expressed CD39 (Fig. 9B, C). In contrast, the $CD39^+$ subset comprised 30-50% of CD8 T cells in the bone marrow (Fig. 9D). Remarkably, $CD39^+$ cells represented the larger part of the CD8 T cell population in Panc02 tumors (Fig. 9A).

In line with our data on activated CD4 Tcons, *in vitro* activation of CD8 T cells led to a two-fold increase in the percentage of cells expressing CD39 (Fig. 10). This suggested that activated CD8 T cells upregulate CD39 in order to hydrolyze the excess of ATP released upon activation. Furthermore, similar to Tcons, memory CD8 T cells were enriched in $CD39^+$ cells (Fig. 11). In particular, effector memory CD8 T cells contained the highest percentage of $CD39^+$ cells, and in the central memory compartment, the frequency of CD39-expressing cells was higher than in

naïve cells (Fig. 11A). Furthermore, the proportions of $CD39^+$ and $CD73^+$ subsets were significantly increased within IFN-γ-producing CD8 T cells. (Fig. 12A, B).

Taken together, these data suggest that CD39 and CD73 ectonucleotidase may play an important role in CD4 and CD8 T cell activation and memory. In particular, in recently activated T cells, they may mediate a negative feedback mechanism restricting excessive activation, proliferation and effector function. In memory T cells, these enzymes may serve to prevent adverse activation of recall response.

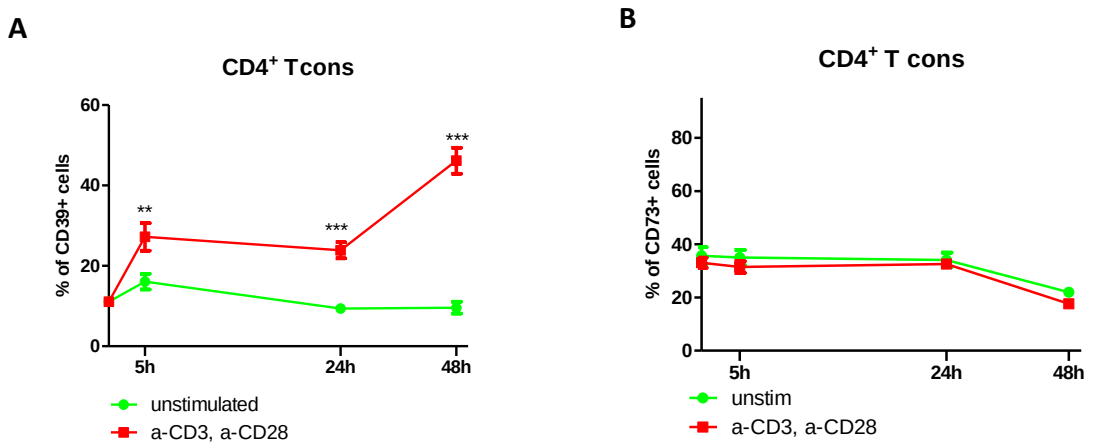


**Figure 5.** Analysis of the expression of CD39 **(A)** and CD73 **(B)** on CD4 Tcons from spleens of healthy mice after the activation of T cells with antibodies against CD3 and CD28 (measured by flow cytometry; n=6; **p <0.01; ***p<0.001).

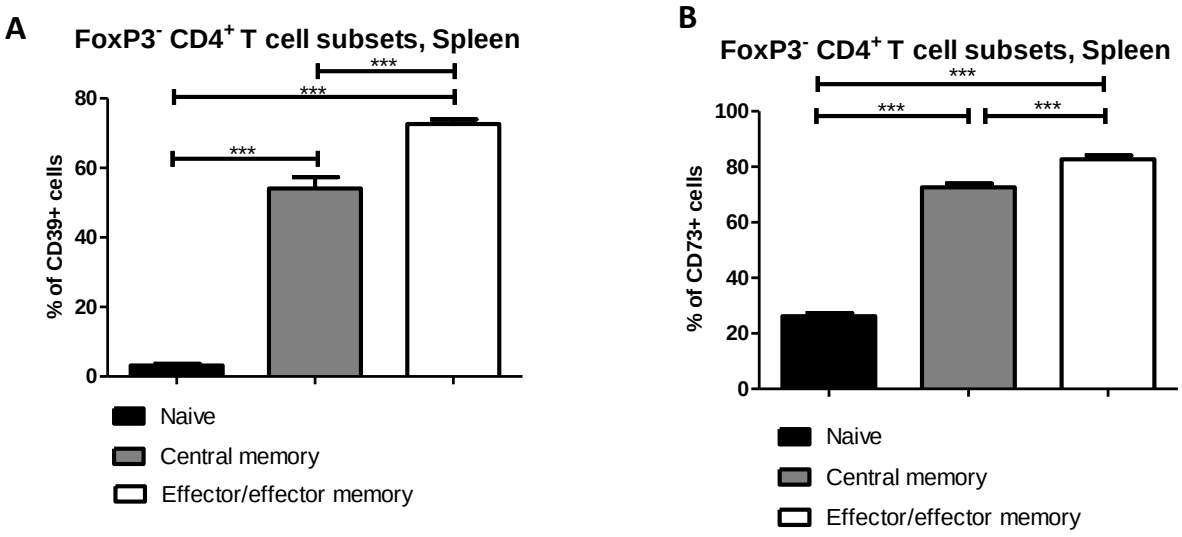


**Figure 6.** Expression of CD39 **(A)** and CD73 **(B)** on memory and naïve FoxP3$^-$ CD4 T cells in spleens of healthy mice (measured by flow cytometry; n=10; ***p<0.001).

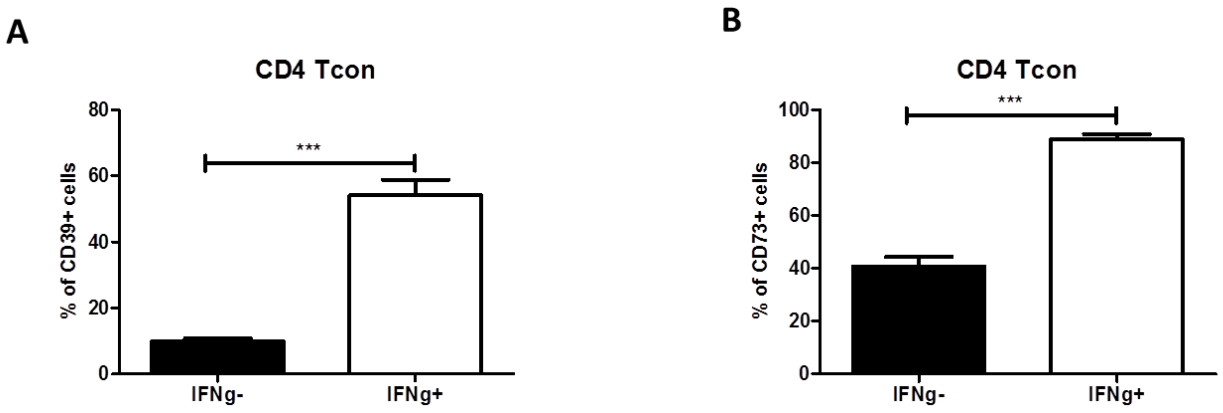


**Figure 7.** Expression of CD39 **(A)** and CD73 **(B)** on IFN-γ-producing CD4 Tcons from spleens of healthy mice (measured by flow cytometry; n=10; ***p<0.001).

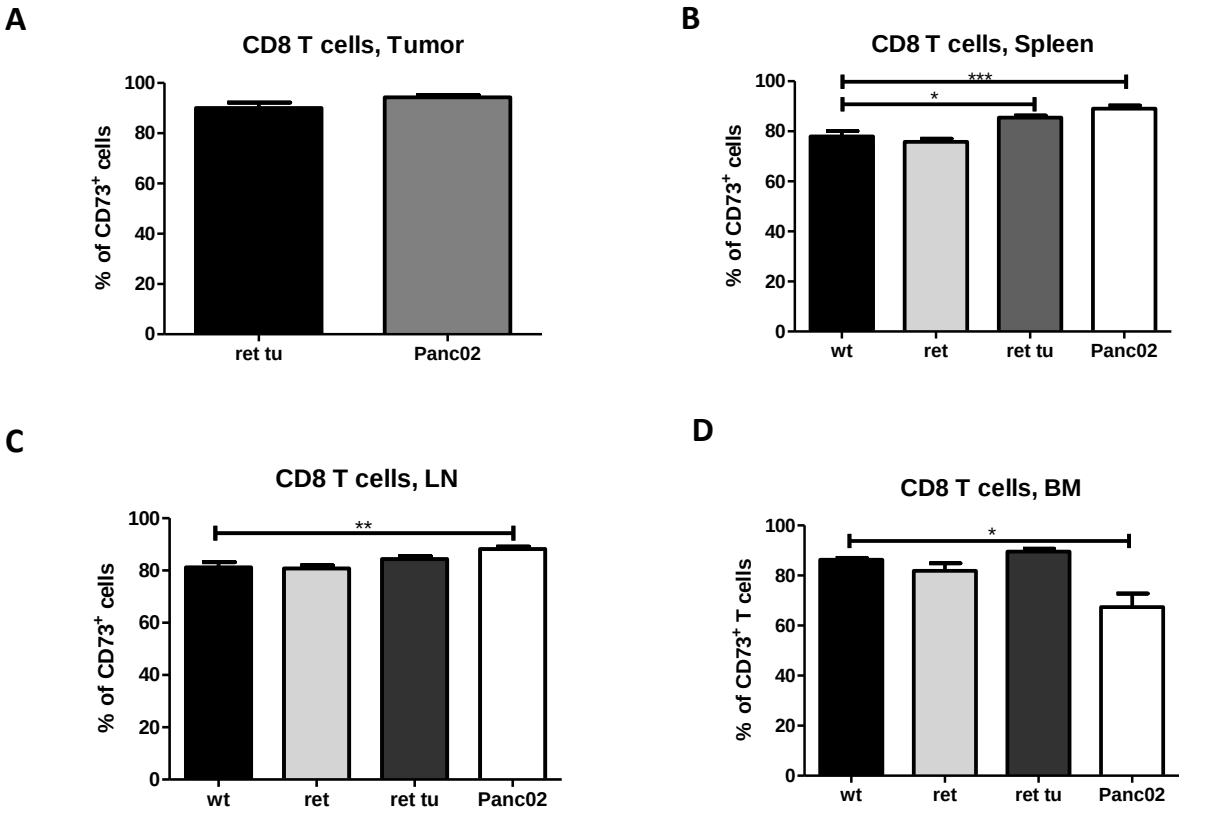


**Figure 8.** Analysis of CD73 expression on CD8 T cells in tumors **(A)** as well as in spleens **(B)**, lymph nodes **(C)** and bone marrow **(D)** of healthy wild type (wt) and Panc02 mice as well as *ret* tg mice with (ret tu) or without tumors (ret) (measured by flow cytometry; n=12; *p<0.05; **p<0.01; ***p<0.001).

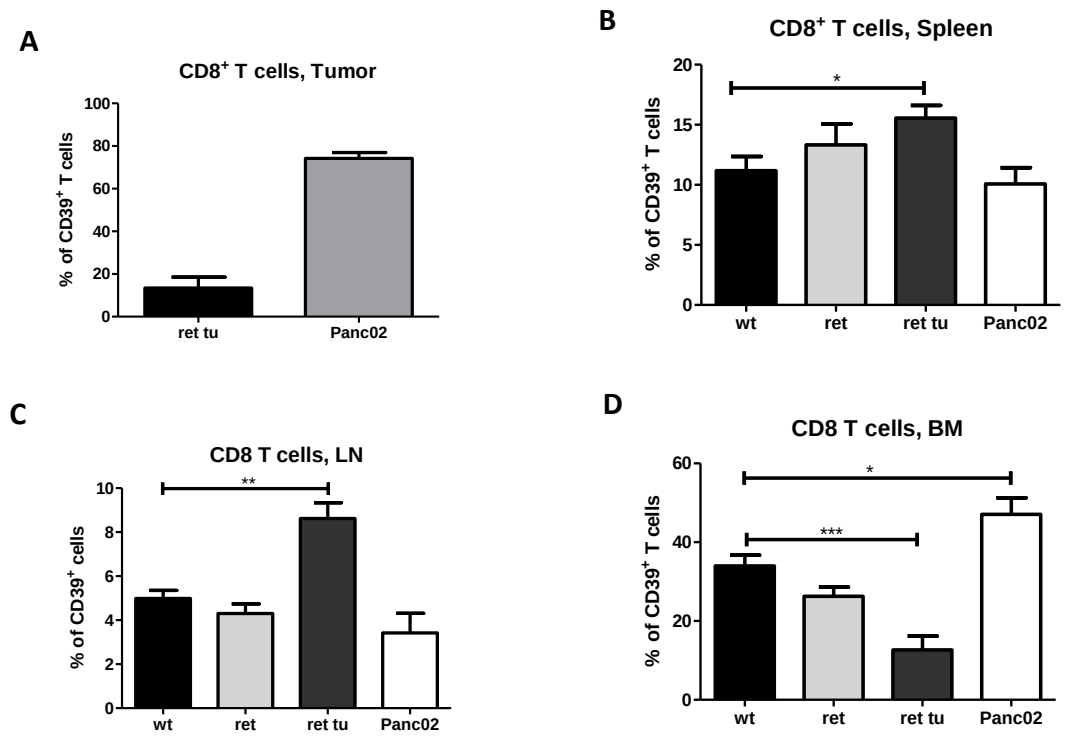


**Figure 9.** Analysis of CD39 expression on CD8 T cells in tumors **(A)**, spleens **(B),** lymph nodes **(C)** and bone marrow **(D)** of healthy wild type mice (wt), *ret* tg mice with (ret tu) or without tumors (ret) as well as mice with pancreatic adenocarcinoma (Panc02) (measured by flow cytometry; n=12; *p<0.05; **p<0.01; ***p<0.001).

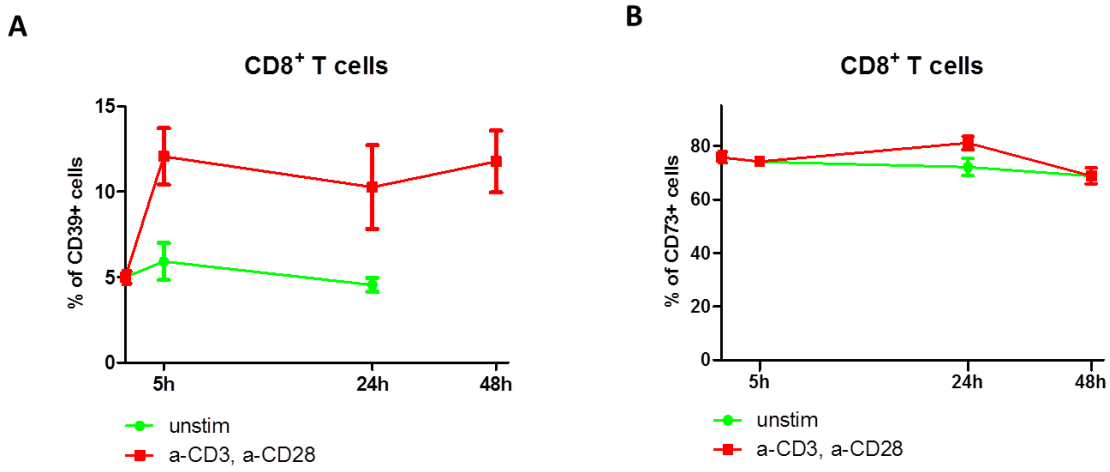


**Figure 10.** Analysis of the expression of CD39 **(A)** and CD73 **(B)** on CD4 Tcons from spleens of healthy mice after the activation of T cells with antibodies against CD3 and CD28 (measured by flow cytometry; n=6; ***p<0.001).

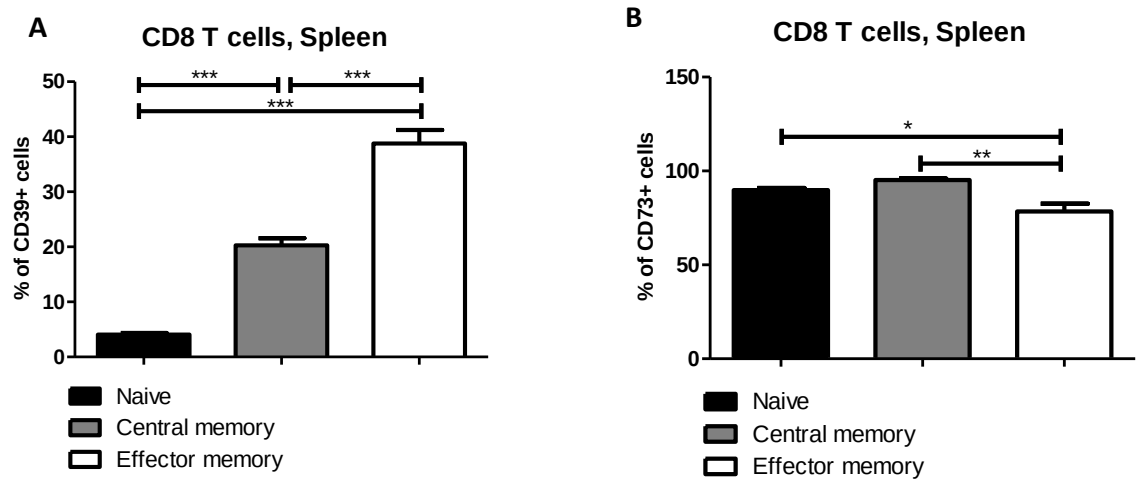


**Figure 11.** Expression of CD39 **(A)** and CD73 **(B)** on memory and naïve CD8 T cells in spleens of healthy mice (measured by flow cytometry; n=10; **p<0.01; ***p<0.001).

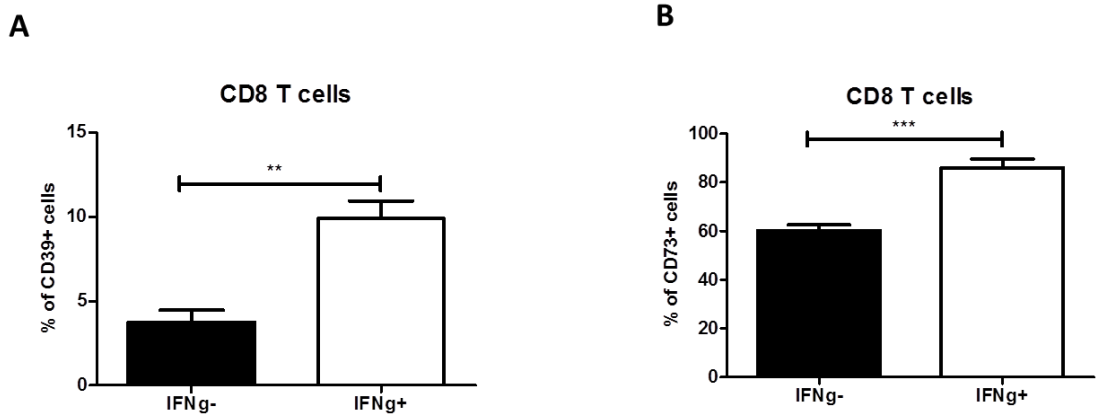


**Figure 12.** Expression of CD39 **(A)** and CD73 **(B)** on IFN-γ-producing CD8 T cells from spleens of healthy mice (measured by flow cytometry; n=10; **p<0.01; ***p<0.001).

### 4.3. Expression of ectonucleotidases on MDSCs

Since the role of CD39 and CD73 expressed on other immunosuppressive cells remains poorly understood, we addressed the question whether MDSCs can also contain the enzymatic cascade of adenosine synthesis (Fig. 13). We found that CD39 is constitutively expressed on both MDSC subpopulations (monocytic $CD11b^{+}Ly6C^{high}Ly6G^{-}$ and granulocytic $CD11b^{+}Ly6C^{-/low}Ly6G^{+}$ cells) from tumor-bearing mice (Fig. 14A). However, CD73 expression differed between granulocytic and monocytic subsets (Fig. 14B-E). In particular, approximately 20% of granulocytic IMCs (which are considered MDSC counterparts in healthy mice) expressed CD73, whereas on the monocytic IMC subset, CD73 was barely detectable (Fig. 14B, C, E). In contrast, $Ly6C^{-/low}$ MDSCs from tumor-bearing mice were highly enriched in $CD73^{+}$ cells (Fig. 14B-D). As regards to the monocytic $Ly6C^{high}$ MDSC subset, we found that the percentages of $CD73^{+}$ cells were slightly increased in spleens and tumors of *ret* transgenic mice, whereas there was a pronounced accumulation of $CD73^{+}$ monocytic MDSCs in Panc02 tumors (Fig. 14E). These data indicate that CD73 expression may represent another distinctive marker of MDSCs, and adenosine production may serve as a highly regulated mechanism of MDSC-mediated immunosuppression.

Thus, the expression of CD39 and CD73 on a variety of immune and malignant cells as well as strong immune suppressive properties of adenosine suggest that this molecule can provide an important mechanism of immune regulation and represent an attractive target for therapeutic modulation. Therefore, we targeted in *ret* transgenic melanoma mouse model either adenosine synthesis or signaling using a CD73 inhibitor (APCP) or an A2a receptor antagonist (SCH58261) respectively (Fig. 15A). Both treatments resulted in a significantly prolonged survival (please include median survival for all arms) of melanoma-bearing animals as compared to the non-treated group (Fig. 15B). To better understand the mechanisms behind the improved survival, we focused on the effects of APCP on the anti-tumor immunity. In tumor-bearing mice, we observed a substantial reduction in the frequency of MDSCs among tumor infiltrating leukocytes (Fig. 16A). In addition, we detected a tendency toward increased numbers of IFN-γ-producing CD8 T cells in metastatic lymph nodes upon the treatment (Fig. 16B). However, the frequency of T cells infiltrating skin tumors or metastatic lymph nodes was not changed.

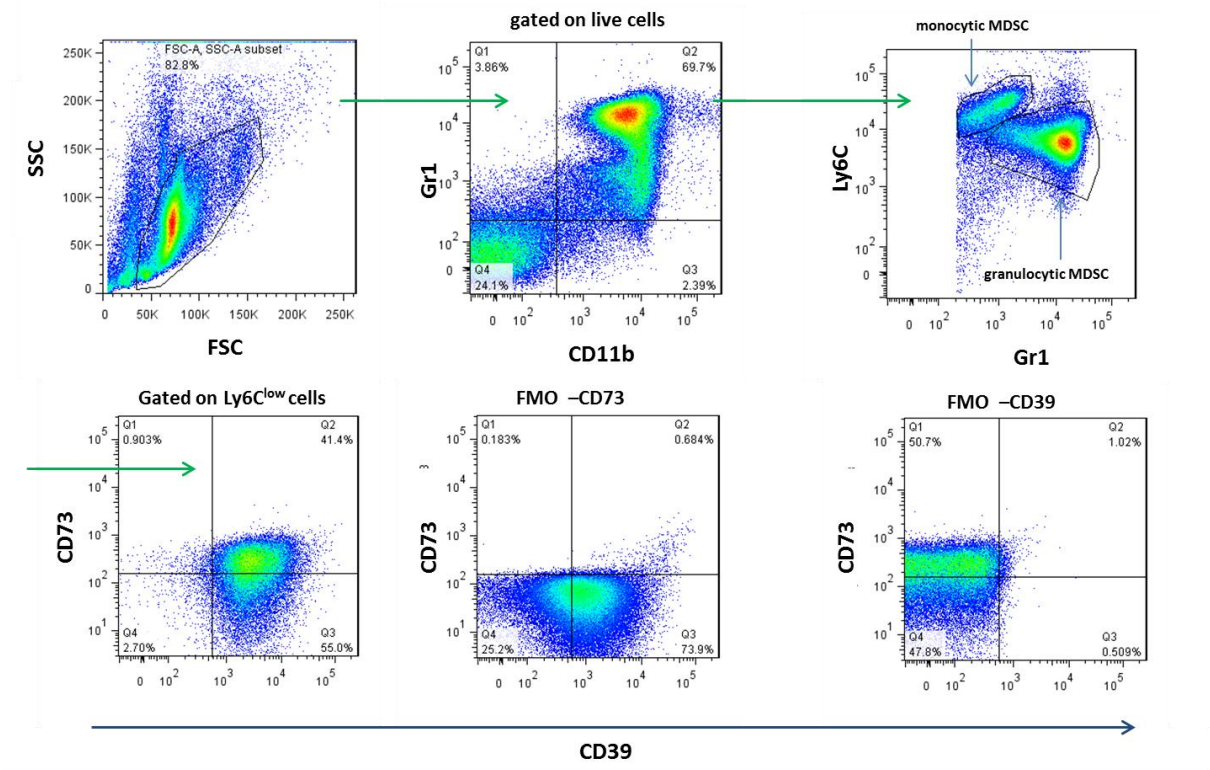


**Figure 13.** Representative dot plots of the bone marrow from *ret* tg melanoma-bearing mice showing the gating strategy for the analysis of CD73 and CD39 expression on MDSCs (measured by flow cytometry).

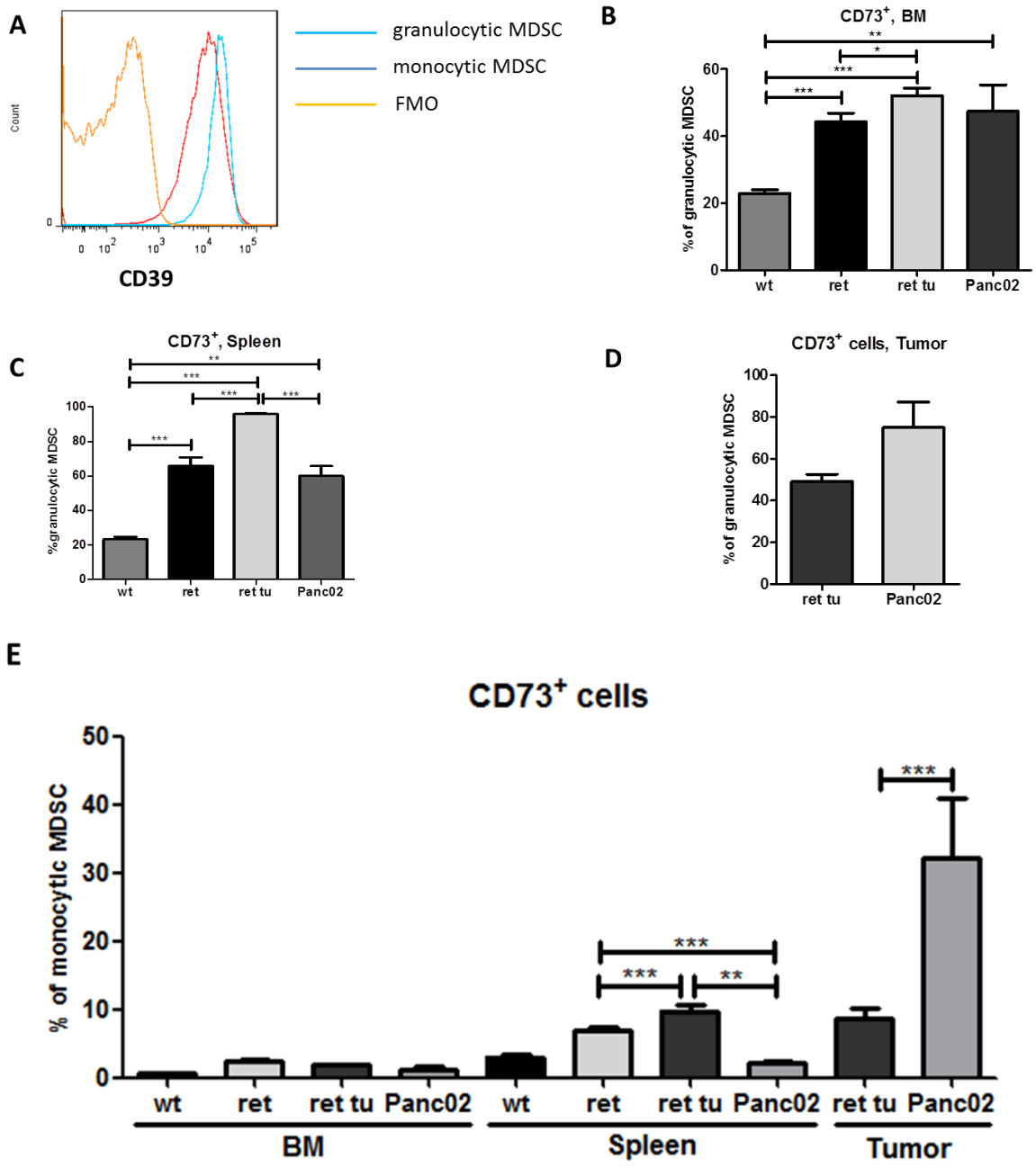


**Figure 14.** Expression of CD39 and CD73 on granulocytic and monocytic MDSC subsets detected by flow cytometry. **(A)** Representative histogram showing CD39 expression on BM-derived MDSCs. **(B-D)** Analysis of CD73 expression on granulocytic MDSCs from the bone marrow **(B)**, spleen **(C)** and tumors **(D).** **(E)** Analysis of CD73 expression on monocytic MDSC in lymphoid organs and tumors (n=10. *p<0.05; **p<0.01; ***p<0.001).

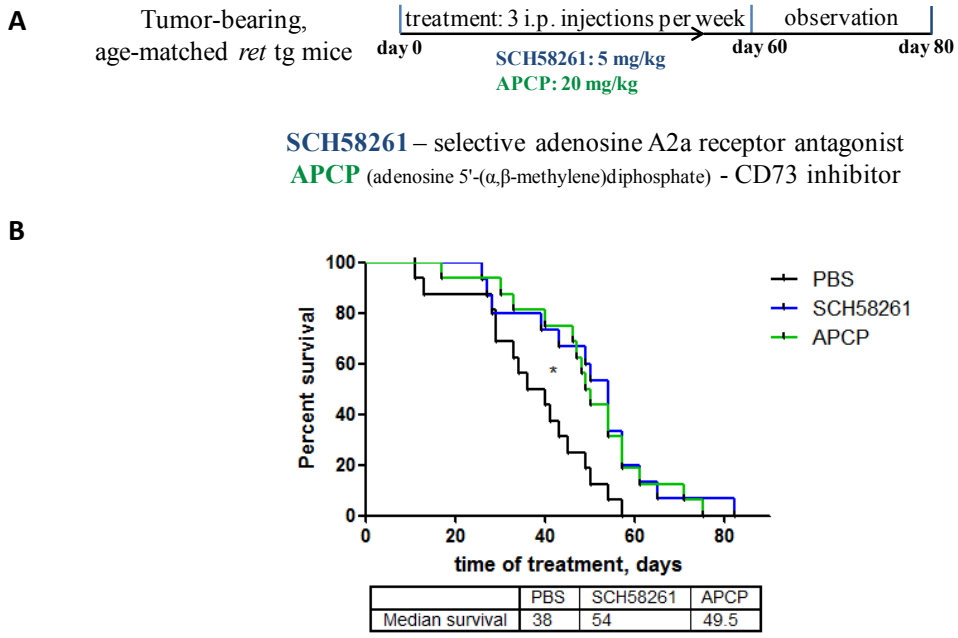


**Figure 15.** Effect of the selective adenosine A2a receptor antagonist SCH58261 and the CD73 inhibitor APCP on the survival of melanoma-bearing transgenic mice. **(A)** Treatment regimens; **(B)** ) Survival of mice (16 mice/group) is shown as a Kaplan-Meier curve (*p<0.05).

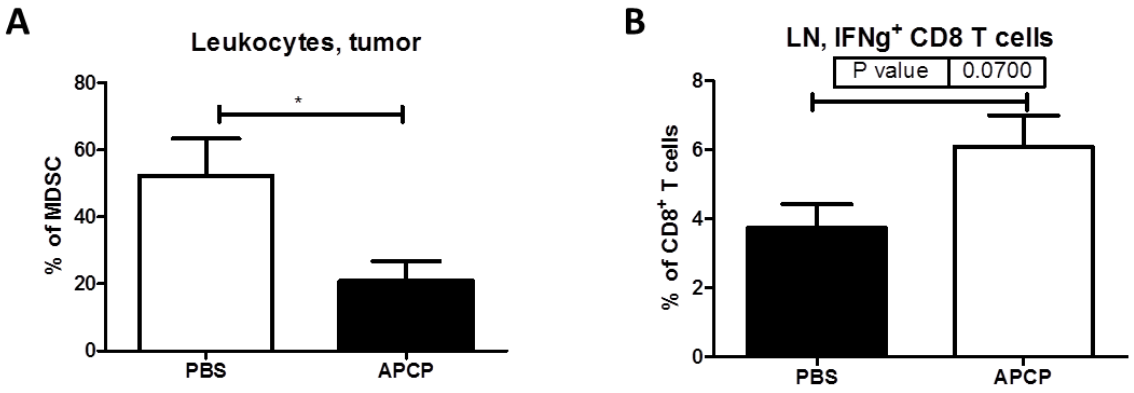


**Figure 16.** Effects of APCP treatment on MDSC frequencies among tumor-infiltrating leukocytes **(A)** and on amounts of IFN-γ-producing CD8 T cells in metastatic lymph nodes **(B)** (measured by flow cytometry; *p<0.05, n=16).

## 5. Discussion

The immune system deploys a variety of suppressive mechanisms to restrain excessive responses against pathogens or preclude harmful autoimmune reactions (35, 83, 183, 184). However, these mechanisms can be hijacked by tumors to subvert anti-cancer immunity or even involve immune cells in promoting tumor growth, angiogenesis and metastasis (1, 6, 39, 40, 82, 132, 174, 176, 185).

Extracellular adenosine is a small molecule, regulating the function of diverse immune cell populations and playing an important role in maintaining the balance between inflammation and tolerance (82, 186). Under homeostatic conditions, adenosine levels in tissues are relatively low (187). However, at the peak of inflammation, the destruction of host tissues by a vigorous immune response combined with damaged microcirculation and hypoxia set off multiple pathways, leading to the accumulation of adenosine in the extracellular space (187). Enhanced adenosine signaling prevents further tissue destruction by inhibiting the effector arm of the ongoing immune response. Tumors are often characterized by hypoxia and severe tissue damage, which trigger adenosine accumulation in the microenvironment and ultimately lead to the inhibition of an anti-tumor immunity (188). Adenosine receptors are expressed on a wide variety of immune cells (82), which makes them subject to adenosine-mediated regulation. Activation of adenosine receptors triggers accumulation of cAMP in target cells, leading to a profound alteration in their activity (82, 188). In particular, the engagement of adenosine receptors on effector T cells and resulting cAMP accumulation strongly inhibits their activation, function and proliferation (82, 188).

One of the main pathways, leading to adenosine accumulation outside the cell is the two-step hydrolysis of extracellular ATP by ectonucleotidases CD39 and CD73 (186). These proteins have been detected on various populations of immune cells as well as on tumor cells. In contrast to many other enzymes, CD39 and CD73 lack regulatory domains. Therefore, their net activity depends on their expression and the concentrations of the substrate, product and bivalent cations (129, 189-192). Importantly, CD73 is inhibited by high concentrations of ATP or ADP, underscoring the highly coordinated action of CD39 and CD73 (191). Ectonucleotidases play an important role in the regulation of the balance between the proinflammatory ATP and immunosuppressive adenosine. However, the specific contribution of particular immune cell

populations to the accumulation of extracellular adenosine is still poorly understood. Moreover, it is remains unclear whether adenosine produced by these enzymes acts in an autocrine or paracrine manner. In the current study, we addressed these questions by analyzing the patterns of ectonucleotidase expression on various immune cell subsets in the context of their phenotype.

### 5.1 Expression of ectonucleotidases on murine Tregs, Tcons and CD8 T cells

Previous studies suggested that extracellular adenosine production represents a major mechanism of Treg-mediated immune suppression (82, 83). Therefore, we decided to analyze the distribution of ectonucleotidases on Tregs from tumors and lymphoid organs to determine if adenosine production is a ubiquitous and constitutive function of Tregs and if its intensity could vary between different lymphoid organs and the tumor site. We found that, both in lymphoid organs and tumors, ectonucleotidases were expressed on the majority of Tregs, although we observed substantial differences between Tregs in spleens and tumors of mice with melanoma and pancreatic cancer. In particular, only 60% of Tregs from melanoma lesions of *ret* transgenic mice were $CD39^{+}CD73^{+}$, whereas more than 90% of Tregs from spleens and Panc02 tumors expressed both enzymes. Moreover, the levels of CD73 on Tregs from spleens of tumor-bearing *ret* transgenic mice were substantially lower than in non-transgenic littermates. These data suggest that ectonucleotidase expression on Tregs is neither constitutive nor ubiquitous, and Tregs from the tumor may strongly differ from Tregs in lymphoid organs in terms of their ability to produce adenosine. This indicates that adenosine production by Tregs can be negatively regulated by the tumor microenvironment.

Another important observation made in the same set of experiments was that ectonucleotidase expression is not restricted to the regulatory compartment of T cells. Although very few Tcons and CD8 T cells from the spleen coexpressed CD39 and CD73, their tumor-infiltrating counterparts were distinctly enriched in $CD39^{+}CD73^{+}$ cells. However, despite the low proportion of CD8 T cells expressing both enzymes, CD73 was ubiquitously expressed on the vast majority of CD8 T cells from lymphoid organs and tumors, suggesting that CD8 T cells may constitutively convert the environmental AMP into adenosine and prevent their own inappropriate activation. On the other hand, this might represent a homeostatic mechanism of maintaining CD8 T cell turnover, proliferation and survival at constant rates, since adenosine signaling has been shown to regulate all these processes (82, 83).

In contrast to the ubiquitously expressed CD73, the expression of CD39 varied significantly between lymphoid organs and tumor tissues as well as between healthy and tumor-bearing animals. Although in spleens and lymph nodes the percentage of $CD39^+$ cells was relatively low (around 5-10%), the CD8 T cell compartment in the bone marrow contained more than 30% or even up to 50% (in the Panc02 model) of CD39-expressing cells. Since the T cell pool in the bone marrow is largely comprised of memory cells, this led us to a hypothesis that ectonucleotidases might be involved in the function of these cells.

Collectively, these observations suggested a previously unappreciated role of CD39 and CD73 expression on effector T cells in the regulation of anti-tumor immunity and implied possible mechanisms of autocrine adenosine signaling in non-regulatory T cells.

## 5.2 Patterns of ectonucleotidase expression on Tregs and Tcons from the peripheral blood of cancer patients and healthy donors

To understand if the data on ectonucleotidase expression obtained in mouse models could be of relevance for the human immune system, we analyzed the expression of CD39 and CD73 in the peripheral blood of melanoma patients and healthy donors. We found that CD39 and CD73 expression on human Tregs and Tcons strongly varied not only among patients but also in healthy donors. Between some individuals, the differences in frequencies of $CD39^+$ or $CD73^+$ populations were more than ten-fold. Furthermore, the patterns of ectonucleotidase expression on human CD4 T cells showed little similarity to those observed in mice. In particular, the mean frequency of $CD39^+$ cells among human Tregs was around 50%. Moreover, CD73 was absent from the majority of Tregs. This was in agreement with the report by Mandapathil et al. (93), showing the lack of CD73 on Treg surface. Thus, most Tregs from human peripheral blood lack the enzymatic machinery that performs the two step conversion of extracellular ATP into adenosine. Therefore, the function of human Tregs is probably restricted to the hydrolysis of the pro-inflammatory ATP. In melanoma patients, we observed a significant expansion of the $CD39^+$ subpopulation of Tregs, which might reflect the enhanced capacity of Tregs to decrease ATP levels in the peripheral blood and thereby dampen the inflammation.

In contrast to Tregs, only 5-10% of human Tcons expressed CD39. The frequency of such cells doubled in patients with pancreatic cancer, compared to healthy donors. As regards to CD73, this

enzyme was expressed on 10-20% of Tcons from the peripheral blood. Notably, the percentage of $CD73^+$ cells in Tcons from melanoma patients was two times larger than in healthy donors.

Thus, ectonucleotidase expression on regulatory and conventional T cells is substantially increased in cancer patients, which may lead to an enhanced adenosine accumulation and contribute to the tumor associated immune suppression.

### 5.3 Activation-dependent induction of ectonucleotidases on T cells

To gain a better understanding of mechanisms governing ectonucleotidase expression on non-regulatory T cells, we investigated the patterns of CD39 and CD73 expression, depending on the phenotype, activation status and functional activity of T cells. We hypothesized that ectonucleotidases on Tcons and CD8 T cells might serve two distinct functions: 1) keep resting T cells in check by preventing an inappropriate activation; 2) provide an autocrine negative feedback loop to restrain a T cell response, once T cells have been activated. This would suggest that similar to CTLA-4 and PD-1 molecules, ectonucleotidases represent an immune checkpoint maintaining tolerance and regulating the duration and intensity of the immune response (6). Accordingly, we found that the $CD25^+$ subset of Tcons, presumably representing activated T cells (35), contain significantly higher percentages of $CD39^+$ and $CD73^+$ cells than their resting $CD25^-$ counterparts both in lymphoid organs (bone marrow, spleen, lymph nodes) and tumors. This was the case both for healthy and tumor-bearing animals. Notably, we observed that even resting $CD25^-$ Tcons in the bone marrow were highly enriched in $CD39^+$ and $CD73^+$ cells.

In addition, we observed that in pancreatic tumors, CD73 was expressed by virtually all Tcons, both $CD25^+$ and $CD25^-$. Moreover, CD39 was also expressed on a considerably higher proportion of Tcons than in spleens or lymph nodes. This indicates that microenvironmental factors might also induce ectonucleotidase expression, even on resting T cells. Indeed, we previously showed that Panc02 tumors contain increasingly high amounts of the immunosuppressive cytokine TGF-β. Furthermore, several recent studies demonstrated that this cytokine might be a major inducer of the ectonucleotidase expression on T cells. In particular, Chalmin et al. (193) showed that T cell polarization under the Th17-skewing conditions (the activation in the presence of TGF-β and IL-6), triggered the ectonucleotidase expression and adenosine production. The results obtained in that study suggested the necessity of both TGF-β and IL-6 for the upregulation of the ectonucleotidase expression. Remarkably, the authors showed that Th17 cells suppressed antigen-

specific T cell proliferation and function in an ectonucleotidase-dependent manner *in vitro* and *in vivo*. However, Regateiro et al. (194) demonstrated that TGF-β alone is sufficient for an activation-dependent upregulation of CD39 and CD73 both in $FoxP3^+$ and $FoxP3^-$ CD4 T cells. Importantly, *de novo* ectonucleotidase induction proved independent of FoxP3. Moreover, this study showed that CD73 is expressed on about 80% of CD8 T cells. Similarly to Tcons, activated CD8 T cells displayed a TGF-β-dependent upregulation of CD73 levels and a strong increase in the frequency of $CD39^+$ cells. Interestingly, although CD39 expression on CD8 T cells could be induced *de novo* by activation alone, the presence of TGF-β substantially increased the expression of the enzyme. In contrast to the results reported by Chalmin et al. (193), the study by Regaterio et al. (194) suggested that inflammatory cytokines, in particular IL-6, can antagonize the ectonucleotidase induction. Altogether, the results of these two studies suggested that activation signals and TGF-β strongly affect the ectonucleotidase expression on T cells.

Therefore, we next analyzed the impact of activation on the expression of CD39 and CD73. To this end, we stimulated splenocytes from healthy mice through the engagement of CD3 and CD28. Since we sought to determine the net effect of activation on ectonucleotidase expression, we did not add any polarizing cytokines. In contrast to the report of Regaterio et al. (194), we observed no significant difference in the percentage of $CD73^+$ T cells between the activated and intact cells. This apparent discrepancy between our *ex vivo* (increased percentage of $CD73^+$ cells among $CD25^+$ Tcons) and *in vitro* findings may be due to the artificial and non-physiological conditions of an *in vitro* T cell activation, which cannot recapitulate the *in vivo* TCR stimulation. However, in line with the study by Regaterio et al. (194) and our own *ex vivo* data, we found that the frequencies of $CD39^+$ cells among both CD4 and CD8 T cells significantly increased upon activation. It has been shown that activated T cells release increased amounts of ATP in order to promote their own activation through autocrine purinergic signaling (186, 195). Indeed, T cells express both ionotropic (P2X) and metabotropic (P2Y) ATP receptors (186). Recently, Schenk et al. (196) demonstrated that the TCR stimulation triggered a release of cellular ATP through pannexin-1 channels. Pannexin-1 translocates to the immune synapse where it releases ATP, which provides an autocrine positive feedback loop amplifying T cell activation (196). However, high concentration of extracellular ATP may trigger apoptosis of T cells (82). Therefore the mechanisms to dispose of excessive ATP are necessary. The rapid induction of CD39 upon the T cell activation may provide an effective means of controlling extracellular ATP levels during the

course of the T cell response. Furthermore, CD73 induction in activated T cells may dampen their function through accumulation of extracellular adenosine. For instance, the data of Romio et al. (90) indicated that NF-κB signaling (a major signaling pathway triggered by T cell activation) in stimulated T cells is regulated by the activity of CD73. A knockdown or inhibition of CD73 resulted in an augmented release of the pro-inflammatory cytokines IL-2, TNF-α and IFN-γ.

Thus, the rapid induction of CD39 and CD73 upon TCR stimulation may have profound effects on the development of the T cell response. In particular, CD39 can serve to degrade the excess of ATP released upon activation, thereby fine-tuning the stimulatory signals and preventing T cell apoptosis. Moreover, since the released ATP can also affect bystander cells such as DCs or NK cells, the activity of CD39 on activated T cells might also modulate the function of a local network of immune cells. In addition, the cleavage of ATP by CD39 yields AMP which is the substrate of CD73. Therefore, an activation-triggered CD39 induction on T cells can also increase the rate of adenosine production leading to the suppression of T cell functions. This can be ensured by the concomitant upregulation of CD73 on the same cells.

### 5.4 Ectonucleotidases on memory T cells

Immunological memory constitutes an essential feature of adaptive immunity, whereby an organism ‘remembers’ the original pathogen encountered and mounts more robust humoral and cellular responses to rapidly control reinfection and reduce the severity of disease (184). Compared to naïve lymphocytes, memory T and B cells display increased longevity and they do not require costimulatory signals for their activation (184).

Memory T cells develop from naive T cells after a stimulation with cognate antigen and exert a rapid and vigorous response to the previously encountered antigen (183, 184). The memory T cell compartment comprises two main subsets: central memory T cells and effector memory T cells (184). Central memory T cells express CD62L (L-selectin) and the CC-chemokine receptor 7 (CCR7), which enables them to circulate through lymphoid organs. In contrast, effector memory T cells lack both CD62L and CCR7, and they circulate in non-lymphoid tissues. In addition, these cells express high levels of effector molecules such as cytokines or granzyme B and perforin, which allows them to rapidly exert effector functions upon activation (184). However, despite numerous studies conducted over the last decades, the mechanisms governing the development and homeostasis of memory lymphocytes are still largely obscure.

Since extracellular adenosine profoundly dampens T cell activation, we hypothesized that it may also play a role in the regulation of T cell memory and effector function. To test this supposition, we evaluated the CD39 and CD73 expression on naïve as well as central and effector memory CD4 and CD8 T cells. We found that central and especially effector memory T cells (both Tcons and CD8) were highly enriched in CD39-expressing cells. The same pattern was observed for CD4 T cells in the case of CD73 expression, whereas all three CD8 T cell subsets were mostly comprised of $CD73^+$ cells. Next, we sought to determine if these correlations of ectonucleotidase with the T cell phenotype may have some functional implications. Since the rapid production of effector molecules upon stimulation is a cardinal feature of memory cells (197), we analyzed the expression of CD39 and CD73 on CD4 and CD8 T cells in the context of their ability to produce IFN-γ, a cytokine driving the Th1-type response (198). We found that both CD4 and CD8 T cells that rapidly produced IFN-γ after restimulation contained a higher percentage of ectonucleotidase-expressing cells as compared to the population unable to produce IFN-γ.

Thus, we showed that ectonucleotidase expression in T cells is largely associated with the memory compartment. Therefore, memory cells might constitutively produce adenosine from ATP to prevent their adverse activation and an outbreak of destructive autoimmunity. In this regard, it is logical that the effector memory subset contains a higher frequency of ectonucleotidase-expressing cells than the central memory cell population, since an inappropriate or unspecific activation of effector memory cells might entail more immediate and severe damages than those of central memory cells. In the same way, the increased percentage of ectonucleotidase-expressing subsets in IFN-γ-producing T cells subpopulations might be essential for controlling the activity of these cells through autrocrine adenosine signaling.

## 5.5 The role of ectonucleotidases on MDSCs

The heterogeneous population of MDSCs exploits a vast array of mechanisms to suppress the anti-tumor immunity (132). Some of these mechanisms are shared by all MDSC fractions while others are rather restricted to specific MDSC subsets (39, 40). Each of these suppressive pathways profoundly inhibits the activation, proliferation, migration and effector functions of T cells.

It has previously been demonstrated that autocrine purinergic signaling plays an important role in the regulation of myeloid cell chemotaxis (186, 199-202). For instance, ectonucleotidases have

been shown to regulate neutrophil chemotaxis by converting extracellular ATP into adenosine (201). Upon their activation with bacterial formylated peptides, neutrophils release ATP through pannexin-1 hemichannels (199, 200). ATP signaling through P2Y2 receptors augments the neutrophil activation and enhances their sensitivity to chemotactic gradients (186, 199, 200). Moreover, P2Y2-signaling triggers cell polarization within the gradient field and induces the translocation of pannexin-1, ectonucleotidases and A3 receptors to the leading edge of the cell (186, 199-201). The clustering of these proteins focuses ATP release and adenosine production at the leading edge and results in the autocrine signaling through A3 receptors, promoting cell migration towards the source of chemoattractants (186, 199-201).

A recent study by Ryzhov et al. (151) suggested that both auto- and paracrine purinergic signaling substantially contribute to the MDSC function. First, the authors detected CD73 on *in vitro* differentiated MDSCs. The expression of CD73 on a per-cell level was the highest in the $CD11b^{+}Gr1^{high}$ subset followed by the $CD11b^{+}Gr1^{int}$ and $CD11b^{+}Gr1^{low}$ fractions. Importantly, the expression of CD73 on the *in vitro* differentiated MDSCs positively correlated with its enzymatic activity. This was in line with the analyses of CD73 crystal structure and *ex vivo* assays showing that the activity of CD73 depends primarily on its expression and the substrate/product ratio and can be described by conventional Michaelis-Menten kinetics (129, 189-192). Furthermore, Ryzhov et al. (151) showed that A2BR-signaling strongly augments the expansion and immunosuppressive activity of $CD11b^{+}Gr1^{high}$ cells both *in vitro* and *in vivo.* This implied that adenosine production by MDSCs contributes to their suppressive properties in a dual manner. On one hand, it can modulate the activity of other cells in the local microenvironment, inhibiting effector T cells and promoting Tregs through the accumulation of intracellular cAMP. On the other hand, autocrine adenosine production can increase the numbers and activity of granulocytic MDSCs. However, this study demonstrated only CD73 expression and activity on MDSCs that were differentiated *in vitro*. Furthermore, CD39 expression on MDSCs was not investigated.

Therefore, we asked if these findings are relevant to the *in vivo* situation. To this end, we studied the expression of CD39 and CD73 on MDSCs from tumor-bearing mice, as well as on $CD11b^{+}Gr1^{+}$ IMCs from healthy animals. Since the granulocytic ($Ly6G^{high}Ly6C^{low/-}$) and monocytic ($Ly6G^{-}Ly6C^{high}$) subsets of MDSCs have been shown to differ in their suppressive

mechanisms, we separately analyzed the ectonucleotidase expression in each of these subsets. We observed that both IMCs and MDSCs constitutively and ubiquitously express CD39, suggesting that all such cells have the capacity to hydrolyze ATP. This corresponds to the findings indicating the important role of CD39 in myeloid cell migration and function (186, 199-203). In contrast, CD73 expression showed considerable heterogeneity. Analyzing the granulocytic subset of $CD11b^+Gr1^+$ cells, we found that IMCs from the spleen and bone marrow contained a substantial population of CD73-expressing cells. However, MDSCs from respective lymphoid organs harbored distinctly higher percentages of $CD73^+$ cells. This could be linked to the observed expansion of $CD11b^+Gr1^+$ cells in both tumor models (168; Karakhanova et al., upublished data). Therefore, these data suggest that ectonucleotidases not only contributes to MDSC suppressive function but can also promote MDSC expansion through autocrine signaling.

Granulocytic MDSCs both from melanoma lesions and pancreatic tumors were also highly enriched in $CD73^+$ cells. Given the high numbers of tumor-infiltrating granulocytic MDSCs in both models (168), these cells can contribute to the accumulation of extracellular adenosine in the tumor microenvironment. Of note, we observed a particularly high frequency of $CD73^+$ cells among the granulocytic MDSCs from Panc02 tumors. These tumors contain high levels of TGF-β, which has been shown to induce CD73 expression on T cells through the downregulation of the transcription factor Gfi-1 (193). It has been reported that this factor plays in important role in the differentiation of neutrophils and its genetic ablation leads to the expansion of immature $CD11b^+Gr1^+$ cells (204). Therefore, the abundance of TGF-β may account for the high frequency of the $CD73^+$ granulocytic MDSCs as well as for the overall expansion of MDSCs in Panc02 tumors.

As regards to the monocytic subset of MDSCs, we showed that only a minor proportion (less than 10%) of these cells expressed $CD73^+$. In contrast, the $CD73^+$ subset comprised up to 40% of the monocytic MDSCs in Panc02 tumors, which might also be a consequence of the high TGF-β levels in the tumor microenvironment. Thus, we showed that MDSCs are highly enriched in CD73-expressing cells, as compared to $CD11b^+Gr1^+$ IMC, and CD73 expression is largely restricted to the granulocytic MDSC subset. In addition, the CD73 expression can be induced on tumor-infiltrating monocytic MDSCs.

Since MDSCs have previously been shown to strongly suppress the anti-tumor response in *ret* transgenic mice (168), we asked if the inhibition of the CD73 activity *in vivo* might hamper MDSC expansion and function, thereby restoring anti-tumor immune responses. To this end, we treated *ret* transgenic tumor-bearing mice with a CD73 inhibitor APCP and analyzed its effects on the survival and the immune system. Similarly to previous studies based on transplantable mouse tumor models (127, 129, 131, 205), the treatment significantly improved the survival of tumor-bearing animals. In addition, this therapy substantially reduced the frequency of MDSCs among tumor-infiltrating leukocytes, suggesting a possible role of adenosine signaling in the MDSC expansion. However, the treatment had no statistically significant effects on T cell frequencies in tumor lesions or lymphoid organs, on the TCR ζ-chain expression in these cells or on the IFN-γ production by these T cells. Therefore, the beneficial effect of the therapy can be partly due to the decrease in the numbers of MDSCs since the latter lave been shown to directly promote tumor development, metastasis and angiogenesis (39). However, other effects of the CD73 inhibitor APCP cannot be excluded and need further investigation.

To summarize, we showed that CD39 and CD73 are highly expressed by murine regulatory T cells. However, on human Tregs these ectonucleotidases are expressed to the less extent than on mouse cells. We have also detected ectonucleotidase expression on $CD4^{+}FoxP3^{-}$ conventional T cells and CD8 T cells. Importantly, $CD25^{+}$ Tcon, tumor-infiltrating T cells and memory T cells were all highly enriched in $CD39^{+}$ and $CD73^{+}$ cells, which implies an important role of ectonucleotidases in the regulation of T cell activation, effector function and memory.

Furthermore, we demonstrated for the first time that the majority of granulocytic MDSCs coexpress CD39 and CD73, which suggests that the production of extracellular adenosine might contribute to MDSC immunosuppressive properties. Finally, using a clinically relevant *ret* transgenic model of melanoma we showed that the inhibition of adenosine production or signaling prolonged survival of tumor-bearing mice and reduced MDSC infiltration into tumors, indicating that this might be a promising strategy for cancer therapy.

# REFERENCES


1. Schreiber RD, Old LJ, Smyth MJ. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331: 1565-70
2. Clark WH, Jr., Elder DE, Guerry Dt, Braitman LE, Trock BJ, Schultz D, Synnestvedt M, Halpern AC. 1989. Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst* 81: 1893-904
3. Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H. 1998. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 58: 3491-4
4. Oleinika K, Nibbs RJ, Graham GJ, Fraser AR. 2013. Suppression, subversion and escape: the role of regulatory T cells in cancer progression. *Clin Exp Immunol* 171: 36-45
5. Trinchieri G. 2012. Cancer and inflammation: an old intuition with rapidly evolving new concepts. *Annu Rev Immunol* 30: 677-706
6. Pardoll DM. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12: 252-64
7. Restifo NP, Dudley ME, Rosenberg SA. 2012. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol* 12: 269-81
8. Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mule JJ, Rosenberg SA, Bennink JR. 1993. Identification of human cancers deficient in antigen processing. *J Exp Med* 177: 265-72
9. Hadrup S, Donia M, Thor Straten P. 2012. Effector CD4 and CD8 T Cells and Their Role in the Tumor Microenvironment. *Cancer Microenviron*
10. Mendez R, Aptsiauri N, Del Campo A, Maleno I, Cabrera T, Ruiz-Cabello F, Garrido F, Garcia-Lora A. 2009. HLA and melanoma: multiple alterations in HLA class I and II expression in human melanoma cell lines from ESTDAB cell bank. *Cancer Immunol Immunother* 58: 1507-15
11. Offringa R. 2009. Antigen choice in adoptive T-cell therapy of cancer. *Curr Opin Immunol* 21: 190-9
12. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE, Dudley ME. 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* 17: 4550-7
13. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C, Rosenberg SA. 2011. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 29: 917-24
14. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254: 1643-7
15. Overwijk WW, Restifo NP. 2000. Autoimmunity and the immunotherapy of cancer: targeting the "self" to destroy the "other". *Crit Rev Immunol* 20: 433-50
16. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman SA, Maric I, Raffeld M, Nathan DA, Lanier BJ, Morgan RA, Rosenberg SA. 2010. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* 116: 4099-102
17. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, Miki T, Rosenberg SA. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc Natl Acad Sci U S A* 91: 3515-9

18. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM, Rosenberg SA. 2009. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114: 535-46
19. Yeh S, Karne NK, Kerkar SP, Heller CK, Palmer DC, Johnson LA, Li Z, Bishop RJ, Wong WT, Sherry RM, Yang JC, Dudley ME, Restifo NP, Rosenberg SA, Nussenblatt RB. 2009. Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. *Ophthalmology* 116: 981-9 e1
20. Davies MA, Samuels Y. 2010. Analysis of the genome to personalize therapy for melanoma. *Oncogene* 29: 5545-55
21. Matsushita H, Vesely MD, Koboldt DC, Rickert CG, Uppaluri R, Magrini VJ, Arthur CD, White JM, Chen YS, Shea LK, Hundal J, Wendl MC, Demeter R, Wylie T, Allison JP, Smyth MJ, Old LJ, Mardis ER, Schreiber RD. 2012. Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature* 482: 400-4
22. Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, Rosenberg SA. 1996. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med* 183: 1185-92
23. Hofmann O, Caballero OL, Stevenson BJ, Chen YT, Cohen T, Chua R, Maher CA, Panji S, Schaefer U, Kruger A, Lehvaslaiho M, Carninci P, Hayashizaki Y, Jongeneel CV, Simpson AJ, Old LJ, Hide W. 2008. Genome-wide analysis of cancer/testis gene expression. *Proc Natl Acad Sci U S A* 105: 20422-7
24. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, Chen YT, Chua R, Gurung S, Gnjatic S, Jungbluth AA, Caballero OL, Bairoch A, Kiesler E, White SL, Simpson AJ, Old LJ, Camargo AA, Vasconcelos AT. 2009. CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. *Nucleic Acids Res* 37: D816-9
25. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. 2005. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 5: 615-25
26. Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW. 2011. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer* 105: 93-103
27. Azimi F, Scolyer RA, Rumcheva P, Moncrieff M, Murali R, McCarthy SW, Saw RP, Thompson JF. 2012. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. *J Clin Oncol* 30: 2678-83
28. Fridman WH, Galon J, Pages F, Tartour E, Sautes-Fridman C, Kroemer G. 2011. Prognostic and predictive impact of intra- and peritumoral immune infiltrates. *Cancer Res* 71: 5601-5
29. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313: 1960-4
30. Andersen MH, Sorensen RB, Brimnes MK, Svane IM, Becker JC, thor Straten P. 2009. Identification of heme oxygenase-1-specific regulatory CD8+ T cells in cancer patients. *J Clin Invest* 119: 2245-56
31. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. 2010. Regulatory T cells in cancer. *Adv Cancer Res* 107: 57-117
32. Klebanoff CA, Gattinoni L, Restifo NP. 2006. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunol Rev* 211: 214-24

33. Cho YA, Yoon HJ, Lee JI, Hong SP, Hong SD. 2011. Relationship between the expressions of PD-L1 and tumor-infiltrating lymphocytes in oral squamous cell carcinoma. *Oral Oncol* 47: 1148-53
34. Gao Q, Wang XY, Qiu SJ, Yamato I, Sho M, Nakajima Y, Zhou J, Li BZ, Shi YH, Xiao YS, Xu Y, Fan J. 2009. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. *Clin Cancer Res* 15: 971-9
35. Josefowicz SZ, Lu LF, Rudensky AY. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30: 531-64
36. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. 2010. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 10: 490-500
37. Badoual C, Hans S, Fridman WH, Brasnu D, Erdman S, Tartour E. 2009. Revisiting the prognostic value of regulatory T cells in patients with cancer. *J Clin Oncol* 27: e5-6; author reply e7
38. Byrne WL, Mills KH, Lederer JA, O'Sullivan GC. 2011. Targeting regulatory T cells in cancer. *Cancer Res* 71: 6915-20
39. Umansky V, Sevko A. 2012. Tumor Microenvironment and Myeloid-Derived Suppressor Cells. *Cancer Microenviron*
40. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. 2012. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 12: 253-68
41. Erdman SE, Rao VP, Olipitz W, Taylor CL, Jackson EA, Levkovich T, Lee CW, Horwitz BH, Fox JG, Ge Z, Poutahidis T. 2010. Unifying roles for regulatory T cells and inflammation in cancer. *Int J Cancer* 126: 1651-65
42. Erdman SE, Poutahidis T. 2010. Cancer inflammation and regulatory T cells. *Int J Cancer* 127: 768-79
43. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, Mosseri V, Laccourreye O, Bruneval P, Fridman WH, Brasnu DF, Tartour E. 2006. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res* 12: 465-72
44. Campbell DJ, Koch MA. 2011. Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nat Rev Immunol* 11: 119-30
45. Pere H, Tanchot C, Bayry J, Terme M, Taieb J, Badoual C, Adotevi O, Merillon N, Marcheteau E, Quillien VR, Banissi C, Carpentier A, Sandoval F, Nizard M, Quintin-Colonna F, Kroemer G, Fridman WH, Zitvogel L, Oudard SP, Tartour E. 2012. Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer. *Oncoimmunology* 1: 326-33
46. Tanchot C, Terme M, Pere H, Tran T, Benhamouda N, Strioga M, Banissi C, Galluzzi L, Kroemer G, Tartour E. 2012. Tumor-Infiltrating Regulatory T Cells: Phenotype, Role, Mechanism of Expansion In Situ and Clinical Significance. *Cancer Microenviron*
47. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6: 1219-27
48. Kretschmer K, Heng TS, von Boehmer H. 2006. De novo production of antigen-specific suppressor cells in vivo. *Nat Protoc* 1: 653-61
49. Verginis P, McLaughlin KA, Wucherpfennig KW, von Boehmer H, Apostolou I. 2008. Induction of antigen-specific regulatory T cells in wild-type mice: visualization and targets of suppression. *Proc Natl Acad Sci U S A* 105: 3479-84
50. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS. 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature* 478: 250-4

51. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. 2007. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol* 178: 4022-6
52. Burchill MA, Yang J, Vang KB, Moon JJ, Chu HH, Lio CW, Vegoe AL, Hsieh CS, Jenkins MK, Farrar MA. 2008. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity* 28: 112-21
53. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, Shevach EM, O'Shea J J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26: 371-81
54. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6: 1142-51
55. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 212: 28-50
56. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737-42
57. Shalev I, Schmelzle M, Robson SC, Levy G. 2011. Making sense of regulatory T cell suppressive function. *Semin Immunol* 23: 282-92
58. Zhou G, Levitsky HI. 2007. Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance. *J Immunol* 178: 2155-62
59. Bilate AM, Lafaille JJ. 2012. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu Rev Immunol* 30: 733-58
60. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* 463: 808-12
61. Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, Umetsu DT, Rudensky AY. 2012. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature* 482: 395-9
62. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. 2012. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell* 150: 29-38
63. Miyao T, Floess S, Setoguchi R, Luche H, Fehling HJ, Waldmann H, Huehn J, Hori S. 2012. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* 36: 262-75
64. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203: 1701-11
65. Allan SE, Alstad AN, Merindol N, Crellin NK, Amendola M, Bacchetta R, Naldini L, Roncarolo MG, Soudeyns H, Levings MK. 2008. Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. *Mol Ther* 16: 194-202
66. Simonetta F, Chiali A, Cordier C, Urrutia A, Girault I, Bloquet S, Tanchot C, Bourgeois C. 2010. Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells. *Eur J Immunol* 40: 2528-38
67. Kleinewietfeld M, Starke M, Di Mitri D, Borsellino G, Battistini L, Rotzschke O, Falk K. 2009. CD49d provides access to "untouched" human Foxp3+ Treg free of contaminating effector cells. *Blood* 113: 827-36

68. Peters JH, Preijers FW, Woestenenk R, Hilbrands LB, Koenen HJ, Joosten I. 2008. Clinical grade Treg: GMP isolation, improvement of purity by CD127 Depletion, Treg expansion, and Treg cryopreservation. *PLoS One* 3: e3161
69. Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, Nomura T, Sakaguchi S. 2007. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity* 27: 145-59
70. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 8: 1353-62
71. Bachmann MF, Kohler G, Ecabert B, Mak TW, Kopf M. 1999. Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. *J Immunol* 163: 1128-31
72. Miyara M, Sakaguchi S. 2007. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med* 13: 108-16
73. Curti A, Pandolfi S, Valzasina B, Aluigi M, Isidori A, Ferri E, Salvestrini V, Bonanno G, Rutella S, Durelli I, Horenstein AL, Fiore F, Massaia M, Colombo MP, Baccarani M, Lemoli RM. 2007. Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25- into CD25+ T regulatory cells. *Blood* 109: 2871-7
74. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, Hipkiss EL, Ravi S, Kowalski J, Levitsky HI, Powell JD, Pardoll DM, Drake CG, Vignali DA. 2004. Role of LAG-3 in regulatory T cells. *Immunity* 21: 503-13
75. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, Flores M, Li N, Schweighoffer E, Greenberg S, Tybulewicz V, Vignali D, Clynes R. 2008. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol* 180: 5916-26
76. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D, Grogan JL. 2009. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* 10: 48-57
77. von Boehmer H. 2005. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 6: 338-44
78. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR, Jr., Muller W, Rudensky AY. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28: 546-58
79. Marie JC, Liggitt D, Rudensky AY. 2006. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity* 25: 441-54
80. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, Stoll S, Schild H, Staege MS, Stassen M, Jonuleit H, Schmitt E. 2007. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204: 1303-10
81. Allard B, Turcotte M, Stagg J. 2012. CD73-generated adenosine: orchestrating the tumor-stroma interplay to promote cancer growth. *J Biomed Biotechnol* 2012: 485156
82. Stagg J, Smyth MJ. 2010. Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 29: 5346-58
83. Beavis PA, Stagg J, Darcy PK, Smyth MJ. 2012. CD73: a potent suppressor of antitumor immune responses. *Trends Immunol* 33: 231-7
84. Fredholm BB, Chern Y, Franco R, Sitkovsky M. 2007. Aspects of the general biology of adenosine A2A signaling. *Prog Neurobiol* 83: 263-76

85. Novitskiy SV, Ryzhov S, Zaynagetdinov R, Goldstein AE, Huang Y, Tikhomirov OY, Blackburn MR, Biaggioni I, Carbone DP, Feoktistov I, Dikov MM. 2008. Adenosine receptors in regulation of dendritic cell differentiation and function. *Blood* 112: 1822-31
86. Majumdar S, Aggarwal BB. 2003. Adenosine suppresses activation of nuclear factor-kappaB selectively induced by tumor necrosis factor in different cell types. *Oncogene* 22: 1206-18
87. Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MK, Huang X, Caldwell S, Liu K, Smith P, Chen JF, Jackson EK, Apasov S, Abrams S, Sitkovsky M. 2006. A2A adenosine receptor protects tumors from antitumor T cells. *Proc Natl Acad Sci U S A* 103: 13132-7
88. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204: 1257-65
89. Whiteside TL, Mandapathil M, Schuler P. 2011. The role of the adenosinergic pathway in immunosuppression mediated by human regulatory T cells (Treg). *Curr Med Chem* 18: 5217-23
90. Romio M, Reinbeck B, Bongardt S, Huls S, Burghoff S, Schrader J. 2011. Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells. *Am J Physiol Cell Physiol* 301: C530-9
91. Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, Franchi L, Vandenabeele P, Nunez G. 2007. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 26: 433-43
92. Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. 2009. Purinergic signalling in the nervous system: an overview. *Trends Neurosci* 32: 19-29
93. Mandapathil M, Hilldorfer B, Szczepanski MJ, Czystowska M, Szajnik M, Ren J, Lang S, Jackson EK, Gorelik E, Whiteside TL. 2010. Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. *J Biol Chem* 285: 7176-86
94. Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. 2012. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol* 13: e32-42
95. Le DT, Jaffee EM. 2012. Regulatory T-cell modulation using cyclophosphamide in vaccine approaches: a current perspective. *Cancer Res* 72: 3439-44
96. Polak L, Turk JL. 1974. Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. *Nature* 249: 654-6
97. Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, Okoye FI, Jaffee EM. 2001. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res* 61: 3689-97
98. Ghiringhelli F, Larmonier N, Schmitt E, Parcellier A, Cathelin D, Garrido C, Chauffert B, Solary E, Bonnotte B, Martin F. 2004. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 34: 336-44
99. Ge Y, Domschke C, Stoiber N, Schott S, Heil J, Rom J, Blumenstein M, Thum J, Sohn C, Schneeweiss A, Beckhove P, Schuetz F. 2012. Metronomic cyclophosphamide treatment in metastasized breast cancer patients: immunological effects and clinical outcome. *Cancer Immunol Immunother* 61: 353-62
100. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. 2005. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood* 105: 2862-8

101. Camisaschi C, Filipazzi P, Tazzari M, Casati C, Beretta V, Pilla L, Patuzzo R, Maurichi A, Cova A, Maio M, Chiarion-Sileni V, Tragni G, Santinami M, Vergani B, Villa A, Berti E, Umansky L, Beckhove P, Umansky V, Parmiani G, Rivoltini L, Castelli C. 2013. Effects of cyclophosphamide and IL-2 on regulatory CD4(+) T cell frequency and function in melanoma patients vaccinated with HLA-class I peptides: impact on the antigen-specific T cell response. *Cancer Immunol Immunother* 62: 897-908
102. Weiss VL, Lee TH, Song H, Kouo TS, Black CM, Sgouros G, Jaffee EM, Armstrong TD. 2012. Trafficking of high avidity HER-2/neu-specific T cells into HER-2/neu-expressing tumors after depletion of effector/memory-like regulatory T cells. *PLoS One* 7: e31962
103. Ercolini AM, Ladle BH, Manning EA, Pfannenstiel LW, Armstrong TD, Machiels JP, Bieler JG, Emens LA, Reilly RT, Jaffee EM. 2005. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J Exp Med* 201: 1591-602
104. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. 1999. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 59: 3128-33
105. Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN. 2004. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J Exp Med* 200: 771-82
106. Nair S, Boczkowski D, Fassnacht M, Pisetsky D, Gilboa E. 2007. Vaccination against the forkhead family transcription factor Foxp3 enhances tumor immunity. *Cancer Res* 67: 371-80
107. Matsushita N, Pilon-Thomas SA, Martin LM, Riker AI. 2008. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. *J Immunol Methods* 333: 167-79
108. Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, Toes RE, Offringa R, Melief CJ. 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 194: 823-32
109. Ramirez-Montagut T, Chow A, Hirschhorn-Cymerman D, Terwey TH, Kochman AA, Lu S, Miles RC, Sakaguchi S, Houghton AN, van den Brink MR. 2006. Glucocorticoid-induced TNF receptor family related gene activation overcomes tolerance/ignorance to melanoma differentiation antigens and enhances antitumor immunity. *J Immunol* 176: 6434-42
110. Jacobs JF, Punt CJ, Lesterhuis WJ, Sutmuller RP, Brouwer HM, Scharenborg NM, Klasen IS, Hilbrands LB, Figdor CG, de Vries IJ, Adema GJ. 2010. Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients. *Clin Cancer Res* 16: 5067-78
111. Rech AJ, Mick R, Martin S, Recio A, Aqui NA, Powell DJ, Jr., Colligon TA, Trosko JA, Leinbach LI, Pletcher CH, Tweed CK, DeMichele A, Fox KR, Domchek SM, Riley JL, Vonderheide RH. 2012. CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients. *Sci Transl Med* 4: 134ra62
112. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, Zhang A, Dahm P, Chao N, Gilboa E, Vieweg J. 2005. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 115: 3623-33
113. Attia P, Maker AV, Haworth LR, Rogers-Freezer L, Rosenberg SA. 2005. Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. *J Immunother* 28: 582-92
114. Telang S, Rasku MA, Clem AL, Carter K, Klarer AC, Badger WR, Milam RA, Rai SN, Pan J, Gragg H, Clem BF, McMasters KM, Miller DM, Chesney J. 2011. Phase II trial of the regulatory T cell-depleting agent, denileukin diftitox, in patients with unresectable stage IV melanoma. *BMC Cancer* 11: 515

115. Powell DJ, Jr., Attia P, Ghetie V, Schindler J, Vitetta ES, Rosenberg SA. 2008. Partial reduction of human FOXP3+ CD4 T cells in vivo after CD25-directed recombinant immunotoxin administration. *J Immunother* 31: 189-98
116. Powell DJ, Jr., Felipe-Silva A, Merino MJ, Ahmadzadeh M, Allen T, Levy C, White DE, Mavroukakis S, Kreitman RJ, Rosenberg SA, Pastan I. 2007. Administration of a CD25-directed immunotoxin, LMB-2, to patients with metastatic melanoma induces a selective partial reduction in regulatory T cells in vivo. *J Immunol* 179: 4919-28
117. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322: 271-5
118. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. 2009. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med* 206: 1717-25
119. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363: 711-23
120. Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, Shimizu J, Nomura T, Chiba T, Sakaguchi S. 2005. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells. *J Exp Med* 202: 885-91
121. Nishikawa H, Kato T, Hirayama M, Orito Y, Sato E, Harada N, Gnjatic S, Old LJ, Shiku H. 2008. Regulatory T cell-resistant CD8+ T cells induced by glucocorticoid-induced tumor necrosis factor receptor signaling. *Cancer Res* 68: 5948-54
122. Jensen SM, Maston LD, Gough MJ, Ruby CE, Redmond WL, Crittenden M, Li Y, Puri S, Poehlein CH, Morris N, Kovacsovics-Bankowski M, Moudgil T, Twitty C, Walker EB, Hu HM, Urba WJ, Weinberg AD, Curti B, Fox BA. 2010. Signaling through OX40 enhances antitumor immunity. *Semin Oncol* 37: 524-32
123. Valzasina B, Guiducci C, Dislich H, Killeen N, Weinberg AD, Colombo MP. 2005. Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood* 105: 2845-51
124. Piconese S, Valzasina B, Colombo MP. 2008. OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection. *J Exp Med* 205: 825-39
125. Kjaergaard J, Tanaka J, Kim JA, Rothchild K, Weinberg A, Shu S. 2000. Therapeutic efficacy of OX-40 receptor antibody depends on tumor immunogenicity and anatomic site of tumor growth. *Cancer Res* 60: 5514-21
126. Hirschhorn-Cymerman D, Budhu S, Kitano S, Liu C, Zhao F, Zhong H, Lesokhin AM, Avogadri-Connors F, Yuan J, Li Y, Houghton AN, Merghoub T, Wolchok JD. 2012. Induction of tumoricidal function in CD4+ T cells is associated with concomitant memory and terminally differentiated phenotype. *J Exp Med* 209: 2113-26
127. Stagg J, Divisekera U, Duret H, Sparwasser T, Teng MW, Darcy PK, Smyth MJ. 2011. CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. *Cancer Res* 71: 2892-900
128. Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer KM, Smyth MJ. 2010. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci U S A* 107: 1547-52

129. Wang L, Fan J, Thompson LF, Zhang Y, Shin T, Curiel TJ, Zhang B. 2011. CD73 has distinct roles in nonhematopoietic and hematopoietic cells to promote tumor growth in mice. *J Clin Invest* 121: 2371-82
130. Jin D, Fan J, Wang L, Thompson LF, Liu A, Daniel BJ, Shin T, Curiel TJ, Zhang B. 2010. CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression. *Cancer Res* 70: 2245-55
131. Stagg J, Beavis PA, Divisekera U, Liu MC, Moller A, Darcy PK, Smyth MJ. 2012. CD73-deficient mice are resistant to carcinogenesis. *Cancer Res* 72: 2190-6
132. Gabrilovich DI, Nagaraj S. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 9: 162-74
133. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. 2009. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother* 58: 49-59
134. Solito S, Falisi E, Diaz-Montero CM, Doni A, Pinton L, Rosato A, Francescato S, Basso G, Zanovello P, Onicescu G, Garrett-Mayer E, Montero AJ, Bronte V, Mandruzzato S. 2011. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood* 118: 2254-65
135. Mandruzzato S, Solito S, Falisi E, Francescato S, Chiarion-Sileni V, Mocellin S, Zanon A, Rossi CR, Nitti D, Bronte V, Zanovello P. 2009. IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. *J Immunol* 182: 6562-8
136. Vuk-Pavlovic S, Bulur PA, Lin Y, Qin R, Szumlanski CL, Zhao X, Dietz AB. 2010. Immunosuppressive CD14+HLA-DRlow/- monocytes in prostate cancer. *Prostate* 70: 443-55
137. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, Manns MP, Greten TF, Korangy F. 2008. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. *Gastroenterology* 135: 234-43
138. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, Castelli C, Mariani L, Parmiani G, Rivoltini L. 2007. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol* 25: 2546-53
139. Sica A, Bronte V. 2007. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* 117: 1155-66
140. Gallina G, Dolcetti L, Serafini P, De Santo C, Marigo I, Colombo MP, Basso G, Brombacher F, Borrello I, Zanovello P, Bicciato S, Bronte V. 2006. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* 116: 2777-90
141. Huang B, Lei Z, Zhao J, Gong W, Liu J, Chen Z, Liu Y, Li D, Yuan Y, Zhang GM, Feng ZH. 2007. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. *Cancer Lett* 252: 86-92
142. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V, Viola A. 2011. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med* 208: 1949-62
143. Greifenberg V, Ribechini E, Rossner S, Lutz MB. 2009. Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development. *Eur J Immunol* 39: 2865-76
144. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. 2009. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16: 183-94

145. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. 2007. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res* 67: 4507-13
146. Zhang HG, Grizzle WE. 2011. Exosomes and cancer: a newly described pathway of immune suppression. *Clin Cancer Res* 17: 959-64
147. Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S. 2009. Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells. *J Immunol* 183: 937-44
148. Dardalhon V, Anderson AC, Karman J, Apetoh L, Chandwaskar R, Lee DH, Cornejo M, Nishi N, Yamauchi A, Quintana FJ, Sobel RA, Hirashima M, Kuchroo VK. 2010. Tim-3/galectin-9 pathway: regulation of Th1 immunity through promotion of CD11b+Ly-6G+ myeloid cells. *J Immunol* 185: 1383-92
149. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, Lehner F, Manns MP, Greten TF, Korangy F. 2009. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. *Hepatology* 50: 799-807
150. Hoechst B, Gamrekelashvili J, Manns MP, Greten TF, Korangy F. 2011. Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. *Blood* 117: 6532-41
151. Ryzhov S, Novitskiy SV, Goldstein AE, Biktasova A, Blackburn MR, Biaggioni I, Dikov MM, Feoktistov I. 2011. Adenosinergic regulation of the expansion and immunosuppressive activity of CD11b+Gr1+ cells. *J Immunol* 187: 6120-9
152. Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, Schwartz M, Divino CM, Pan PY, Chen SH. 2009. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. *Cancer Res* 69: 2514-22
153. Ko JS, Rayman P, Ireland J, Swaidani S, Li G, Bunting KD, Rini B, Finke JH, Cohen PA. 2010. Direct and differential suppression of myeloid-derived suppressor cell subsets by sunitinib is compartmentally constrained. *Cancer Res* 70: 3526-36
154. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, Golshayan A, Rayman PA, Wood L, Garcia J, Dreicer R, Bukowski R, Finke JH. 2009. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res* 15: 2148-57
155. Mirza N, Fishman M, Fricke I, Dunn M, Neuger AM, Frost TJ, Lush RM, Antonia S, Gabrilovich DI. 2006. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* 66: 9299-307
156. Michels T, Shurin GV, Naiditch H, Sevko A, Umansky V, Shurin MR. 2012. Paclitaxel promotes differentiation of myeloid-derived suppressor cells into dendritic cells in vitro in a TLR4-independent manner. *J Immunotoxicol* 9: 292-300
157. Sevko A, Kremer V, Falk C, Umansky L, Shurin MR, Shurin GV, Umansky V. 2012. Application of paclitaxel in low non-cytotoxic doses supports vaccination with melanoma antigens in normal mice. *J Immunotoxicol* 9: 275-81
158. Shurin MR, Naiditch H, Gutkin DW, Umansky V, Shurin GV. 2012. ChemoImmunoModulation: immune regulation by the antineoplastic chemotherapeutic agents. *Curr Med Chem* 19: 1792-803
159. Sevko A, Michels T, Vrohlings M, Umansky L, Beckhove P, Kato M, Shurin GV, Shurin MR, Umansky V. 2013. Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. *J Immunol* 190: 2464-71

160. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. 2005. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res* 11: 6713-21
161. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, Martin F, Apetoh L, Rebe C, Ghiringhelli F. 2010. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res* 70: 3052-61
162. Boelte KC, Gordy LE, Joyce S, Thompson MA, Yang L, Lin PC. 2011. Rgs2 mediates pro-angiogenic function of myeloid derived suppressor cells in the tumor microenvironment via upregulation of MCP-1. *PLoS One* 6: e18534
163. Sawanobori Y, Ueha S, Kurachi M, Shimaoka T, Talmadge JE, Abe J, Shono Y, Kitabatake M, Kakimi K, Mukaida N, Matsushima K. 2008. Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. *Blood* 111: 5457-66
164. Zollo M, Di Dato V, Spano D, De Martino D, Liguori L, Marino N, Vastolo V, Navas L, Garrone B, Mangano G, Biondi G, Guglielmotti A. 2012. Targeting monocyte chemotactic protein-1 synthesis with bindarit induces tumor regression in prostate and breast cancer animal models. *Clin Exp Metastasis* 29: 585-601
165. Capuano G, Rigamonti N, Grioni M, Freschi M, Bellone M. 2009. Modulators of arginine metabolism support cancer immunosurveillance. *BMC Immunol* 10: 1
166. Serafini P, Mgebroff S, Noonan K, Borrello I. 2008. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res* 68: 5439-49
167. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I. 2006. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med* 203: 2691-702
168. Meyer C, Sevko A, Ramacher M, Bazhin AV, Falk CS, Osen W, Borrello I, Kato M, Schadendorf D, Baniyash M, Umansky V. 2011. Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. *Proc Natl Acad Sci U S A* 108: 17111-6
169. Ugel S, Delpozzo F, Desantis G, Papalini F, Simonato F, Sonda N, Zilio S, Bronte V. 2009. Therapeutic targeting of myeloid-derived suppressor cells. *Curr Opin Pharmacol* 9: 470-81
170. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, Melani C, Guiducci C, Colombo MP, Iezzi M, Musiani P, Zanovello P, Bronte V. 2005. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc Natl Acad Sci U S A* 102: 4185-90
171. Veltman JD, Lambers ME, van Nimwegen M, Hendriks RW, Hoogsteden HC, Aerts JG, Hegmans JP. 2010. COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived suppressor cells in mesothelioma. Celecoxib influences MDSC function. *BMC Cancer* 10: 464
172. Fujita M, Kohanbash G, Fellows-Mayle W, Hamilton RL, Komohara Y, Decker SA, Ohlfest JR, Okada H. 2011. COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res* 71: 2664-74
173. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. 2007. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. *Clin Cancer Res* 13: 721s-6s
174. Umansky V, Sevko A. 2012. Overcoming immunosuppression in the melanoma microenvironment induced by chronic inflammation. *Cancer Immunol Immunother* 61: 275-82
175. Umansky V, Sevko A. 2012. Melanoma-induced immunosuppression and its neutralization. *Semin Cancer Biol* 22: 319-26

176. Umansky V, Sevko A. 2013. Ret transgenic mouse model of spontaneous skin melanoma: focus on regulatory T cells. *Pigment Cell Melanoma Res*
177. Kato M, Takahashi M, Akhand AA, Liu W, Dai Y, Shimizu S, Iwamoto T, Suzuki H, Nakashima I. 1998. Transgenic mouse model for skin malignant melanoma. *Oncogene* 17: 1885-8
178. Umansky V, Abschuetz O, Osen W, Ramacher M, Zhao F, Kato M, Schadendorf D. 2008. Melanoma-specific memory T cells are functionally active in Ret transgenic mice without macroscopic tumors. *Cancer Res* 68: 9451-8
179. Kimpfler S, Sevko A, Ring S, Falk C, Osen W, Frank K, Kato M, Mahnke K, Schadendorf D, Umansky V. 2009. Skin melanoma development in ret transgenic mice despite the depletion of CD25+Foxp3+ regulatory T cells in lymphoid organs. *J Immunol* 183: 6330-7
180. Corbett TH, Roberts BJ, Leopold WR, Peckham JC, Wilkoff LJ, Griswold DP, Jr., Schabel FM, Jr. 1984. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice. *Cancer Res* 44: 717-26
181. Morikane K, Tempero RM, Sivinski CL, Nomoto M, Van Lith ML, Muto T, Hollingsworth MA. 1999. Organ-specific pancreatic tumor growth properties and tumor immunity. *Cancer Immunol Immunother* 47: 287-96
182. Shevchenko I, Karakhanova S, Soltek S, Link J, Bayry J, Werner J, Umansky V, Bazhin AV. 2013. Low-dose gemcitabine depletes regulatory T cells and improves survival in the orthotopic Panc02 model of pancreatic cancer. *Int J Cancer* 133: 98-107
183. Cui W, Kaech SM. 2010. Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunol Rev* 236: 151-66
184. Weng NP, Araki Y, Subedi K. 2012. The molecular basis of the memory T cell response: differential gene expression and its epigenetic regulation. *Nat Rev Immunol* 12: 306-15
185. Sevko A, Umansky V. 2013. Myeloid-derived suppressor cells interact with tumors in terms of myelopoiesis, tumorigenesis and immunosuppression: thick as thieves. *J Cancer* 4: 3-11
186. Junger WG. 2011. Immune cell regulation by autocrine purinergic signalling. *Nat Rev Immunol* 11: 201-12
187. Sitkovsky MV, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A, Thiel M. 2004. Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu Rev Immunol* 22: 657-82
188. Sitkovsky MV, Kjaergaard J, Lukashev D, Ohta A. 2008. Hypoxia-adenosinergic immunosuppression: tumor protection by T regulatory cells and cancerous tissue hypoxia. *Clin Cancer Res* 14: 5947-52
189. Knapp K, Zebisch M, Pippel J, El-Tayeb A, Muller CE, Strater N. 2012. Crystal structure of the human ecto-5'-nucleotidase (CD73): insights into the regulation of purinergic signaling. *Structure* 20: 2161-73
190. Wang TF, Ou Y, Guidotti G. 1998. The transmembrane domains of ectoapyrase (CD39) affect its enzymatic activity and quaternary structure. *J Biol Chem* 273: 24814-21
191. Strater N. 2006. Ecto-5'-nucleotidase: Structure function relationships. *Purinergic Signal* 2: 343-50
192. Zimmermann H. 1992. 5'-Nucleotidase: molecular structure and functional aspects. *Biochem J* 285 ( Pt 2): 345-65
193. Chalmin F, Mignot G, Bruchard M, Chevriaux A, Vegran F, Hichami A, Ladoire S, Derangere V, Vincent J, Masson D, Robson SC, Eberl G, Pallandre JR, Borg C, Ryffel B, Apetoh L, Rebe C, Ghiringhelli F. 2012. Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. *Immunity* 36: 362-73

194. Regateiro FS, Howie D, Nolan KF, Agorogiannis EI, Greaves DR, Cobbold SP, Waldmann H. 2011. Generation of anti-inflammatory adenosine by leukocytes is regulated by TGF-beta. *Eur J Immunol* 41: 2955-65
195. Yip L, Woehrle T, Corriden R, Hirsh M, Chen Y, Inoue Y, Ferrari V, Insel PA, Junger WG. 2009. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. *FASEB J* 23: 1685-93
196. Schenk U, Frascoli M, Proietti M, Geffers R, Traggiai E, Buer J, Ricordi C, Westendorf AM, Grassi F. 2011. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. *Sci Signal* 4: ra12
197. Pepper M, Jenkins MK. 2011. Origins of CD4(+) effector and central memory T cells. *Nat Immunol* 12: 467-71
198. Zhu J, Yamane H, Paul WE. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28: 445-89
199. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG. 2006. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* 314: 1792-5
200. Chen Y, Yao Y, Sumi Y, Li A, To UK, Elkhal A, Inoue Y, Woehrle T, Zhang Q, Hauser C, Junger WG. 2010. Purinergic signaling: a fundamental mechanism in neutrophil activation. *Sci Signal* 3: ra45
201. Corriden R, Chen Y, Inoue Y, Beldi G, Robson SC, Insel PA, Junger WG. 2008. Ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. *J Biol Chem* 283: 28480-6
202. Hyman MC, Petrovic-Djergovic D, Visovatti SH, Liao H, Yanamadala S, Bouis D, Su EJ, Lawrence DA, Broekman MJ, Marcus AJ, Pinsky DJ. 2009. Self-regulation of inflammatory cell trafficking in mice by the leukocyte surface apyrase CD39. *J Clin Invest* 119: 1136-49
203. Kukulski F, Bahrami F, Ben Yebdri F, Lecka J, Martin-Satue M, Levesque SA, Sevigny J. 2011. NTPDase1 controls IL-8 production by human neutrophils. *J Immunol* 187: 644-53
204. Hock H, Hamblen MJ, Rooke HM, Traver D, Bronson RT, Cameron S, Orkin SH. 2003. Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. *Immunity* 18: 109-20
205. Stagg J. 2012. The double-edge sword effect of anti-CD73 cancer therapy. *Oncoimmunology* 1: 217-8

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