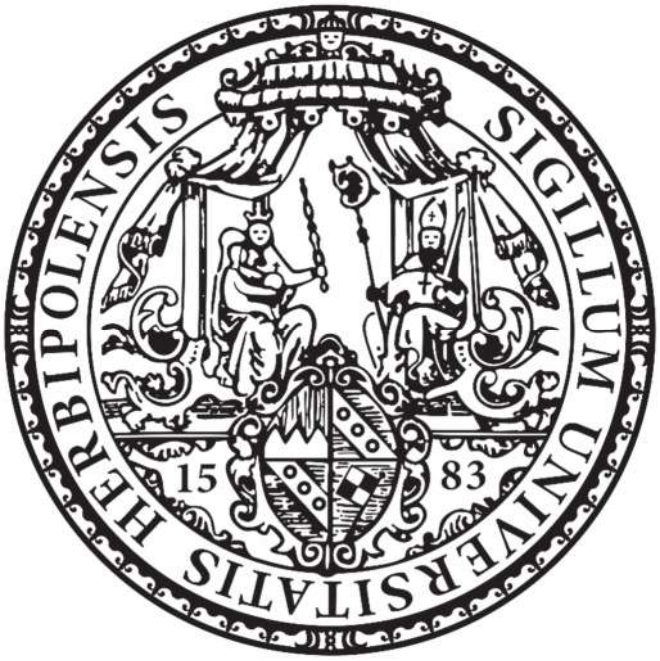
Immunosuppressive role of adenosine produced by ectonucleotidases CD39 and CD73 in ovarian cancer, tumor associated macrophages and the host immune system



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I hereby declare that my thesis entitled „ Immunosuppressive role of adenosine produced by ectonucleotidases CD39 and CD73 in ovarian cancer, tumor associated macrophages and the host immune system" is the result of my own work. I did not receive any help or support from commercial consultants or others. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I verify that this thesis has not been submitted as part of another examination process, neither in identical nor in similar form.

Würzburg, 2015 Itsaso Montalbán del Barrio

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0.1 SUMMARY

Ovarian cancer (OvCa) is the tumor with the most unfavourable prognosis among all gynaecological malignancies causing more than 6000 deaths per year in Germany alone. Patients with OvCa show symptoms at very advanced stages of tumor progression when the only available treatments consist on tumor debulking surgery and administration of platinum based chemotherapeutics and anthracyclins. There is an urgent need to develop new therapeutical strategies since the actual 5 year survival rate of OvCa patients does not exceed 20-40%.

Immunotherapy is a promising approach for treatment of ovarian cancer, since it has been observed that immunological parameters can influence the outcome of the patient. The aim of our research is to overcome tumor immune escape by counteracting the immunosuppressive mechanisms developed by the tumor. In particular, this work studies the influence of adenosine generated by the ectonucleotidases CD39 and CD73 in the tumor microenvironment. Cellular expression of CD39 and CD73 contributes to immunosupression as these ectonucleotidases convert immune-stimulatory extracellular ATP into immunosuppressive adenosine. This was primarily described as effector mechanism for regulatory T cells, but may also be important in the tumor microenvironment.

Having found that tumor cells from OvCa-patients express high levels of ATP-depleting ectonucleotidases CD39 and CD73 we set out to investigate a potential immunosuppressive mechanism via adenosine production in the tumor microenvironment. We could measure 30-60 times higher adenosine production by OvCa cell lines and ascites-derived cancer cells as compared to physiological normal conditions. To confirm this putative immune escape mechanism we investigated its effect on several immune cell populations. CFSE-based assays, for example, showed an inhibition of $CD4^+$ T cell proliferation by OvCA cell-derived adenosine. In this context, we have further established an *in-vitro* assay, where OvCa cells modulate the function of macrophages towards a M2 or tumor associated (TAM) phenotype. Together with the

phenotype modulation adenosine exerts chemotactic effects on human monocytes and is thus likely to attract myeloid precursor cells towards the tumor tissue. Moreover, in a microenvironment that is shaped by OvCa cells, human monocytes differentiate into M2 macrophages or TAMs which themselves express significant levels of the adenosine-generating ectonucleotidases CD39 and CD73.

Investigating the regulation of ectonucleotidase expression, we also observed that approaches clinically used to treat OvCa (namely application of doxorubicine or irradiation) influence CD73 and CD39 levels of OvCa and immune cells *in vitro*. In this study we show how this treatment-induced change in the ATP/adenosine ratio modulates the effector function of different immune cells. Furthermore, we investigate the potential benefit of clinically available small molecule inhibitors for CD39 and CD73 that could relieve immunosuppression in the tumor microenvironment especially in combination with common treatment regimes.

0.2 ZUSAMMENFASSUNG

Eierstockkrebs ist der Tumor mit der schlechtesten Heilungsprognose unter allen gynäkologischen Malignomen. Allein in Deutschland verursacht er über 6000 Tote pro Jahr. Patienten mit Ovarialkarzinom zeigen erst in einem sehr fortgeschrittenen Stadium charakteristische Symptome. Die einzig möglichen Behandlungsmethoden sind dann die operative Tumorentfernung und die Verabreichung von platinbasierter Chemotherapien sowie von Anthrazyklinen. Da die aktuelle 5-Jahres-Überlebensrate lediglich 20-40% beträgt, besteht ein dringender Bedarf an neuen therapeutischen Optionen.

Seit herausgefunden wurde, dass immunologische Parameter das Überleben der Patienten beeinflussen, ist Immuntherapie zu einer der vielversprechendsten Behandlungsarten des Eierstockkrebs geworden. Das Ziel unserer Forschung ist die Überwindung der Immunevasion des Tumors durch ein Verhindern der immun-unterdrückenden Mechanismen des Tumors. Im Speziellen befasst sich diese Arbeit mit dem Einfluss von Adenosin, das durch die Ectonukleotidasen CD39 und CD73 in der Mikroumgebung des Tumors gebildet wird. Die CD39- und CD73-Expression der Zellen führt zu Immunosuppression da diese Ectonukleotidasen immun-stimulierendes, extrazelluläres ATP in immunsuppressives Adenosin umwandeln. Dies wurde zuerst als Effektormechanismus für regulatorische T-Zellen beschrieben, kann aber auch im Tumormikromilieu von Bedeutung sein.

Mit dem Wissen, dass Tumorzellen von Eierstockkrebs-Patientinnen große Mengen der ATP-unterdrückenden Ectonukleotidasen CD39 und CD73 bilden, analysierten wir die adenosinvermittelte Unterdrückendung von Immunantwortenin der Mikroumgebung der Tumorzellen. Im Vergleich zu regulatorischen T Zellen konnten wir bei Eierstockkrebs-Zelllinien und bei aus Aszites gewonnenen Krebszellen eine 30- bis 60-fache Adenosinproduktion messen. Um diesen mutmaßlichen Immunevasions-Mechanismus zu bestätigen, untersuchten wir seine Auswirkungen auf mehrere Immunzellenpopulationen. CSFE-basierte Experimente zeigten zum Beispiel eine Hemmung der $CD4^+$ T-Zell-Proliferation durch Adenosin, welches von Eierstockkrebs-Zellen produziert wurde. In diesem Zusammenhang haben wir auch eine *in-vitro*

Methode entwickelt, mit der wir die Beeinflussung von Makrophagen durch Eierstockkrebszellen analysieren und modulieren konnten. Neben seiner suppressiven Wirkung übt Adenosin auch chemotaktische Effekte auf menschliche Monozyten aus und lockt wahrscheinlich myeloide Vorläuferzellen zum Tumorgewebe. Anschließend differenzieren sich menschliche Monozyten in einer von Eierstockkrebszellen geformten Mikroumgebung zu M2 Makrophagen oder tumor-assoziierten Makrophagen (TAMs), die ihrerseits erhebliche Mengen der Adenosin-produzierenden Ectonukleotidasen CD39 und CD73 bilden.

Während wir die Regulierung der Ectonukleotidasen-Expression untersuchten, entdeckten wir auch, dass klinisch genutzte Techniken zur Behandlung von Eierstockkrebs (zum Beispiel die Anwendung von Doxorubicin oder Bestrahlung) *in vitro* das CD73- und CD39-Level von Eierstockkrebs- und Immunzellen beeinflussen. In dieser Studie zeigen wir, wie dieser behandlungsbedingte Wechsel des ATP/Adenosine-Verhältnisses die Effektorfunktion verschiedener Immunzellen moduliert. Darüber hinaus untersuchen wir den potentiellen Vorteil von klinisch verfügbaren, niedermolekularen Inhibitoren für CD39 und CD73, die die Immunsuppression in der Mikroumgebung des Tumors partiell aufheben könnten, und die vor allem in Kombination mit gängigen Behandlungsschemata von großem Interesse sein könnten.

1. INTRODUCTION

1.1. OVARIAN CANCER

1.1.1. Epidemiology

Ovarian cancer represents the sixth most common cancer among women in the world (Riman, Persson, & Nilsson, 1998) and is the leading casue of death among gynecological malignancies (Fehrmann et al., 2007). Worldwide, the estimated annual incidence of ovarian cancer is 204,000, with 125,000 deaths (Rauh-Hain, Krivak, Del Carmen, & Olawaiye, 2011). The lack of early symptoms together with the the lack of cancer screening tests make it difficult to diagnose and therefore, ovarian carcinoma is usually detected at late stages. The 5-year survival rate is approximately 45%, however, if it is diagnosed at an early stage the 5-year survival rate jumps to 95% whereas tumors detected at late stage will in >80% of all cases cause the death of the patient within 5 years.

1.1.2. Symptoms

Symptoms for ovarian cancer can be grouped into genitourinary, gastrointestinal, abdominal and pelvic response symptoms (Partridge & Barnes, 1999). Most common symptoms are: increased abdominal size, abdominal pain, fatigue, abdominal bloating, urinary frequency, indigestion, nausea, pelvic pain, constipation, urinary incontinence, back pain, early satiety, pain with intercourse, weight loss, diarrhea, bleeding with intercourse and deep venous thrombosis (Martin, 2002). However, at early stages, ovarian carcinoma presents nearly no symptoms and thus they are not eligible for an early detection and diagnosis.

1.1.3. Types

Different types of ovarian tumors can be classified depending on the cells of origin; most of them fall into one of these three major categories: surface epithelial tumors, sex cord-stromal tumors and germ cell tumors. Roughly, 10-15% of ovarian cancer cases are sex cord-stromal tumors. More than 50% of stromal cell tumors appear in postmenopausal women over 50, however, some cases have also been observed in younger girls.

Common types of malignant stromal cell tumors include granulosa cell tumors, theca cell tumors, sertoli-leydig cell tumors, and hilar cell tumors.

Around 5-10% of ovarian cancer cases are germ cell tumors, which arise from the oocytes. These tumors are more common among adolescents and represent approximately 60% of ovarian tumors under 20 years (Norris & Jensen, 1972). Some examples of germ cell tumors are: teratomas, dysterminomas, endodermal sinus tumors, and choriocarcinomas.

Epithelial ovarian cancer (EOC) is the most genuine gynaecological tumor and according to latest research tumors originate from a STIC (serous tubal intraepithelial carcinoma) close to the fimbriated end of the fallopian tube (Dietl, Wischhusen, & Hausler, 2011). EOC represents up to 80% of all ovarian carcinomas (Auersperg, Edelson, Mok, Johnson, & Hamilton, 1998; Herbst, 1994).

EOC can be benign or malignant; benign tumors do not propagate from the ovaries and are not linked to serious disease. Benign tumors include serous adenomas, mucinous adenomas, and Brenner tumors (T. I. Williams et al., 2007). Malignant tumors of the ovarian surface epithelium are known as carcinomas. These malignancies have the potential to spread into the proximal and distal areas of the body and therefore represent a life-threatening disease. According to tissue morphology, EOC can be classified into four major types: Serous, mucinous, endometrioid, and clear-cell carcinomas. In addition there are other minor types of EOC such as malignant Brenner tumors and undifferentiated carcinomas (Slotman & Rao, 1988).

1.1.4. Etiology

Several hypotheses have been proposed regarding the beginning of EOC. The incessant ovulation hypothesis is one of them; it was postulated after realising that women with a higher number of ovulatory cycles have an increased risk of suffering from ovarian cancer (Fathalla, 1971). According to this hypothesis, uninterrupted ovulation leads to a sustained cycle of damage and repair of the surface epithelium. Failures in the repair mechanisms increase the risk of generating mutations and a subsequent development of cancer. Furthermore, increased ovulatory activity is linked with inclusion cysts and other changes in the ovary surface, such as invaginations. These inclusion cysts might generate an appropriate environment for ovarian cancer development (Feeley & Wells,

2001). Concordant with this hypothesis it has been observed that women with multiple pregnancies, increased time of lactation and oral contraceptive use have a lower incidence of ovarian cancer (Gwinn, Lee, Rhodes, Layde, & Rubin, 1990; Hough, Cho, Zonderman, Schwartz, & Morin, 2001; Nasca, Greenwald, Chorost, Richart, & Caputo, 1984). However, this theory is weakened by the fact that progesterone-based oral contraceptives which do not inhibit ovulation show the same protective effect as ovulation inhibiting contraceptives (Risch, 1998). Furthermore, women with polycystic ovarian syndrome whose ovulatory cycles are reduced, have a high risk of developing EOC (Schildkraut, Schwingl, Bastos, Evanoff, & Hughes, 1996).

After the failure of the incessant ovulation hypothesis to explain certain aspects of ovarian cancer generation, the gonadotropin hypothesis was postulated. This hypothesis suggests that persistent high levels of gonadotropins (required for initiating ovulation) can induce malignant formation by stimulating the ephitelial cells from the ovary surface (Cramer & Welch, 1983; Ness & Cottreau, 1999; Ozols et al., 2004). In addition, gonadotropins can induce the loss of ovarian surface epithelial basement membrane (Roland et al., 2003). Furthermore, ovulation is an inflammation-like process which involves multiple cytokines and proteolytic enzymes, and their actions ultimately lead to tissue rupture (Ozols et al., 2004). Since inflammation is a precursor to cancer development, the chronic inflammatory processes of the ovarian surface epithelium could explain the mechanism by which gonadotropin stimulation and ovulation contribute to ovarian cancer formation (Akhmedkhanov et al., 2001; Ness & Cottreau, 1999). The most recent theory hypothesizes that ovarian cancer does not begin in the ovary, but rather at the distal fallopian tube. This hypothesis is supported by the fact that the majority of early diagnosed serous malignancies, detected by risk-reducing bilateral salpingo-oophorectomies (BSO), were found in the distal fallopian tube and not in the ovary (Erickson, Conner, & Landen, 2013). In addition, analysis of mutations in TP53 in those early serous malignancies of the distal fallopian tube and adjacent bulky carcinomas of the ovary showed shared mutations (Kupryjanczyk et al., 1993). This theory could be valid for serous carcinomas; however, it fails to explain endometrioid, mucinous or clear cell types of ovarian cancer.

1.1.5. Screening, Detection, Treatment and Prognosis:

Up to date, no more than 19% of ovarian cancers are diagnosed at early stages (stage I or II) while the tumor is still confined to the ovary. Around 7% of the cases are detected with regional (pelvic) spread and the majority (68%) are diagnosed with distant spread (abdomen and extra-abdominal) (Jemal et al., 2009). The high mortality rates among patients suffereing from ovarian cancer is due to the fact that most cases are diagnosed at late stages. To date, neither specific nor sensitive screening method capable of detecting ovarian cancer has been developed. Current screening methods for ovarian cancer diagnose combine pelvic examinations, measurement of serum cancer antigen-125 (CA 125) levels, and transvaginal or pelvic ultrasonography. These are annually performed in women with a family record of ovarian cancer (Goff, Mandel, Muntz, & Melancon, 2000; Jemal et al., 2009; Ozols et al., 2004). When a patient is suspected to suffer from ovarian cancer (according to symptoms and physical pelvic examination), the level of CA125 in serum is measured along with transvaginal and abdominal ultrasonography. In addition, a computed axial tomography (CT) scan of the abdomen and pelvis is performed. Once the ovarian cancer has been diagnosed, an exploratory laparotomy is performed leading, in many cases, to the resection of one or both ovaries, fallopian tubes and/or uterus. In addition, lymph nodes, liver and suspicious sites within the abdomen are sampled and studied for possible metastasis. Only surgery can provide a definitive diagnosis, identify the histology and stage of the tumor, and remove the majority of the tumor (Cannistra, 2004; Martin, 2005).

1.1.6. Clinical Management

Further treatment after surgery will depend on the stage of the disease. Surgically removed ovarian cancer at stages IA or IB usually requires no further treatment. In the case of a poorly differentiated tumor at stages IC, II, III and IV, chemotherapy treatment is prescribed.

The postoperative chemotherapy regimen consists of a platinum compound such as cisplatin or carboplatin, or a taxane/platinum combination. The most common drug combination is carboplatin and paclitaxel (du Bois et al., 2012) and in case of patients with relapsed OvCa, anthracyclins like pegylated, lipsosomal doxorubicin (Caelyx®) are administered (Pignata et al., 2009). In the recommended dosages (e.g. Carboplatin AUC5

with Paclitacel 175 mg/m2 q21d versus e.g. 50 mg/m2 Caelyx® q28d or 30 mg/m2 Caelyx® plus Carboplatin AUC5 (du Bois et al., 2012; Gordon et al., 2001; Pignata et al., 2009) these drugs cause severe substance-related side effects like suppression of haematopoiesis (followed by increased susceptibility to infections, bleeding or fatigue), neurological disorders (taxoids) or cardiomyopathy (anthracyclines like doxorubicin) besides the common chemotherapeutic effects such as nausea/vomiting, diarrhea or mucositis (Bookman, 2011; Chatterjee, Zhang, Honbo, & Karliner, 2010; Wollmer & Neubauer, 2011). Worst of all, these approaches are only moderately effective and the actual 5 year survival rate does not exceed 20-40% (Dietl & Wischhusen, 2011) – which has remained unchanged for over 30 years. Hence, new therapeutic approaches are required.

1.1.6.1. Immunotherapy

Several lines of evidence support the importance of immunotherapy as a treatment for ovarian carcinoma. The first and most remarkable observation was the association of infiltrating T cells in ovarian tumors with an improved prognosis of the patient. In 2003, 186 patients with stage III or IV OvCa were studied by Zhang et al. Results revealed an improved 5-year overall survival in patients whos tumors contained infiltrated t-cells (L. Zhang et al., 2003). Among 74 patients who showed a complete clinical response after debulking and platinum-based therapy, the 5-year survival rate was 73.9% for patients with $CD3^+$ T cell infiltrates within their tumor, compared to only 11.9% of the patients without infiltrating T cells (L. Zhang et al., 2003).

In this study, the authors also observed that tumors with a high number of infiltrating T cells present a significantly increased expression of monokines induced by IFN-γ, macrophage-derived chemokines and secondary lymphoid-tissue chemokines, compared to those of tumors lacking T cells. This observation indicates that these chemokines could play an important role in the antitumor response (L. Zhang et al., 2003).

While effector T cells (helper and cytotoxic T cells) have an antitumor immune function, regulatory T-cells can suppress immunity (Knutson, Disis, & Salazar, 2007). In the last years many studies have tried to elucidate whether effector T-cells are linked to a good prognosis of the patients and if so, what their exact role and phenotype is. For example,

in a study conducted by Sato and colleagues, 117 ovarian cancer cases were studied and they could observe that a high number of infiltrated intraepithelial $CD8^+$ T cells was linked with an improved survival, thus, patients with infiltrated $CD8^+$ T cells lived an average of 29 month longer (Sato et al., 2005). These findings were further confirmed by the research group of Leffers and colleagues in an independent study (Leffers et al., 2009). Cytotoxic $CD8^+$ T cells are classically believed to be the foremost antitumor mediators; they can recognize antigens presented on MHC (HLA) class I molecules on ovarian cancer cells and upon recognition of their cognate antigen, the cytotoxic cells express FasL and TRAIL and release apoptosis-inducing mediators, such as granzyme and perforine, thereby causing the death of the target cells (Ashton-Rickardt, 2005).

Nevertheless, the association of $CD8^+$ T cells with a high concentration of granzyme B within the tumor points out that the majority of $CD8^+$ T cells may play a cytotoxic role (Milne et al., 2009). Several studies have tried to elucidate the mechanisms of lymphocyte recruitment to the tumor site by using gene expression profiling techniques to compare gene expression of tumors with high and low numbers of infiltration. Thanks to this technique two different genes have been identified as possibly responsible for lymphocyte recruitment to the tumor site: interferon regulatory factor (IRF)-1 and chemokine receptor (CXCR) 6 (Callahan et al., 2008; Leffers et al., 2010).

These studies provide evidence of specific genes and pathways involved in cytotoxic T cells recruitment in ovarian tumors and postulate possible strategies for new immune therapies designed to modulate recruitment of T-cell subsets.

On the other hand, the role of infiltrating $CD4^+$ helper T-cell in ovarian tumors is not clear yet due to the prevalence of the CD4 marker on Tregs. Research conducted by the groups of Sato and Milne observed comparable outcomes among patients with or without $CD4^+$ T-cell staining of tumors (Milne et al., 2009; Sato et al., 2005). The group of Kryczek showed and association between high levels of IL-17 and improved patient outcome, postulating that a subset of $CD4^+$ Th cells, Th17 , may play an important role in eliminating tumors (Kryczek et al., 2009).

The role of other antitumor immune cell subsets such as NK cells has also been studied in OvCa. NK cells are a group of cytotoxic lymphocytes found either in the periphery, mainly as $CD16^+CD56^{dim}$ NK cells, or in secondary lymphoid tissue, where $CD16^-CD56^{bright}$

NK cells predominate. $CD16^{+}CD56^{dim}$ NK cells display a much higher cytolytic activity compared to the $CD16^{-}CD56^{bright}$ cells (Perussia, Chen, & Loza, 2005). A high NK cell activity in the peripheral blood of OvCa patients at the time of surgery has been related with an improved progression-free survival (PFS) (Garzetti, Cignitti, Ciavattini, Fabris, & Romanini, 1993). However, presence of NK cells in peritoneal and pleural effusions of metastatic ovarian carcinoma has been associated with a poor prognosis (Dong et al., 2006).

In OvCa patients, generation of antibody responses is a typical observation which suggests an antitumoral role for B cells (Goodell et al., 2006; Knutson et al., 2006; Tchabo et al., 2009). B cells do not need to be at the tumor site to exert their antibody-secreeting antitumor activity, however, studies associating the infiltration of B cells with prognosis show diverse results (Dong et al., 2006; Miller, Cervenka, Lund, Okazaki, & Moss, 1999; Milne et al., 2009). Of note, B cells can also act in a tolerogenic way.

Tumors evade immune attack by several mechanisms of immunosuppression, many of which operate in parallel. Some of these mechanisms involve paracrine mediators such as adenosine, prostaglandin E2, TGF-β and VEGF-A which may exert several direct and indirect immunosuppressive activities. These mediators may suppress dendritic cell activity, inhibit T-cell infiltration into the tumor microenvironment or suppress effector T-cell activation while enhancing the activity of regulatory T cells (Shin, Yoon, Kim, Kim, & Park, 2009). Tumor cells can also escape T-cell recognition by downregulation of MHC class I or by impairing components of the antigen processing machinery. Release of soluble NKG2D ligands (MIC-A or MIC-B) compromises the capacity of effector T cells to act in the tumor microenvironment. Moreover, tumor cells may upregulate different surface ligands, which may mediate T-cell anergy, such as PD-L1.

Although tumor immunology is a fast developing research area a better understanding of the tumor immunosuppressive mechanisms is needed to improve clinical effectiveness of immunotherapy.

Current cancer immunotherapeutic approaches against OvCa can be classified in three major categories: immune checkpoint inhibitors; therapeutic vaccines and adoptive T cell transfer.

These therapies are still in early-phase testing (phase I and II), but their success in other cancer types suggests that they may be effective against OvCa as well.

Immune checkpoint inhibitors target molecules that serve as checks and balances in the regulation of immune responses. This strategy is based upon an enhancement of the already existing anti-cancer immune-responses by blockade of inhibitory molecules which can be combined with activation of stimulatory molecules. For example, Ipilimumab (Yervoy™) targets the CTLA-4 checkpoint molecule on activated immune cells. Ipilimumab (approved for that indication in 2011) was the first treatment proven to prolong survival in patients with metastatic melanoma, the most deadly form of skin cancer. This therapeutic approach is now being tested in a variety of other cancer types including ovarian cancer in which a phase II study is currently being performed. Two other immune checkpoint inhibitors are tremelimumab (anti-CTLA-4) and MEDI4736 (anti-PD-L1). These treatments are being tested in combination for patients with advanced solid tumors.

On the other hand, several ovarian cancer-related antigens have been identified — i.e. molecules on or in OvCa cells which are capable of eliciting an immune response and which can serve as targets for immune recognition and attack. One of these antigens is NY-ESO-1; which is being intensively investigated by researchers in the CRI/Ludwig Clinical Trials Network. Research conducted by Kunle Odunsi has shown that NY-ESO-1 expression may be found in up to 43% of ovarian cancers (Ademuyiwa et al., 2012).

Other antigens that are expressed by ovarian cancer cell are CA-12, HER2/neu, MUC1, MAGE, OA3, membrane folate receptor, TAG-72, mesothelin, sialyl-Tn, p53, survivin, and hTERT. There are several strategies to target these antigens such as antibodies targeting CA-125 which is elevated in 79% of all patients with ovarian cancer, as well as antigen-specific vaccines targeting HER2, NY-ESO-1, p53, folate binding protein (E39 peptide), and dendritic cell vaccines targeting defined tumor antigens, such as hTERT/survivin, or patient- or tumor-specific antigens, including treatments such as CVac (personalized immunocellular therapy). Most recently the VEGF-A inhibitor bevacizumab has been introduced in combination with chemotherapie for OvCa treatment (Cannistra, 2004).

1.2. ADENOSINE

1.2.1. Adenosine- structure and *de novo* synthesis

Adenosine (Adenosine) is a purin nucleoside consisting of an adenine molecule linked to a ribose through a β-N9-glycosidic bond (fig.1). De novo synthesis of purine nucleosides starts with ribose -5'- phosphate; a derivate from the pentose-phosphate pathway. The amino acids glycine, aspartic acid and glutamine, as well as activated derivatives of tetrahydrofolate and CO_2 supply the carbon and nitrogen atoms that form the purine ring. The first product, which has a complete purine ring, is inosine monophosphate (IMP). IMP is a precursor of AMP; adenylate is formed in the presence of aspartate and fumarate. Finally, dephosphorylation of the ribose 5'- phosphate leads to the formation of Adenosine.

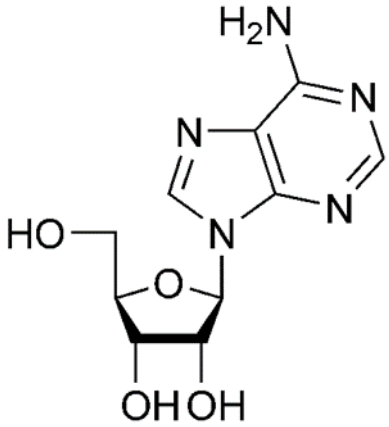


Figure 1: Molecular structure of adenosine

Adenosine is an important component of the nucleic acids DNA and RNA, as well as a component of high-energy compounds such as ATP and NADH. Moreover, Adenosine regulates a variety of physiological functions in almost all organs of the human body (Klotz 2007).

1.2.2. Production

Under normal conditions, adenosine is continuously produced by cells through the dephosphorylation of AMP by the activity of cytosolic 5'-nucleotidases. In hypoxic conditions, ATP synthesis is inhibited and AMP levels rise which causes a large increase in adenosine production. Substantial amounts of adenosine are also produced by the hydrolysis of adenine nucleotides released from the granules of neutrophils, mast cells and endothelial cells as a result of cellular damage (Linden, 2001; Ramkumar, Hallam, & Nie, 2001; M. V. Sitkovsky et al., 2004). Ecto-apyrases such as CD39 hydrolyse ATP or ADP to AMP which is then converted to adenosine by the extracellular 5'-ectonucleotidase CD73 (Zimmermann, 2000). Both of these enzymes are induced during hypoxia to enhance adenosine production (Niemela et al., 2004). Extracellular accumulation of adenosine is further enhanced during hypoxia by inhibition of the enzyme adenosine kinase which converts excess of adenosine back into AMP (M. V. Sitkovsky et al., 2004).

1.2.3. Adenosine- biological function

Adenosine (fig. 1) is a ubiquitous purine nucleoside present in all tissues and body cells. Adenosine has diverse relevant effects in multiple biological processes such as smooth muscle contraction, modulation of cardiac function, neurotransmission, exocrine and endocrine secretion, modulation of the immune response, inflammation, platelet aggregation and pain among others (Abbracchio et al., 1997; Ralevic & Burnstock, 1998). The first description of adenosine as a signaling molecule was provided by Dury et al. in 1929. They showed several biological effects of adenosine including cardiac arrest, vasodilatation and inhibition of intestinal contraction. Later in 1934, Gillespie (Gillespie 1934) observed for the first time the relationship between the activity and the structure of adenine compounds. He showed how the removal of phosphates can affect the type of response. They proved that dephosphorylation could increase the vasodilatory effect of adenine compounds whereas deamination reduced the pharmacological activity. Moreover, this study showed that ATP was more potent than AMP and adenosine in inducing contractions of guinea-pig ileum and uterus (Gillespie 1934). This observation

implied the existence of different purine receptors, which were not yet described at the time of the study.

It was not until 1972 when Burnstock set the basis on purinergic signaling knowledge. Ever since, purinergic signaling has been on the focus of many research groups which found out purines to play a key role in tissue pathophysiology.

1.2.4. Physiological roles of adenosine

Adenosine receptors were discovered in 1970 when adenosine was found out to stimulate cAMP formation in brain cells (Sattin & Rall, 1970) . This finding opened a new field of research which lead to the discovery of many new physiological roles of adenosine. In the heart, adenosine was shown to have protective effects; it can increase blood circulation, reduce rate and force of contraction and preconditions the heart against injury by prolonged ischemia. Adenosine has been shown to have and important role in the central nervous system (CNS). It can protect against ischemic effects in this tissue too and acts as a crucial modulator of neuro transmission (Sattin & Rall, 1970). In nephritic tissue, by the control of sodium excretion, adenosine can reduce glomerular filtration (Tagawa & Vander, 1970). Adenosine has also been reported to induce bronchoconstriction (Pauwels & van der Straeten 1986). In adipose tissue adenosine acts as a regulator of metabolism. It functions as a local insulin-like receptor which can increase glucose uptake in adipocytes and therefore inhibit lipolysis (Vannucci, Klim, Martin, & LaNoue, 1989). Blood cell activity is also affected by the physiological concentration of adenosine. Thus, platelet aggregation can be inhibited by adenosine (Dawicki, Agarwal, & Parks, 1986). Moreover, lymphocyte and lymphoblast proliferation is inhibited by adenosine (Hirschhorn, Grossman, & Weissmann, 1970; Van der Weyden & Kelley, 1976), while red cell production is stimulated (Schooley & Mahlmann, 1975). Finally, as we will see later in further detail adenosine is also a potent immunomodulator. These specific functions of adenosine are believed to be mediated through specific adenosine receptors, which have been cloned and classified as A1, A2A, A2B and A3 receptors (Fredholm et al., 1994). Adenosine receptors are expressed in all tissues of mammalian bodies; this suggests a role on maintenance of normal conditions. Apart from its regulatory function, adenosine is also a part of the structure of several

bioactive compounds, namely, ATP, RNA, coenzymes such as NAD FAD, CoA, cAMP, and S-adenosyl methionine.

1.2.5. Adenosine deaminases and regulation of adenosine concentration

Under normal physiological conditions, adenosine concentration is maintained at very low levels. Extracellular adenosine is quickly taken up by cells. Its main regulator is, however, the enzyme adenosine deaminase (ADA) which catalyzes an irreversible conversion of adenosine to inosine. There are two types of enzymes with adenosine deaminase activity: ADA1 and ADA2-like proteins (or ADGFs). ADA1 or "classical adenosine deaminase" is present in all prokaryotes and eukaryotes. ADA1 is an intracellular soluble monomer present in all cells (most studies are done for this form, especially on lymphocytes; (Aldrich, Blackburn, & Kellems, 2000). This enzyme may also appear as an ecto-ADA (bound to the membrane glycoprotein CD- 26/dipeptidil peptidase IV) which is thought to regulate the level of extracellular adenosine (Richard et al., 2003). ADA1 catalyzes deamination of adenosine and deoxyadenosine to inosine and deoxyinosine. Deoxyadenosine is a cytotoxic metabolite secreted by cell populations that undergo apoptosis. This metabolite can kill cells via a mechanism that disrupts deoxynucleotide metabolism. Congenital lack of human or murine ADA1 leads to elevated levels adenosine and deoxyadenosine, causing developmental defects. This could be a plausible cause for Severe Combined Immunodeficiency Syndrome (SCID; deciphering) (Aldrich et al., 2000).

The ADA2 subfamily is somewhat different from the "classical adenosine deaminases". Mammalian ADA2 is a secreted enzyme and its only substrate is adenosine. Mammalian ADA2 is encoded in the gene CECR1 (cat eye syndrome critical region protein 1; (Riazi et al., 2000).

Six genes with sequence similarity to CECR1 subfamily have been identified in Drosophila: ADGF-A, -A2, -B, -C, -D and -E. ADGF-A and ADGF-D exhibit strong adenosine deaminase activity and have a mitogenic effect on certain cells by depletion of the extracellular adenosine, which has been shown to block proliferation (Zurovec, Dolezal, Gazi, Pavlova, & Bryant, 2002).

1.2.6. Adenosine receptors and signaling

1.2.6.1. Adenine nucleotide degradation cascade and purinergic receptors

Earlier, dying cells were seen as the only source of extracellular nucleotides. However, it was shown that many cells release ATP as a result of physiological stimuli (Bodin & Burnstock, 2001; Burnstock, 2006). Figure 2 gives an overview of the various purine compounds and their receptors. Adenosine can influence on several signaling pathways, and therefore the cell function by binding purine receptors (P1). On the extracellular surface there are also specific purine (P2) receptors for ATP and ADP. P2 receptors can bind uridin nucleotides as well as adenine nucleotides. Activation of P2X receptors leads to a change in ion permeability. For example, activation of P2X7 receptors in T-lymphocytes has been shown to increase Ca^{+2} levels (Schenk et al., 2008). In contrast, metabotropic P2Y receptors are G-protein coupled and function similar to the adenosine-P1 receptors via an intracellular signaling pathway.

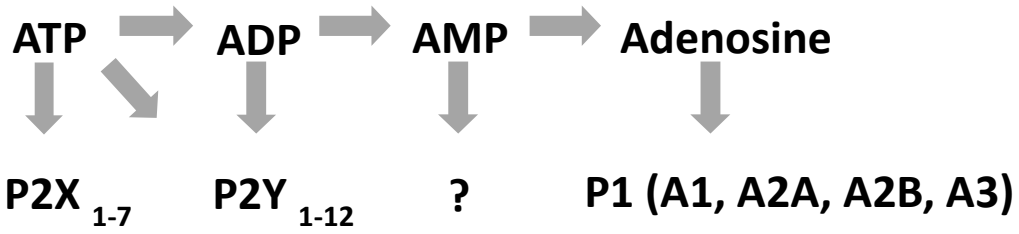


Figure 2: Adenine-purine and its receptors (modified from Klotz 2007)

Apart from binding the above mentioned P2 receptors, adenine-nucleotides can also be desphosphorylated and degraded to adenosine at the extracellular milieu before being converted to inosine in a very last step.

The first step of this degradation cascade is fulfilled by members of the the NTPDase family. This family is formed by eight different subtypes distinguished from each other based on their cellular localization and their substrate affinities. NTPDases 1-3 and 8 are typical membrane -associated nucleotidases. In contrast, NTPDases 4 and 7 are found only in the intracellular space; and NTPDases 5 and 6 are found both in the cytosol and interstitially in the intercellular space. NTPDase 1 shows the same affinity for ATP and ADP, whereas the NTPDases 2, 3 and 8 prefer ATP. NTPDase 1 (ecto-apyrase) is thus the main source of AMP (Robson et al., 1997). At present, there is no receptor known for extracellular AMP, though it has been postulated that GPR80/99 could act as receptor

for AMP. (Takedachi et al. 2007). Thus, it is assumed that AMP is converted to adenosine mainly via the ecto -5' -nucleotidase (ecto -5' -NT, CD73). Adenosine can also be formed from AMP by action of the alkaline phosphatase (AP), though AP is not as specific as CD73 since it can also degrade other biological compounds such as purine/pyrimidine mono- phosphates (GMP, UMP, CMP). Figure 3 shows a scheme of the mentioned process.

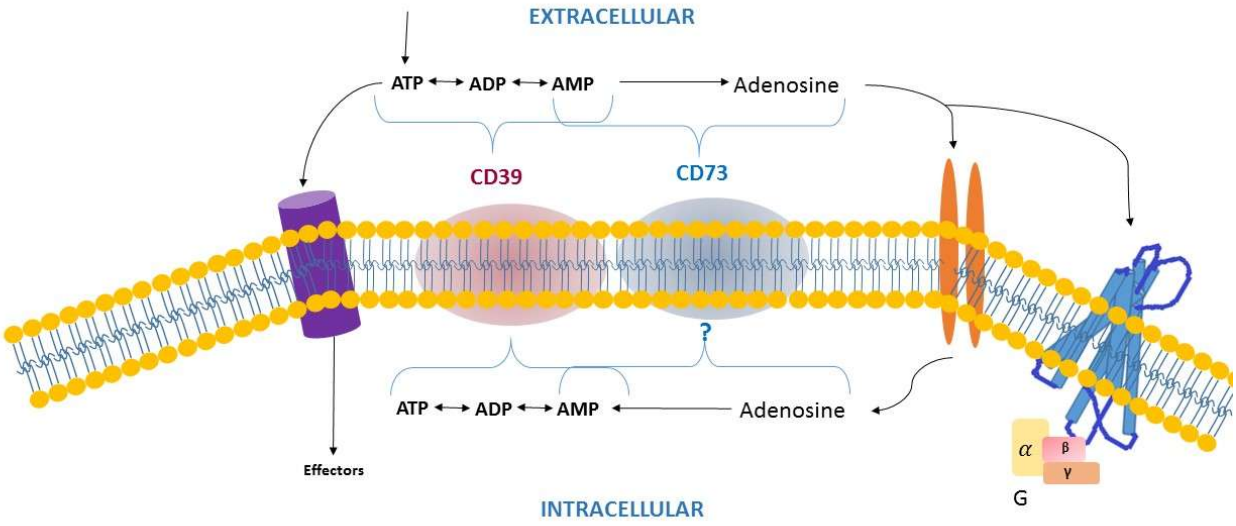


Figure 3: Adenine-purine: degradation and receptors

1.2.6.2. Adenosine receptors and their signal transduction

Adenosine receptors are G (guanine nucleotide-binding) protein coupled in the intracellular space and are composed of three subunits: α, β and γ. Signal transduction is subtype-dependent. There are four different G-protein coupled receptors (GPCRs) which specifically bind Adenosine: A1 (A1R), A2A (A2AR), A2B (A2BR) and A3 (A3R). These receptors are classified by the different G-protein subunits and the resultant intracellular signaling mechanisms. An overview of the Adenosine receptors, their G-protein subunits and their initial cellular effects are summarized in figure 4. In addition to the receptor-specific G-proteins, receptors also have different ligand affinities for Adenosine.

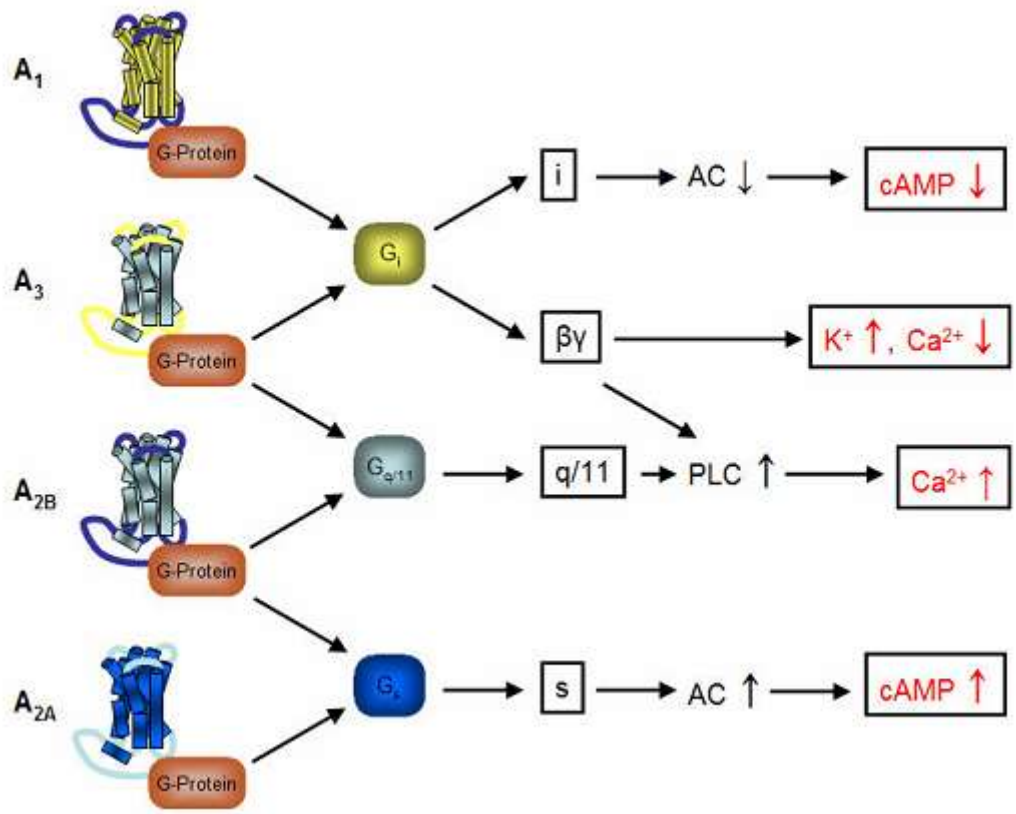


Figure 4: Adenosine receptors and initial intracellular effects.

1.2.6.3. G-proteins and adenosine signaling

The A1R has a αi subunit, which prevents the formation of cAMP through inhibition of adenylate cyclase. A1R activation plays a proinflammatory role; it particularly enhances the adherence of neutrophils to the endothelium (Cronstein, Naime, & Firestein, 1995). On the other hand, activation of the A2AR, having a αs subunit, increases the activity of adenylate cyclase and promotes the formation of cAMP. The A2BR can also increase intracellular cAMP levels. A2 receptors prevent, among others, oxidative burst of neutrophils (Bouma, van den Wildenberg, & Buurman, 1996). It is also known that activation of the A2BR can lead to an increase in the activity of phospholipase C (PLC). This results in an increase of intracellular Ca^{2+} levels. This Ca^{2+} increase is mediated by the αq/11 subunit and, among other things, is required for activation of lymphocytes. Thus, A2BR also has a proinflammatory potential. A3R, similar to A1R, has a negative effect on cAMP production via the αi subunit. Anti-inflammatory effects are well known

for the A3R, as it regulates the degranulation of neutrophils (Bouma et al., 1996) and migration of eosinophils (Knight et al., 1997).

1.2.6.4. Ligand affinities of adenosine receptors

Apart from the different signaling pathways, adenosine receptors also show different affinities for adenosine. A1R and A2AR get activated in concentration ranges from 10 - 100nM. In contrast, the A2BR and A3R need concentrations in the µM range for activation (Fredholm et al., 1994). Under normal physiological conditions Adenosine concentration in the extracellular milieu is in the nM range, or lower. This basal adenosine concentration is enough to activate the A1 and A2A repectors at least if they are abundantly expressed. On the other hand, A2B receptors need higher concentrations of adenosine to be significantly activated (Johansson et al., 2001). Under hypoxic conditions, however, the interstitial adenosine concentration increases in the tissue considerably up to 100 fold (MacLean, Sinoway, & Leuenberger, 1998; Siaghy et al., 2000). Changes in the extracellular adenosine content lead to a change in nucleoside transport activity (Baldwin et al., 2004). These transport processes depend on the concentration difference of adenosine between intra- and extracellular space. Furthermore, extracellular degradation of ATP affects the interstitial adenosine concentration. Here, the ecto-5'-nucleotidase (CD73) is of particular importance as it is the last enzyme of the degradation cascade.

1.3. ECTOENZYMES CD73 AND CD39

1.3.1. CD73

CD73 (ecto-5'-nucleotidase, 5'NT) is a glycosyl phosphatidylinositol-linked, 70-kDa membrane-bound ectoenzyme expressed in most tissues (Resta, Yamashita, & Thompson, 1998; M. V. Sitkovsky et al., 2004). CD73 catalyzes dephosphorylation of AMP to adenosine (Zimmermann, 2000). CD73 exists also as a soluble enzyme detectable in circulation and it is released upon stress (Yegutkin, Henttinen, Samburski, Spychala, & Jalkanen, 2002). CD73 is responsible for controlling the balance between pro- and anti-inflammatory purines (Hunsucker et al., 2005). Adenosine 5'-triphosphate (ATP) is constantly released into extracellular space and this release increases significantly during inflammation. ATP is immediately degraded into adenosine 5'-diphosphate (ADP) and further to adenosine 5'-monophosphate (AMP) by CD39 (ectoapyrase, NTPDase). Further on CD73 catalyzes dephosphorylation of AMP to adenosine. Both ATP and ADP have been reported to have pro-inflammatory effects whereas adenosine is a potent anti-inflammatory molecule (Di Virgilio et al., 2001). Once adenosine is generated by endothelial CD73, it binds to one of the four already mentioned adenosine receptors: A1, A2A, A2B and A3 (Linden, 2001). These receptors are ubiquitously expressed and signal intracellularly via different pathways. CD73 can regulate many different physiological responses by extracellular generation of adenosine and subsequent activation of adenosine receptors.

1.3.1.1. The role of CD73 in normal conditions

Several studies suggest that platelet function can be regulated by purinergic signaling. While intrinsic platelet function studied *ex vivo* appeared to be unaltered in CD73 knock-out mice, *in vivo* studies, however, showed various differences (Koszalka et al., 2004). Platelets from CD73 deficient mice presented lower levels of intracellular cyclic adenosine monophosphate (cAMP) compared to the wild-type, probably due to the lower levels of adenosine present in the plasma of the knock-out mice and the consequent lack of adenosine receptor activation. This observation is linked to a reduced bleeding time after tail tip resection and vessel occlusion induced by free radical injury

(Koszalka et al., 2004). Adenosine is also known to be an important regulator of glomerular filtration by acting as a messenger between macula densa and the underlying smooth muscle cells. Another study with CD73 deficient mice, conducted by Castrop et al. revealed that under physiological normal conditions, there was no difference in renal function between knock-out and wild-type controls. However, when tubular perfusion flow was increased in mice lacking CD73, significantly lower superficial nephron glomerular filtration rates were measured. Furthermore, CD73 deficient animals present almost no residual feedback response during prolonged perfusion of the loop of Henle (Castrop et al., 2004). Epithelial cells in the lungs and intestine actively transport water and ions in order to maintain the epithelial surface hydrated. Also under normal conditions, high CD73 activity can be detected in these mucosal organs (Thompson et al., 2004). Later on, adenosine was demonstrated to be responsible for the activation of electrogenic chloride transport and fluid secretion (Gamba, 2005). However, there is still no direct proof of the importance of CD73 activity in ion transport and hydration of the mucosas.

1.3.1.2. The role of CD73 in disease

Hypoxia has been proven to induce high levels of endogenous adenosine (Gnaiger, 2001; O'Farrell, 2001). On one hand, this phenomenon can be explained through the well known vasodilatory effects of adenosine and its consequent supply of oxygen to the hypoxic tissue, since it has been shown that binding of adenosine to the A1 receptor leads to an increase on the blood flow to the hypoxic area (Bryan & Marshall, 1999). On the other hand, limitation of inflammatory response by ATP depletion can also explain the raised adenosine levels in hypoxic conditions. It has been well documented that increased adenosine production under hypoxic conditions is attributable to CD73 (Eltzschig et al., 2003; Kobayashi, Zimmermann, & Millhorn, 2000; Ledoux et al., 2003; Synnestvedt et al., 2002; Thompson et al., 2004). Until now two different mechanisms have been shown to upregulate CD73. First, hypoxia induces transcription of CD73 via hypoxia inducible factor-1 (Synnestvedt et al., 2002). This induction of CD73 expression will lead to an activation of the high affinity adenosine receptors A2A or A2B and consequently, intracellular levels of cAMP will be raised. Since CD73 gene promoter

presents a cAMP response element, the product of CD73-catalyzed reaction leads to a further transcriptional up-regulation of CD73 (Hansen, Resta, Webb, & Thompson, 1995). As mentioned above, hypoxia can also induce an increase in vascular permeability with a subsequent extravasation of protein-rich fluids and neutrophils. This vascular leakage can be enhanced upon blockade of CD73 enzymatic activity (Eltzschig et al., 2004; Thompson et al., 2004).

Adenosine is well known to have protective effects in ischemic myocardium and it can give rise to tolerance to ischemia in myocardium through a process known as preconditioning (de Jong, Elzinga, McCammon, Grivell, & van der Spek, 2000; Headrick et al., 2003). Adenosine will exert its protective effect by binding to adenosine receptors on several cell types, namely cardiomyocytes, endothelium and/or immune cells (Eltzschig et al., 2003).

CD73 activity is the major source of extracellular adenosine production in the heart and it has been proven to assist in ischemic preconditioning (Eckle et al., 2007; Koszalka et al., 2004). Adenosine can reduce an ischemia-reperfusion injury by conjugation and consequent activation of the A2A receptors on inflammatory cells (Linden, 2001).

During an inflammatory progress, activated neutrophils extravasate from circulation to the tissues. These cells play an important role in the development of inflammation-induced injury which leads to cell death and tissue edema. Adenosine can reduce neutrophil activation and so prevent its potentially harmful effects in the tissue (Cronstein, Kramer, Weissmann, & Hirschhorn, 1983). This effect is exerted via activation of adenosine receptors on neutrophils. Similar effects have been observed when the receptors were activated by agonists, which are being intensively studied for their therapeutic potential (McCallion, Harkin, & Gardiner, 2004; Mubagwa & Flameng, 2001; Rosengren, Arfors, & Proctor, 1991). The importance of endogenous adenosine generation in inflammation was recognized only recently using gene-targeted animals (Eckle et al., 2007; Eltzschig et al., 2004; Grenz et al., 2007; Koszalka et al., 2004; Thompson et al., 2004). Inflammation is accompanied by extracellular release of adenine nucleotides. Endothelial cells, activated neutrophils, platelets and dead cells all contribute to the nucleotide release. ATP, ADP and AMP are readily metabolized by CD39 and CD73 into adenosine. This mechanism abolishes excessive accumulation of

neutrophils in the inflamed tissues and prevents excessive tissue injury (Eltzschig et al., 2004; Grenz et al., 2007; Guckelberger et al., 2004; Thompson et al., 2004). Deaglio et al. suggest that the mechanism of CD73-mediated immunosuppression is adenosine generation on the surface of regulatory T cells. The potential role of CD73 in the response of host to microbial infection has only been recognized recently. Thus, intestinal epithelial cells damaged by bacterial infection release ATP, which is immediately metabolized by CD39 and CD73 into adenosine. The adenosine produced in this reaction may increase fluid secretion by the epithelium and produce diarrhea (Crane, Olson, Jones, & Duffey, 2002). Furthermore, viral infection of endothelial cells was shown to increase both expression and activity of CD73 (Kas-Deelen et al., 2001). One of the possible mechanisms behind CD73 induction in viral infection is release of pro-inflammatory cytokines by the infected cells. One of these cytokines, interferon-α (IFN-α), has been shown to induce CD73 activity and adenosine production *in vivo* (Niemela et al., 2004).

In spite of all these studies, the role of CD73 in cancer remains unclear. Previous studies showed that CD73 participates in cell-cell and cell-matrix interactions and related the function of CD73 to drug resistance and tumor progression (Spychala, 2000). This ectoenzyme has been found in many cancer cell lines and its expression is enhanced in tumor tissues (Jin et al., 2010). In concordance with this, genetic data revealed an upregulation of CD73 in several human carcinomas, such as colon, lung, pancreas and ovary. Moreover, high levels of CD73 expression are associated with tumor neovascularization, invasiveness and metastasis among others (Spychala et al., 2004). Importantly, this ectoenzyme has been confirmed to promote migration, invasion and adhesion of human breast cancer cells (L. Wang et al., 2008). Accordingly, high levels of CD73 have been found in highly invasive human melanoma cells lines.

CD73 has been proposed as a diagnostic tool for papillary thyroid carcinomas due to its strong expression. Furthermore, its activity could also help in the diagnosis of colon carcinoma. This way, an increase of CD73 activity during tumor progression might be the result of a physiologic attempt to provide more substrate to intensify purine salvage pathway activity (Spychala, 2000).

1.3.1.3. Therapeutic potential of targeting CD73

The regulatory effects of adenosine signaling in numerous diseases make it an attractive candidate as a therapeutic target. However, the short life span of the molecule and the severe side effects, namely hypotension and arrhythmias, limit the clinical use of adenosine.

Adenosine receptor agonists and antagonists represent another set of potential therapeutic candidates. In this case, specificity and pharmacodynamics have been serious obstacles (Linden, 2001). Manipulation of endogenous adenosine generation is an approach which has a great therapeutic potential. CD73 directed therapies have not been well-developed. The use of the CD73 inhibitor αβ-methylene ADP (APCP) has been well documented in various murine models (Eltzschig et al., 2004; Synnestvedt et al., 2002; Thompson et al., 2004). APCP has been proven to be well tolerated, biologically active through oral administration and nontoxic in mice up to 60mg/kg/day.

Given the established relation among angiogenesis and adenosine concentration, blockade of adenosine production by CD73 inhibition could be a therapeutic target for the prevention of tumor progression.

1.3.2. CD39

CD39 is a glycoprotein expressed on the surface of different cell types. It was first identified as a marker of B-cell activation and since then, CD39 has been found to be present on numerous cells; ranging from natural killer cells, T cells, monocytes (Kansas & Tedder, 1991), neutrophils (Koziak, Sevigny, Robson, Siegel, & Kaczmarek, 1999), endothelium (Kansas & Tedder, 1991), cardiomyoctes, neurons, smooth muscle cells, Langerhans cells and mesangial cells.

After the cloning of CD39, a great similarity was found out with yeast (Sacharomyces cerevisae) guanosine diphosphate GDAI. This observation led to the idea that CD39 might have an enzymatic activity. It was later proven, by Guidotti and Wang, that CD39 has an ecto-apyrase activity. CD39 can catalyze the dephosphorylation of ATP and ADP to AMP in a Ca^{2+} and Mg^{2+} dependent manner. The affinity of this ecto-enzyme is slightly higher for ATP than for ADP (Boeynaems, Communi, Gonzalez, & Robaye, 2005; Marcus

et al., 1991). When CD39 sequentially metabolizes ATP to AMP, this happens in a concerted step. The intermediate ADP will not be released in spite of the different phosphohydrolysis rates for ATP and ADP.

CD39 is the major vascular apyrase, thus it is thought to play a critical role in the control of nucleotide concentration in blood. CD39 is located in the cell membrane; with intracellular amino- and carboxy- terminal ends, two transmembrane domains, and an extracellular loop. It is not yet clear what the function of the amino and carboxy-terminals are, but it has been shown that the amino-terminal Cys13 can be palmitoylated and that this lipidic modification is important for localization of CD39 to caveolaes. Caveolaes are pouch-like and highly specialized membrane invagination sites where signaling domains integrate external stimuli and transduce them into internal activation through a signaling cascade. CD39 is colocalized with the AMPase CD73 in the caveolae, this might coordinate the transfer of the final product of CD39, AMP, to CD73; allowing this way an efficient generation of adenosine. Other purinergic receptors such as P2Y1-receptor can be found at close proximity in the same caveolae. This may imply that the funtion of CD39 and CD73 could be coordinated to regulate purinergic signaling at the surface of a cell. As already mentioned, ATP and ADP specific receptors (P2) arouse a pro-inflammatory response, whereas adenosine receptors (P1) elicit an anti-inflammatory response. This way, CD39 can convert inflammatory stimuli into anti-inflammatory signal.

1.3.2.1. CD39: Structure and Function

CD39 belongs to the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) family of ectonucleotidases. Every member of the ENTPDase gene family shows a specific distribution of expression and enzymatic activity (Grinthal & Guidotti, 2002). CD39 degrades ATP slightly more efficiently than ADP, while CD39L1, another E-NTPDase family member, degrades ATP much more efficiently than ADP (Grinthal & Guidotti, 2002).

CD39 activity will vary according to membrane flexibility, fluidity and composition. For example, monounsaturated and saturated fatty acids increase ATPDase activity of CD39 whereas polyunsaturated fatty acids diminish it (Robson et al., 1997). Furthermore,

CD39 activity is also modulated by the intracellular signaling molecule Ran Binding Protein M (RanBPM). RanBPM is thought to alter CD39's activity by binding its amino terminus, and thus change its structural conformation (Y. Wu et al., 2006). The extracellular loop of CD39 contains 7 N-glycosolation sites allowing substantial post-translational modifications of the enzyme. Deglycosylation with N-glycanase reduces the molecular weight of CD39 to 54 kD the glycosyl while the glycosylated form has a size ranging from 70 to 100 kD. On the one hand, these N-linked glycosolations are required for enabling export from the endoplasmic reticulum to the cell surface (Zhong, Kriz, Kumar, & Guidotti, 2005; Zhong, Malhotra, Woodruff, & Guidotti, 2001). On the other hand, certain N-glycosolation sites (1, 4, and 7) have been reported to be critical for conferring specificity to CD39. These specific glycosylation sites may affect protein structure but not surface expression (J. J. Wu, Choi, & Guidotti, 2005). As mentioned above, RanBPM can bind CD39; this binding suggests that CD39 might play a role in signal transduction (Y. Wu et al., 2006). This idea has been supported by results revealing the binding of B cell surface CD39 to an antibody leads to homotypic B cell adhesion (Kansas & Tedder, 1991). The intracellular pathway by which the extracellular signal is transmited is still unclear, though it is known that B-cell clustering is tyrosine kinase dependent (Kansas & Tedder, 1991). Moreover, CD39 is thought to have an ATPase channel function. The structure of CD39 is unique among apyrases for having two transmembrane domains with one extracellular loop, which makes it similar to numerous cell membrane pores and channels (North, 1996).

1.3.2.2. CD39 and Thromboregulation

Adenosine diphosphate (ADP) was the first molecule identified as an activator of circulating platelets (Born, 1962). Later on, other agonists have been proven to activate platelets such as collagen and thrombin (Smyth et al., 2009). However, ADP signaling is required for the auto-activation of platelet thrombus formation. Activated platelets will release granules containing ADP, which in turn activate other platelets initiating a second wave of platelet activation and thus stabilizing thrombus formation (Gachet, 2008).

1.3.2.3. CD39 and Immunomodulation

CD39 was thought to play a role in the regulation of immune responses after being found on the surface of B cells (Kansas & Tedder, 1991; Kansas, Wood, & Tedder, 1991). It was also observed that the expression of CD39 on the B cells was greatly increased after an infection with Epstein-Barr virus. Moreover, homotypic adhesion of immune cells was induced by adding anti-CD39 antibody to lymphocytes (Kansas & Tedder, 1991). The first observation pointing out the functional relevance of CD39 came from studies performed in Langerhans cells, since CD39 is used as a marker for Langerhans cells in skin biopsies (Wolff & Winkelmann, 1967). CD39 knock out mice were studied in a model of contact dermatitis, and they could show that CD39 limits ATP signaling between Langerhans cells and keratinocytes (Granstein, 2002; Mizumoto et al., 2002). This communication is pivotal for leukocyte recruitment and its failure could lead to an aggravation of inflammation. In addition, other experiments demonstrated that desensitization of immune cells can reduce allergy-induced dermatitis in $CD39^{-/-}$ mice (Mizumoto et al., 2002).

CD39 has been shown to be present on the surface of many immune cells ranging from monocytes to neutrophils (Pulte et al., 2007). It has been observed in a whole tissue myeloperoxidase assay, in some ischemia and reperfusion models that the innate immune system response was enhanced in $CD39^{-/-}$ mice (Guckelberger et al., 2004). The inflammation mechanism of this model is not clear, although they could observe that ischemic knock-out mice had an increased vascular permeability thereby allowing enhanced leukocyte infiltration (Guckelberger et al., 2004).

CD39 has also been shown to control endothelial activation and cytokine release induction. Together with the vascular permeability increase, CD39 provides a plausible mechanism to suppress inflammation (Goepfert et al., 2000; Imai, Goepfert, Kaczmarek, & Robson, 2000). The link between CD39 and immunity has been extensively studied in T cells. A T cell subset with very high CD39 expression is a population of regulatory T cells called T_{regs} (Deaglio et al., 2007). It has been shown that T cells release ATP during antigen presentation. Thus, degradation of this ATP to adenosine is thought to play an immunosuppressive role which involves T cells. Interestingly, T_{reg} cells also express CD73 which suggests a rational mechanism by which ATP is converted to adenosine by T_{regs} to

promote immunosuppression (Deaglio et al., 2007). Furthermore, CD39 knock out T_{reg} cells have dysfunctional immunosuppressive capabilities and cannot stop allograft rejection (Deaglio et al., 2007).

1.4 THE ROLE OF ADENOSINE IN INFLAMMATION AND CANCER

The link between cancer and inflammation was first described by the Greek physician Galenus over 2000 years ago (Trinchieri, 2012). Much later, in the ninenteenth century, Rudolf Virchow observed "lymphoreticular infiltrates" in tumor tissues and considered it as a confirmation of Galenus's hypothesis which affirmed that tumors arise at sites of inflammation (Balkwill & Mantovani, 2001). Further decades of research have proven an association between a chronic inflammatory environment and tumor growth (Grivennikov, Greten, & Karin, 2010; Kamp, Shacter, & Weitzman, 2011). Moreover, 25% of cancer have been related with chronic inflammatory conditions sustained by diverse infectious agents such as, helicobacter pylori, human papilloma virus and human herpes virus among others (Arzumanyan, Reis, & Feitelson, 2013; Bhandari & Crowe, 2012; Kemeny, Gyulai, Kiss, Nagy, & Dobozy, 1997), as well as other inflammatory conditions: inflammatory bowel disease (Hartnett & Egan, 2012), chronic obstructive pulmonary disease (Houghton, 2013) or prostatitis (De Marzo et al., 2007).

The chronic inflammation provides both the intrinsic (oncogenes, tumor suppressor factors) and the extrinsic (immune and stromal factors) conditions which support tumor growth (Coussens, Zitvogel, & Palucka, 2013; Spychala, 2000). Cell infiltration in neoplastic tissue can be different according to the inflammation type due to the co-evolution of tumor cells and the composition of their microenvironment (Balkwill, Charles, & Mantovani, 2005). These specific inflammatory infiltrates create a tumor favorable scenario by secreting diverse factors and cytokines such as, TGF-ß, TNF-α, chemokines, interleukins among others (Coussens et al., 2013). These factors cause functional changes in immune cells and impairs cytotoxic cell-mediated killing of the tumor cells (Coussens et al., 2013).

1.4.1. Adenosine: a stress signaling molecule

Under metabolic stress conditions such as hypoxia, ischaemia, inflammation or trauma adenosine is produced in higher amounts and released into the extracellular milieu (Hasko, Linden, Cronstein, & Pacher, 2008). For example, in hypoxic conditions oxygen demand and supply are not balanced and so is the ATP utilization and regeneration rate, leading to a higher adenosine production. The most common adenosine production source is AMP dephosphorylation. Thus, the cell will try to establish equilibrium by producing more ATP [2 ADP ↔ ATP +AMP] (Bruns 1991).

Adenosine functions as a signaling metabolite from plants to animals (Abbracchio et al., 1997). Its main role is to protect tissue from metabolic stress damage. The function of adenosine can generally be seen as homeostatic and protective, since it can increase blood flow during hypoxia (Berne, 1963) and control energetic balance by diminishing energy demand and increasing its supply (Bruns 1991).

Extracellular adenosine is considered to be a very relevant signaling molecule under stress conditions. Its concentration drastically increases in stress conditions such as multiple organ failure, which is responsible for 50-80% of all deaths in surgical intensive care units (Hasko & Cronstein, 2004). In systemic stress conditions, adenosine is secreted from the sympathetic nervous system (Sperlagh, Doda, Baranyi, & Hasko, 2000) and under hypoxic conditions, it can be released from various tissues (Buck, 2004). Extracellular adenosine behaves so similar to a hormone that it has also been called the "stress hormone" (Hasko & Cronstein, 2004). However, adenosine has a very short life span and therefore it can only act locally. In mammals adenosine has an effect in every tissue (Linden, 2001). For example, Drury and Szent-Györgyi already in 1929 observed its vasoconstrictory effect. Apart from this, adenosine can also modify hormone release (Nyce, 1999) and therefore oxygen and metabolic supply/demand in tissues (Berne, 1963; Costa & Biaggioni, 1998), immune responses (M. Sitkovsky & Lukashev, 2005) and signaling in the nervous system (Dunwiddie & Masino, 2001; Masino & Dulla, 2005).

1.4.2. Adenosine bioavailability and its influence in the innate immune system

As already explained, adenosine mainly exerts its physiological effects by binding adenosine receptors on the cell membrane and thus, activating downstream cellular pathways. Adenosine production, metabolism, secretion and cell-uptake will determine its availability. These processes are highly regulated; thus, during hypoxic conditions or ischemia, dephosphorylation of ATP to adenosine by CD39 and CD73 is paralleled by a suppression of the activity of the salvage enzyme adenosine kinase, thereby preventing the rephosphorylation of adenosine (Fredholm, AP, Jacobson, Klotz, & Linden, 2001). When adenosine has reached a high concentration inside the cell, it is transferred to the extracellular space through specialized nucleoside transporters (Pastor-Anglada et al., 2001). Another pathway leading to an increase of the extracellular adenosine concentration is the already mentioned release of adenine precursor nucleotides (AMP, ADP and ATP) followed by extracellular degradation to adenosine by the ectoenzymes CD39 and CD73. The bioavailability of adenosine is limited by the action of adenosine deaminase which degrades adenosine to inosine. Neutrophils and endothelial cells are known to release high levels of adenosine at sites of stress, inflammation and/or infection (Cronstein, 1994). Furthermore, it has been shown (Sperlagh et al., 2000) that in an ischemic spleen high levels of adenosine are released from nerve terminals. Adenosine is also generated by dephosphorylation of ADP released by platelets at the site of injury (Ralevic & Burnstock, 1998). Normal adenosine concentration do not have any effect on immune responses, however, the increased concentration of adenosine upon inflammation exerts an immunomodulatory effect, more exactly immunosuppressive.

Adenosine signaling in the immune system involves also up- and downstream metabolites. It is known that adenine nucleotides have an immunoregulatory function which is mediated by the P2-purin receptors (Di Virgilio et al., 2001). On the other hand, adenosine degradation products, inosine and uric acid have also many effects on the immune response (Hasko, Kuhel, Nemeth, et al., 2000; Scott et al., 2002). Regarding the levels of purinergic products the tissue manifests its metabolic status, providing information about its “health” to the immune system.

1.4.3. Extracellular adenosine and regulation of immune response

Damaged cells release ATP (e.g. at the site of inflammation) to the extracellular milieu where it is successively dephosphorylated to adenosine by the already mentioned cascade of ecto-enzymes. The decrease of ATP and the increase of adenosine regulate acute inflammatory responses, the fine-tuning of ongoing inflammation and its eventual downregulation through purinergic receptors (Bourset al., 2006). This regulatory process is complex and there are still many open questions regarding the role of ATP and adenosine. It has been hypothesized that ATP could as well be released from endothelial cells as a response to specific inflammatory stimuli, such as bacterial products (Bodin & Burnstock, 2001). The effects of adenosine on immune cells and inflammation, on peripheral blood cells and on regulation of hematopoiesis is reviewed in Hasko et al., (2008), Cronstein et al. (1996), Hofer et al. (2011) and Antonioli et al. (2013). Figure 5 shows a scheme of adenosine production at the tumor microenvironment and its effects on different immune cells.

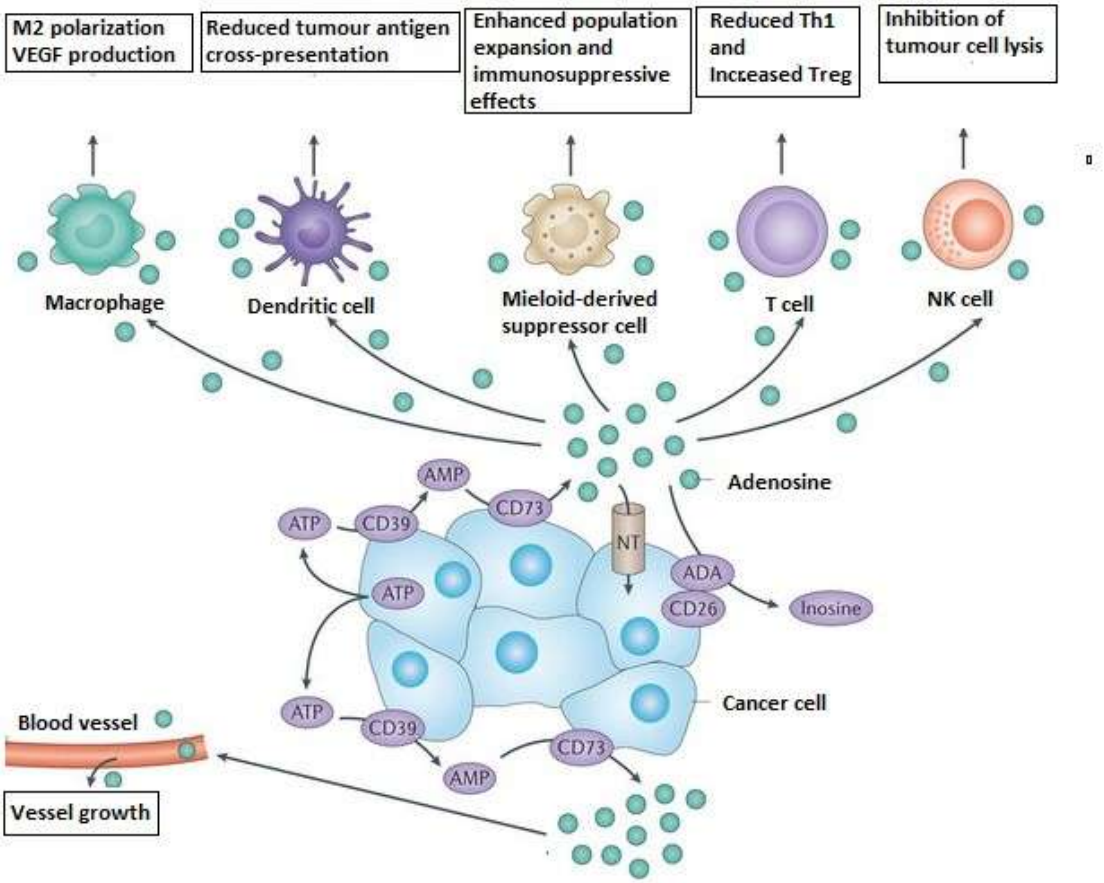


Figure 5: adenosine production in the tumor microenvironment and its effects on immune cells (modified from (Antonioli, Blandizzi, Pacher, & Hasko, 2013).

1.4.4. Effects of adenosine in the Immune system

1.4.4.1. Cell trafficking

The endothelial adenosine receptors can regulate immune cell trafficking among different tissue compartments. The early events of inflammation are orchestrated by endothelial cells in response to different inflammatory mediators such as cytokines and complement components. Upon inflammation, endothelial cells change their surface protein pattern and express adhesion molecules responsible for leukocyte recruitment. Apart from changing their surface molecular expression, endothelial cells also secrete several cytokines (e.g IL-8 and IL-6) which directly promote leukocyte trafficking between tissue compartments.

It is well known that several types of vascular endothelial cells express A2A and A2B adenosine receptors (Eltzschig et al., 2003; Montesinos et al., 1997) and are known to be important sources of adenosine (Lennon, Taylor, Stahl, & Colgan, 1998). Along with the transmigration among the endothelial monolayer, neutrophils release AMP which is phosphorylated to adenosine by the ectoenzymes on endothelial cells. The produced adenosine will then support endothelial barrier function via the A2B receptors (Eltzschig et al., 2003; Montesinos et al., 1997). Adenosine has also been revealed to inhibit the expression of IL-6 and IL-8 as well as the expression of adhesion molecules E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in immunostimulated human umbilical vein endothelial cells (Bouma et al., 1996). This inhibition is a result of the A2B adenosine receptor activation (Bouma et al., 1996).

1.4.4.2. Neutrophils

The first immune cells to be recruited at the site of injury are neutrophils. They kill microorganisms, eliminate cell debris and release factors which attract other inflammatory cells to the site. In 1983 Cronstein et al. first demonstrated that adenosine was able to suppress the production of superoxide anion in neutrophils (Cronstein et al., 1983). Further studies revealed that the binding of adenosine to its A2A receptor could inhibit neutrophil adhesion, killing, bactericidal activity, programmed cell death, expression and secretion of adhesion molecules, cytokine release and growth factor

synthesis (Cronstein et al., 1995; Flamand et al., 2000). Several studies have reported that the effect of adenosine in neutrophils is mediated by the A2A receptor (Sullivan, Linden, Buster, & Scheld, 1999; Thibault et al., 2002). However, neutrophils have been reported to have more than one adenosine receptor (Rose, Hirschhorn, Weissmann, & Cronstein, 1988). For example, the activated A1 receptors may increase neutrophil chemotaxis and phagocytosis (Cronstein, 1994). Nevertheless, the effects of the A1 receptors are eclipsed by the effect of A2A receptors due to their higher affinity for adenosine (Cronstein, 1994).

1.4.4.3. Antigen presenting cells (APCs)

Defense against infection and phagocytosis of apoptotic host cells or injurious molecules are some of the functions of macrophages and dendritic cells (DCs). The APCs are present all throughout the body and are responsible for the capture and processing of antigens and they activate specific effector mechanisms (lymphocyte activation). APCs express several pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), mannose-binding lectins and scavenger receptors (Janeway & Medzhitov, 2002). Recent studies have revealed that pathways activated by PRRs can be interrupted by adenosine signals. For example, secretion of IL-12 after recognition of LPS by TLR4 can be inhibited by adenosine signaling (Hasko, Kuhel, Chen, et al., 2000; Khoa et al., 2001; Link et al., 2000). IL-12 can cause a strong inflamatory response and thus adenosine prevents inflammation at the injury site. Furthermore, adenosine signaling has been reported to diminish secretion of proinflamatory mediators such as TNFα, MIP-1 A, and nitric oxide as well as to increase the secretion of anti-inflammatory IL-10 (Hasko, Nemeth, Vizi, Salzman, & Szabo, 1998; Mayne et al., 2001; Sajjadi, Takabayashi, Foster, Domingo, & Firestein, 1996; Szabo et al., 1998). It is likely that adenosine exerts its anti-inflammatory effects by targeting a common intracellular pathway. However, the nature of such a common mediator is still unclear. Knocking down murine A2A and A3 receptors has revealed that both of them contributed to adenosine inhibition of the inflammatory mediators released after TLR activation (Hasko, Kuhel, Chen, et al., 2000; Ohta & Sitkovsky, 2001; Salvatore et al., 2000). Moreover, the A2B receptor has been found to

cause the downregulation of the expression of NO synthase and MHC II in response to interferon γ (IFNγ) (Xaus et al., 1999).

In addition, adenosine binding to the A3 receptor can promote chemotaxis in immature DCs; this is associated with an increase of intracellular calcium levels and actin reorganization (Fossetta et al., 2003; Panther et al., 2003). Thus, adenosine exerts a protective effect by accumulating immature DCs at sites of microbial invasion, which is crucial for the initiation of an immune-response. However, adenosine suppresses TLR-mediated IL-12 production of mature DCs present at an establish immune response and thus these DCs cannot differentiate T cells towards a Th1 phenotype (Panther et al., 2003). Inhibition of IL-12 hence constitutes another mechanism by which adenosine can suppress inflammation.

1.4.4.4. Mast cells

Mast cells are essential components of the innate immune system. Although traditionally mast cells have been associated with IgE mediated responses against parasites, they also participate in immune responses against bacteria as well as in the pathogenesis of inflammatory arthritis (Feger, Varadaradjalou, Gao, Abraham, & Arock, 2002). Adenosine has been found to be a potent mast cell activator through the activation of the receptor A2B. This activation leads to degranulation of mast cells liberating histamine, chemokines, serotonin and injurious proteases (Feoktistov, Ryzhov, Goldstein, & Biaggioni, 2003; Hannon, Pfannkuche, & Fozard, 1995; Jin et al., 2010; Ramkumar, Stiles, Beaven, & Ali, 1993; Salvatore et al., 2000). It has been postulated that the adenosine mediated release of histamine could provide a negative feedback mechanism in macrophages inhibiting their TNFα production through their H2 histamine receptors (Smith, Denhardt, & Terminelli, 2002). However, the anti-inflammatory potential of adenosine is overruled by its proinflammatory function in cases of prolonged high adenosine concentration. It has been shown that adenosine deaminase deficient mice develop a pulmonary pathology, similar to asthma, due to adenosine-triggered degranulation of mast cells (Blackburn et al., 2003; Blackburn et al., 2000; Chunn, Young, Banerjee, Colasurdo, & Blackburn, 2001).

1.4.4.5. T lymphocytes

$CD4^+$ and $CD8^+$ T lymphocytes recognize major histo- compatibility class (MHC) II- and I associated antigens, respectively, and have been identified in the circulation of cancer patients, as well as in the tumor microenvironment (Albers et al., 2005; Koch et al., 2006; Sotiropoulou et al., 2003). $CD4^+$ Th1 cells produce and secrete type 1 cytokines which lead to an anti-tumor immune response, whereas $CD8^+$ cytotoxic T lymphocytes (CTL) eliminate tumor cells via granule-dependent and -independent cytotoxicity (Knutson & Disis, 2005; Titu, Monson, & Greenman, 2002). $CD4^+CD25^+Foxp3^+$ regulatory T cells have an inhibitory function which is likely involved in tumor evasion of immune responses (H. Y. Wang & Wang, 2007). In 2007, Deaglio et al. showed that regulatory T cells can suppress immune responses through extracellular adenosine production which is catalyzed sequentially by CD39 and CD73 expressed on the surface of regulatory T cells (Deaglio et al., 2007).

$CD4^+$ and $CD8^+$ T cells express each of the adenosine receptor subtypes except A1. Adenosine has been reported to inhibit a wide range of T lymphocyte functions such as antigenic stimulation, proliferation (Hoskin, Butler, Drapeau, Haeryfar, & Blay, 2002; Huang, Apasov, Koshiba, & Sitkovsky, 1997), synthesis of IL-2 and proinflammatory cytokines, namely interferon-γ and TNF-α (Butler et al., 2003; Lappas, Rieger, & Linden, 2005; Raskovalova et al., 2007), up-regulation of CD25 (IL-2 receptor α chain) (Butler et al., 2003; Huang et al., 1997), expression of cytotoxic effector molecules such as perforin and Fas ligand (Hoskin et al., 2002; Koshiba, Kojima, Huang, Apasov, & Sitkovsky, 1997), CTL adhesion to tumor target cells (MacKenzie, Hoskin, & Blay, 1994, 2002) and granule exocytosis by CTL (Koshiba et al., 1997). Thus, a micromolar concentration of adenosine or 5'-N-ethylcarboxamidoadenosine (NECA), a non-selective but stable adenosine receptor agonist, inhibited T cell receptor/CD3- and CD28-induced phosphorylation of tyrosine residues on murine tyrosine kinases p56lck and ZAP-70, which are essential components of the T cell receptor signal transduction pathway (Mustelin & Tasken, 2003). Furthermore, adenosine suppresses DNA synthesis in T cells. Studies in murine models have revealed that adenosine signals in T cells mainly through the A2A and A3 receptors (Hoskin et al., 2002; Huang et al., 1997; Koshiba et al., 1997; Lappas et al., 2005; H. Zhang et al., 2004). For example, expression of CD25 is regulated by A2A, whereas inhibition of T cell proliferation and adhesion in response to activation is

mediated by the A3 receptor (Hoskin et al., 2002; MacKenzie et al., 1994). Activation of the A2A receptor is also responsible for the inhibiton of IL-2 and TNF-α secretion in mouse $CD8^+$ CTL (Erdmann et al., 2005). Inhibition of IL-2 production inhibits clonal expansion of activated antigen-specific T cells. The inhibitory effect of A2A adenosine receptor signaling on T cell function is caused by accumulation of intracellular cAMP (Huang et al., 1997; Koshiba et al., 1997; Lappas et al., 2005).

It has been shown that A2A-receptor signaling can also activate the enzyme tyrosine phosphatase SHP-2, which dephosphorylates IL-2 receptor associated STAT-5 and truncates signal transduction of this receptor (H. Zhang et al., 2004). T cell receptor-driven activation of $CD4^+$ T helper cells requires the participation of antigen-presenting cells (APCs) such as dendritic cells, macrophages and B lymphocytes, which present antigenic peptides to $CD4^+$ T cells in the context of MHC class II molecules and provide an essential source of costimulation for T cells (Harris & Ronchese, 1999; Schneider & Sercarz, 1997). It is likely that adenosine indirectly prevents the activation of tumor-specific $CD4^+$ T cells by interfering with the APC function. As mentioned above, mature dendritic cells express A2A adenosine receptors through which adenosine inhibits IL-12 production (Panther et al., 2001).

Adenosine can as well inhibit IL-12 synthesis by macrophages via A2A adenosine receptor-dependent and independent mechanisms (Hasko, Kuhel, Nemeth, et al., 2000). Since IL-12 plays a critical role in the development of Th1 cells (O'Garra, 1998), decreased IL-12 synthesis by APCs in the presence of extracellular adenosine is thought to interfere with the induction of Th1-dependent cell-mediated immune responses. Furthermore, adenosine has been also reported to induce IL10 secretion by DCs which disrupts Th1 cell development (Panther et al., 2003).

Moreover, the adenosine analogue NECA inhibits the expression of CCR5, MIP-3ß/CCL19, and MDR-1 in mature DCs and slows down migration to draining lymph nodes (Hofer et al., 2003). Thus, adenosine might prevent DCs from promoting T cell-mediated immune responses. Murine B lymphocytes activated with phorbol ester and ionophore revealed a reduced expression of CD80 and CD86 (CD28 ligands on T cells) as well as CD80 in the presence of adenosine or NECA. The effect of adenosine on costimulatory molecule expression on B cells is likely mediated through activation of the A2A receptors

since B cells show a strong A2A adenosine receptor expression but little or no expression of other adenosine receptor subtypes (Lukashev et al., 2003). Apart from interfering with T cell costimulation at APC level, adenosine also inhibits T cell receptor and IL-2 dependent up-regulation of costimulatory CD2 and CD28 in mouse T cells (Butler et al., 2003) which may further impair T cell activation.

1.4.4.6. Natural killer cells

NK cells secrete proinflammatory cytokines, lyse MHC class I-deficient cells and, upon stimulation by cytokines (IL-2, IL-12, and IL-15) they turn into lymphokine-activated killer (LAK) cells which have a stronger cytolytic activity (Zamai et al., 2007). Adenosine and adenosine analogues are potent inhibitors of NK (Miller et al., 1999; Priebe, Platsoucas, & Nelson, 1990; B. A. Williams, Manzer, Blay, & Hoskin, 1997) and LAK cell function (Lokshin et al., 2006; Raskovalova et al., 2005). Intracellular levels of cAMP modulate NK cell function (Goto, Herberman, Maluish, & Strong, 1983). Thus, activation of the A2A receptor on murine NKs suppresses their cytotoxic activity, whereas A1 receptor activation shows a stimulatory effect (Priebe et al., 1990).

Adenosine can also inhibit the killing of 3LL Lewis lung carcinoma cells by LAK cells through the activation of the A2A receptor (Raskovalova et al., 2005). In recent studies, cAMP-dependent activation of protein kinase A type I has been implicated in adenosine-mediated inhibition of proinflammatory cytokine production and cytotoxic activity in mouse LAK cells (Lokshin et al., 2006). Interestingly, administration of the A3 receptor agonist 2-chloro-N6- (3-iodobenzyl)-adenosine-5-N-methyl-uronamide (Cl-IB- MECA) has been suggested to enhance the cytotoxic activity of NK cells, increase levels of IL-12 in serum and reduce in vivo growth of B16-F10 melanoma cells in mouse (Harish, Hohana, Fishman, Arnon, & Bar-Yehuda, 2003). However, IL-12 can strongly stimulate NK cell activity (Lee, Suen, Qian, Knoppel, & Cairo, 1998), and therefore it is equally likely that the apparent potentiating effect of Cl-IB-MECA on NK cell-mediated cytotoxicity is just an indirect result of the increased IL-12 levels in response to the A3 receptor activation. Accumulation of intracellular cAMP has also been related to the suppressive effect of adenosine on IL-2-induced cellular proliferation and TNF α production in human NK cell cultures; however, in this case adenosine could not suppress cytotoxic activity,

probably due to a counteracting effect of the cytotoxicity-promoting IL-2 high concentration that was added to the NK cell cultures (Miller et al., 1999). Furthermore, murine NK cells show abnormal granule secretion in presence of adenosine, although in this case the inhibitory effect is caused by a distinct and yet not characterized adenosine receptor (B. A. Williams et al., 1997).

1.4.5. The effect of adenosine on tumor cell growth

Insufficient microcirculation is a characteristic of fast growing tumor tissue due to the high metabolic rate of proliferating tumor cells (Vaupel et al., 1989). Thus, ischemia and hypoxia develop locally. Adenosine levels are increased in the hypoxic regions via three different ways: breakdown of ATP to adenosine by combined action of CD73 and CD39, diminished AMP formation by inhibition of adenosine kinase and adenosine synthesis by upregulation of S-adenosinhomocysteine hydrolase (Decking, Schlieper, Kroll, & Schrader, 1997; Headrick et al., 2003; Ueland, 1982). Adenosine concentration in tumor tissue can reach up to 2.4 µM (Blay et al., 1997). The observed high adenosine concentration in the tumor microenvironment leads to study its role in lymphomas, leukemia and solid tumors. The effect of adenosine in tumor tissue resulted in cell cycle arrest, inhibition of proliferation and induction of apoptosis (Barry & Lind, 2000; Brown, Cornell, & Cook, 2000; Fishman, Bar-Yehuda, & Vagman, 1998; MacLaughlin, Martinez-Salgado, Eleno, Olivera, & Lopez-Novoa, 1997). Inhibition of tumor growth was observed by increased adenosine concentrations (µm range) in human leukemia cell lines HL-60 and K-562 as well as in rat lymphoma cell line Nb2-11C. Active transport of adenosine into the cell induced apoptosis in some cell types (Fishman et al., 1998; Tanaka, Yoshihara, Tsuyuki, & Kamiya, 1994). It has also been shown that adenosine can decrease telomeric signal and thus arrest cells in the G0/G1 phase of the cell cycle. The telomers consist of repeated DNA sequences guarding the end of the chromosomes and serve as a check point for cell cycle progression (Fishman et al., 2000). Several studies have investigated the effect of adenosine on solid tumors; it has been observed that µM concentration of adenosine can inhibit cell growth in A431 human epidermoid carcinoma cells, LNCaP human prostate carcinoma line as well as in murine B16-F10 melanoma cell line (Fishman et al., 1998; Tey, Khoo, & Tan, 1992). Proliferation of lymphocytes derived from patients with chronic lymphocytic leukemia could be

inhibited by adenosine, while that of normal lymphocytes was suppressed to a lesser extent (Bajaj, Insel, Quagliata, Hirschhorn, & Silber, 1983).

1.5. BLOCKADE OF THE ADENOSINERGIC SYSTEM

Many in vitro studies and animal models (table 1) have already confirmed that targeting the adenosinergic system with pharmacological inhibitors has a vast therapeutical potential against cancer.

In this work, we have used 3 different small molecular inhibitors ARL67156, APCP and SCH 58261 to block CD39, CD73 and A2A receptor respectively.

1.5.1. Small molecular inhibitors

1.5.1.1. ARL 67156

This small molecule is an ecto-ATPase inhibitor; prevents metabolism of P2 purinoceptor agonists. In an attempt to develop specific tools to investigate the complex purinergic signalling system, Fisons Laboratories (now AstraZeneca, Loughborough, UK) produced this nucleotide analogue 6-N,N-diethyl-D-β-γ-dibromomethylene adenosine triphosphate (ARL 67156). Originally, this inhibitor was named FPL 67156 or AR-c67156, and today is known as ARL 67156. It was first described in 1995 by Crack et al. (1995) as a selective inhibitor of ecto-ATPase activity from blood cells. ARL 67156 has been shown to inhibit ecto-ATPase activity in different tissues from various species, such as smooth muscle membranes of mouse, rat, rabbit and guinea-pig vas deferens (Ghildyal & Manchanda, 2004; Westfall, Kennedy, & Sneddon, 1996), rat superior cervical ganglia (Connolly, Demaine, & Duley, 1998), bovine chromaffin cells (Drakulich, Spellmon, & Hexum, 2004) and rat parotid acinar cells (Dowd, Li, & Zeng, 1999). Per contra, ARL 67156 it did not block ATPase activity from guinea-pig hearts (Erga et al., 2000) nor from rat nodose ganglia (Connolly et al., 1998).

1.5.1.2. SCH58261

Chemically, SCH58261 is [7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2, 4-triazolo[1,5-c]pyrimidine]. In binding studies conducted by Zocci et al. on rat and bovine

brain tissues, SCH 58261 showed nanomolar range affinity for the A2A adenosine receptors as well as a good A2A adenosine vs. A1 receptor selectivity (Zocchi et al., 1996). Moreover, SCH 58261 did not show affinity for neither the A3 receptor nor other receptors at concentrations up to 1 µM (Zocchi et al., 1996). Additionally, saturation experiments performed by the same group on rat A1 and A2A adenosine receptors, indicated a competitive kinetic of the antagonism. Moreover, SCH 58261 failed to antagonize 5'-N-ethylcarboxamidoadenosine-induced vasorelaxation in the isolated guinea pig aorta, a response mediated by A2B adenosine receptors. Furthermore, this small molecular inhibitor could only inhibit weakly the A1 receptor-mediated negative chronotropic effect induced by 2-chloro-N6-cyclopentyladenosine in the isolated rat atria (Zocchi et al., 1996).

1.5.1.3. APCP

α,β-Methyleneadenosine 5’-diphosphate (AMP-CP) is a CD73 inhibitor and it is used to study adenosinergic signaling regulation via CD73/ecto-5’-nucleotidase.

Target	Pharmacological Inhibitor	Animals	Tumor	Pharmacological effects	Ref.
A2A	SCH58216	C57BL/6 mice	Murine OvCa cells	Combination of SCH58216 and T cell therapy improved survival of tumor bearing mice	(Jin et al., 2010)
A2A	SCH58216	Balb/c mice	Murine breast cancer cells	Increase of doxorubicin activity against tumor cells	(Loi et al., 2013)
A2A	SCH58216 and ZM241385 (A2A receptor antagonist)	Athymic nude mice	Human lung adenocarcinoma cells	Inhibition of tumor growth	(Mediavilla-Varela et al., 2013)
A2A	SCH58216	C57BL/6 mice	Murine melanoma cells	Inhibition of metastasis	(Beavis et al., 2013)
CD39	ARL67156 and NGXT191 (both CD39 inhibitors)	Immunocompetent C57BL/6 and inmunodeficient mice	Murine sarcoma cells	Increase of therapeutic effect of methotrexate or oxaliplatin	(Michaud et al., 2011)
CD73	CD73-specific mab	Balb/c mice	Murin breast cancer cells	Inhibition of tumor growth and spontaneous methastasis	(Stagg et al., 2010)
CD73	CD73-specific mab	Athymic nude mice	Human breast cancer cells	Inhibition of tumor growth	(Rust et al., 2013)
CD73	CD73-specific mab	C57BL/6 mice	Murine prostate cancer cells	Inhibition of tumor growth and methastasis	(Stagg et al., 2012)
CD73	CD73-specific mab	C57BL/6 mice	MCA-induced fibrosarcoma	Inhibition of tumor growth	(Stagg et al., 2012)
CD73	CD73-specific mab	Balb/c mice		Increase of doxorubicin activity against tumor cells	(Loi et al., 2013)
CD73	CD73-specific mab	Balb/c and C57BL/6 mice	MCA-induced fibrosarcoma, murine prostate,breast and colon cancer cells	Increase of the therapeutic activity of the immune checkpoint inhibitors CTLA-4 specific and PD1-specific mAbs	(Allard, Pommey, Smyth, & Stagg, 2013)
CD73	CD73-specific mab	C57BL/6 mice	Murine melanoma cells	Inhibition of tumor growth	(Stagg et al., 2011)
CD73	CD73-specific mab	C57BL/6 mice	Murine breast cancer cells	Reduction of microvessel density in tumors	(Allard et al., 2013)

CD73	APCP	C57BL/6 mice	Murine breast cancer cells	Inhibition of tumor migration	(Beavis et al., 2013)
CD73	APCP	C57BL/6 mice	Murine melanoma cells	Induce tumor regression by promoting the release of Th1 and Th17 cell-associated cztokines and inducing CD8T cell infiltration in the TME	(Forte et al., 2012)
CD73	APCP	C57BL/6 mice	Murine melanoma cells	Inhibition of tumor growth	(Yegutkin et al., 2002)
CD73	APCP	C57BL/6 mice	Murine OvCa cells	Increased survival of tumor-bearing mice	(Jin et al., 2010)
CD73	APCP	C57BL/6 mice	Murine melanoma cells	Inhibition of lung metastases	(Stagg et al., 2011)
CD73	APCP	C57BL/6 mice	Murine breast cancer cells	Reduction of microvessel density in tumors	(Allard et al., 2013)
CD73	APCP	Nude mice	Human breast adenocarcinoma cells	Inhibition of tumor growth	(Zhou et al., 2007)

Table 1: effects of the adenosinergic system blockade in cancer. Modified from Antonioli et al., 2013.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Instruments and device

INSTRUMENT	MANUFACTURER
ABI TaqMan 7500	Applied Biosystems, Life Technologies Corporation, Carlsbad, California 92008, USA
Autoclave	H P Labortechnik AG, 85764 Oberschleißheim, Germany
Balance	Sartorius AG, 37075 Goettingen, Germany
Centrifuges	Eppendorf, 22339 Hamburg, Germany. Thermo Electron GmbH, 63303 Dreieich, Germany
CO_2 Incubator	Thermo Electron GmbH, 63303 Dreieich, Germany
Counting chamber	Carl Roth GmbH + Co. KG 76185 Karlsruhe, Germany
Cryo box	Nalgene, Thermo Fisher Scientific, 63505 Langenselbold, Germany
ELISA-Reader Sunrise	TECAN, 74564 Crailsheim, Germany
FACSCalibur	Becton Dickinson, Franklin Lakes, NJ 07417, USA
Freezers (-20°C, -86°C)	Germany Liebherr, Germany; Thermo Electron GmbH 63303 Dreieich, Germany. Philipp Kirsch GmbH. 77608 Offenburg,

Heating block	Biometra GmbH, D-37079 Goettingen, Germany
Hot water bath	Julabo, 77960 Seelbach, Germany
Ice machine	Scotsman Ice Systems, Vernon Hills, IL 60061, USA
Inverted Microscope	Leica, 35606 Solms, Germany
Irradiation machine	Philips Electronics, Germany
Laminar flow Hood	Heraeus, Hanau, Germany
Liquid nitrogen tank	Air Liquide, 40235 Düsseldorf, Germany
Luminometer Orion II	Berthold Detection Systems, 75173 Pforzheim, Germany
MACS™ Separators	Miltenyi Biotec GmbH, 51429 Bergisch Gladbach, Germany
Magnetic stirrer	VWR, 64295 Darmstadt, Germany
Microwave	Sharp Electronics (Europe) GmbH, Germany
PCR thermocycler	Biometra GmbH, D-37079 Mettler-Toledo GmbH, 35353 Gießen, Germany
Photometer	Thermo Electron GmbH
Pipettes	Eppendorf AG
Power-Supply	Thermo Electron GmbH
Refrigerator	Liebherr, Germany
Roller mixer	Stuart, Bibby Scientific Limited, Beacon Road, Stone, Staffordshire ST15 0SA, UK
Sample shaker (Vortex)	VWR

SDS gel electrophoresis system	Whatman, GE Healthcare, D-80807 Munich, Germany
Shakers	Heidolph Instruments GmbH & Co. KG, 91126 Schwabach, Germany
Thermo-mixer	Eppendorf AG
Ultra Freezer (-86°C)	Heraeus
UV lamp	Biometra GmbH
X-ray film Cassettes	Carl Roth GmbH + Co. KG

2.1.2. Consumables

CONSUMABLE	MANUFACTURER
X-ray films	Fisher Scientific GmbH - Im Heiligen Feld 17- D58239 Schwerte, Germany
Cell culture dishes	TPP, 8219 Trasadingen, Switzerland
Cell culture flasks (25 cm^2, 75 cm^2)	Greiner bio-one
Cell culture plates (96-well, 24-well, 12-well, 6-wel)	TPP, 8219 Trasadingen, Switzerland
Cryotubes	Nunc, Roskilde, Denmark
FACS tubes	Hartenstein, 97078 Würzburg, Germany
Falcon tubes (15 ml, 50 ml)	Greiner bio-one
Film (TaqMan)	Sarstedt
KODAK developer	KODAK
KODAK fixative	KODAK
LS/LM columns	Miltenyi Biotec GmbH
Luminometer plates	Greiner bio-one
Micro test tubes (Eppendorf caps)	Greiner bio-one
Nylon Cell Strainer 70µm	Nunc
Parafilm	Becton Dickinson
Transwell plates	Corning Incorporated, Building 300 Suite 3401 Tewksbury MA 01876, USA

2.1.3. Chemical Reagents

CHEMICALS, SOLUTIONS AND REAGENTS	MANUFACTURER
ACD-A (sodium citrate solution)	Haemonetics
Acetone	Carl Roth
Acrylamid/ Bisacrylamid	Carl Roth
Agarose	Carl Roth
Ammonium persulfate (APS)	Carl Roth
Aprotinin	Carl Roth
Aqua ad iniectabilia	Braun
Biocoll separation solution	Biochrom
Bovines Serum Albumin (BSA)	Applichem
Bromophenol blue	Carl Roth
CFDA-SE	Invitrogen
Cell Proliferation Dye eFluor 670	Invitrogen
Chloroform	Sigma
Cisplatin	Uniklinik Würzburg
Dispase	Roche
Doxorubicin	Uniklinik Würzburg
Ethylenediaminetetraacetic acid (EDTA)	Applichem
FuGENE HD Transfection Reagent	Roche
GelRed	Biotium
Glycerol	Carl Roth
Glycine	Carl Roth
Goat serum	Caltag
Human serum albumin (HSA)	Behring
Hydrochloric acid	Applichem
Hydrogen peroxide	Carl Roth
Ionomycin	Cayman
Isopropanol	Carl Roth
Liberase DH	Roche
Luciferin	P.J.K.
Methanol	Uniklinik Würzburg

NECA (5'-N-ethylcarboxamidoadenosine)	Tocris
PageBlue protein staining solution	Fermentas
PeqGold Trifast	Peqlab
Ponceau S	Carl Roth
SCH58261	Tocris
Skimmed milk powder	Merck
Sodium Citrate	Calr Roth
Streptavidin-HRP	Dako
Tris hydroxymethyl aminomethane	Carl Roth
(Tris)-Ultra	Carl Roth
Tris-HCl	Carl Roth
Tween20	Carl Roth
β-mercaptoethanol	Carl Roth

2.1.4. Product Kits

PRODUCT KIT	MANUFACTURER
ABsolute Blue QPCR SYBR Green low Rox Mix	Thermo Fisher Scientific
CD326 (EpCam) Microbeads	Miltenyi Biotech
$CD4^+$ T cell isolation kit	Miltenyi Biotech
$CD8^+$ T cell isolation kit	Miltenyi Biotech
Cell event Caspase 3/7 substrate	Life-Technologies
iScript cDNA Synthesis Kit	Biorad
Tumor dissociation kit, Human	Miltenyi Biotech

2.1.5. Cell Culture Reagents

CELL CULTURE REAGENT	MANUFACTURER
Dimethylsulfoxid (DMSO)	Carl Roth
Dulbecco's PBS	PAA
Fetal calf serum (FCS)	PAA/Sigma/Biochrom

Penicillin/Streptomycin	PAA
RPMI 1640	PAA

2.1.6. Detachment Reagents

DETACHMENT REAGENT	MANUFACTURER
Accutase	PAA
Trypsin EDTA	PAA

2.1.7. Desinfectants

DESINFECTANT	MANUFACTURER
Cutasept	Bode
Ethanol (EtOH)	Uniklinik Würzburg

2.1.8. Cytokines

CYTOKINES	MANUFACTURER
IFN-γ	Immunotools
TGF-ß	Peprotech/Cell Signaling

2.1.9. Toxins

TOXINS	MANUFACTURER
LPS	Sigma
Staurosporin	LC Laboratories

2.1.10. Reagents and buffers for FACS

REAGENTS FOR FACS STAINING	MANUFACTURER
Beriglobin solution	Novartis
FACS Rinse	Becton Dickinson
FACS Flow	Becton Dickinson

Monensin	Bio legend

2.1.11. Reagents and buffers for Western Blot

BUFFERS: WESTERN BLOT	COMPOSITION
Blocking Buffer	5% skim milk powder in PBS- 50 mg
ECL solution A	Luminol in 200 ml 0.1 M Tris-HCl pH 6.8
ECL solution B	11 mg para- hydroxycoumarinic acid in 10ml of DMSO
Lysis buffer	50 mM Tris-HCl, pH 8, 120 mM NaCl, 5mM EDTA, 0.5% NP-40, 2µg/ml, Aprotinin, 10µg/ml Leupeptin, 100µg/ml PMSF, 50mM NaF, 200 µM NaV_2O_5
PBS-T	PBS, 0.05% Tween20
Ponceau S solution	0.2% Ponceau S in 3% trichloroacetic acid
Protein loading buffer (5x)	100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% Bromophenol blue, 20% Glycerol, 10% β-Mercaptoethanol
Running buffer	25 mM Tris. 193 mM Glycin, 0.5% SDS pH 8.8
Stripping Buffer	0.2 M Glycine, 0.5 M NaCl, pH 2.8
Transfer buffer	25 mM Tris, 192 mM Glycine, 20% Methanol

2.1.12. Reagents and Buffers for Polyacrylamide gels

BUFFERS: POLYACRYLAMIDE GELS	COMPOSITION
Separating gel 10%	dd H2O 1.9ml, Acrylamide/Bisacrylamide (30%) 1.7 ml , Tris-HCl 1.5 M, pH 8.8 1.3ml , SDS (10%) 50 µl, APS (10%) 50 µl, TEMED 2 µl
Stacking gel 5%	Becton Dickinson dd H 2O 0.68 ml, Acrylamide/Bisacrylamide (30%) 0.17ml, Tris-HCl 1 M, pH 6.8 0.13 ml, SDS (10%) 10 µl, APS (10%) 10 µl, TEMED 1 µl

2.1.13. Reagents and Buffers used in various techniques

GENERAL BUFFERS AND SOLUTIONS	COMPOSITION
FACS-Buffer	PBS, 2% FCS
Freezing medium	70% FCS, 20% RPMI complete, 10 % DMSO Tris-HCl 1 M, pH 6.8 0.13 ml, SDS (10%) 10 µl, APS (10%) 10 µl, TEMED 1 µl
MACS-Buffer	PBS pH 7.2, 0.5% BSA
PBA	2 mM PBS, 1% BSA
Standard medium	PBS, 0.05% Tween20
PBS-T	88.5% RPMI 1640, 10% FCS, 1% Pen./Strep.
TBS	10 mM Tris-HCl, 150 mM NaCl, pH 7.5

2.1.14. Cell lines

CELL LINE	SPECIES	SOURCE
OAW-42	Human, OvCa	European Collection of CellCultures (ECACC)
OVCAR-3	Human, OvCa	ATCC Manassas, VA 20110-2209, USA
SK-OV-3	Human, OvCa	ATCC Manassas, VA 20110-2209,USA
HEK-293	Human, kidney	ATCC Manassas, VA 20110-2209,USA

2.1.15. Plasmids

PLASMID	SOURCE
pRL-CMV	Promega, Madison, WI, USA
RIP1-CRE.luc	George G. Holz, State University of New York

2.1.16. Oligonucleotides

OLIGONUCLEOTIDES	SEQUENCE 5´>3´
18s RNA, forward	CGGCTACCACATCCAAGGAA
18s RNA, reverse	GCTGGAATTACCGCGGCT
GAPDH, forward	ACGACAGTCCATGCCATCAC
GAPDH, reverse	TCCACCACCCTGTTCCTGTA
CD39, forward	GTAAGTGACCTTTACAAGACCC
CD39, reverse	TGCTGGAATGGAAGAGTCATC

CD73, forward	GGCTCCTCTCAATCATGCCG
CD73, reverse	CAGAACATTTCATCCGTGTGT
IL10, forward	GCCTAACATGCTTCGAGATC
IL10, reverse	CTCATGGCTTTGTAGATGCC
IL12, forward	GAAGGCCAGACAACTCTAG
IL12, reverse	CTATCAATAGCTACTGCCC

2.1.17. Antibodies

ANTIBODY	COMPANY	CLONE
CD3	Immunotools	UCHT-1
CD4	Immunotools	EDU-2
CD11B	Immunotools	LT11
CD14	Immunotools	MEM-18
CD28	Immunotools	15E8
CD39	Biolegend	A1
CD39 (WB)	Abcam	22A9
CD73	Biolegend	AD2
CD73 (WB)	Biolegend	AD2
EpCAM	Biolegend	9C4
IL10	Miltenyi -biotech	JES3-9D7
IL12	Miltenyi -biotech	C8.6

2.2. METHODS

2.2.1. Cell culture

2.2.1.1. Routine, feeding and maintenance

Cell cultures were examined regularly and routinely (every two days) both macroscopically and microscopically. The cell morphology and density was evaluated by microscope as well as the presence of contaminants such as fungus. Macroscopically, the color and turbidity of the medium was monitored. Media (plus serum and other additives) was changed regularly and not allowed to become acidic or depleted, depriving cells of specific nutrients. The pH of the medium was easily monitored by color indicators (phenol red) included in the medium. The frequency of media renewal was dependent on the growth rate of the culture; more rapidly growing cultures requiring more regular changes. Cells were kept at 37°C, 5% CO_2 and 95% relative humidity.

2.2.1.2. Subculture

When a culture had occupied the complete surface of a flask (monolayer cultures) or had grown to the point where media was depleted of nutrients (suspension culture), cell cultures were subcultured, allowing a healthy growth of the cell population. Via this process cell density is reduced to levels were they can optimally grow without a fast combustion of nutrients and medium. For subculturing, detachment of the adherent monolyer is required and therefore proteolytic enzymes, such as trypsin-EDTA or accutase, were used. These enzymes break the cell-cell and cell-substrate links creating a single-cell suspension. 5-10 min. after addition of the proteolytic enzymes, the cells were completely detached. Then, the catalytic activity of the enzymes was stopped with 5 ml of FCS-containing medium. Once detached, cells were collected and centrifuged at 1500 rpm for 5 min, resuspended in 10 ml of medium and cells were diluted 1 to 10 and transferred into a new cell culture flask for further culture.

2.2.2. Cryopreservation of cells

Preservation of cell stocks in liquid nitrogen allows long-term storage of cells. For optimizing the viability of cells, cryoprotective agents were used (DMSO) for preventing ice crystals formation and the fragmenting of membranes (see material freezing medium). Optimal freezing rate is 1°C/min; therefore, cells in the freezing vials were first kept for 24h in -80°C freezers, and then placed in the liquid nitrogen tanks.

2.2.3. Cell counting

Determination of cell number is a key measurement for setting up experiments with cancer cell lines as well as monitoring cell responses under experimental conditions. Cells were counted using a hemocytometre (Neubauer chamber). Cells mixed with bromophenol-blue (1:1) were placed in the hemocytomter. The hemocytometer is a glass chamber with a quartz cover slip exactly 0.1 mm above the chamber floor. The counting chamber is precisely etched in a surface area of 9 mm^2. Calculation of cell number is based on counting the number of cells within a defined area underneath the cover slip.

2.2.4. Isolation of PBMCs from whole blood

Separation of PBMCs from whole blood was achieved through density gradient centrifugation. Blood was extracted from healthy volunteers and mixed with 10% of sodium citrate solution to avoid clotting. The blood-citrate mixture was diluted with twice the volume of PBS and distributed into 50 ml centrifuge tubes which had already been filled with 15 ml Biocoll (lymphocyte separation medium). In order to avoid the mixture of both solutions, the citrate-blood-PBS mixture was gently poured on top of the Biocoll layer in order to obtain two phases. Following, the tubes were centrifuged at 600 rpm for 20 minutes (room temperature, no brake and low acceleration). Then, the uppermost 5 ml were aspirated from each tube to remove the platelets and were again centrifuged at 1,400 rpm for 30 minutes.

Differential migration during centrifugation results in the separation of cell types into different layers (figure 6). The bottom layer contains Biocoll-aggregated red blood cells. Immediately above there is a diffuse layer containing mostly granulocytes and unbound Biocoll. Due to a slightly lower density, the lymphocytes (including the monocytic PBMC fraction) sediment at the interface between the Biocoll and plasma/platelet layer and thus remain in the upper part of the falcon tube. PBMCs are removed from the interface and subjected to washes in PBS (x2) to remove any residual Biocoll. Isolated cells are then ready for analysis or culture. All steps were performed under sterile conditions.

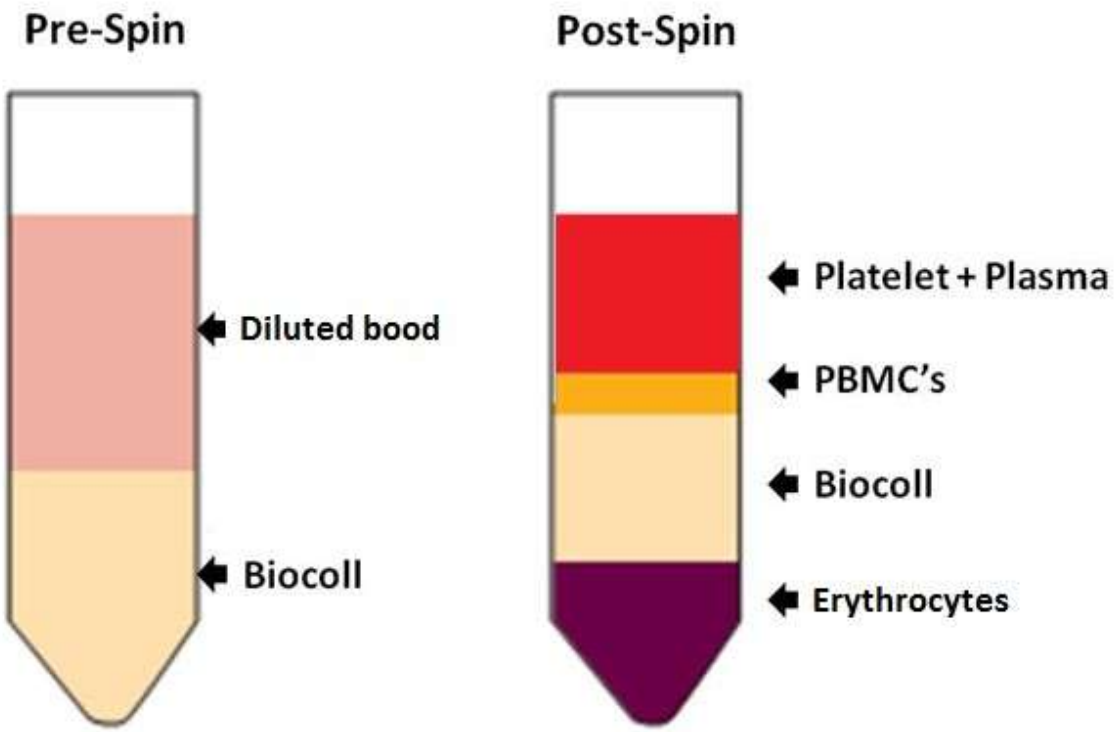


Figure 6: Sample layering before and after density gradient

2.2.5. Preparation of cell lysates from tumor cells and tissues

Five million tumor cells were centrifuged (5 min /450 g/ 4°C) and the cell pellet was washed with 100 µl PBS with protease and phosphate inhibitors (2 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml PMSF, 50 mM NaF, 200 µM NaV_2O_5 and phospho-stop). Then, lysis buffer-P (70 µl) containing protease and phosphatase inhibitors was added to every sample and incubated on ice for 20 min. Once the cells were lysed, samples were centrifuged (15 min /13,000 g / 4°C) and supernatant containing the protein lysates was transferred to clean microcentrifuge-caps.

2.2.6. Flow cytometric analysis of immune cells

2.2.6.1 Extracellular FACS staining

Expression of extracellular proteins was determined via flow cytometry. Cells were transferred into FACS tubes and washed with PBS. The non-specific binding sites for antibodies (Fc-γ receptors) were blocked with human IgG (Beriglobin) solution. After washing with FACS buffer, immune cells were incubated for 30 min in the dark with fluorescence-labeled antibodies against the respective surface antigens, at the appropriate concentration. Cells were then washed twice with FACS buffer and finally, flow cytometric analysis was performed on a flow cytometre (FACScalibur or Attune). Data was evaluated using the analysis programs Summit (Beckman Coulter, Version 4.3) or Attune analysis software.

2.2.6.2. Intracellular FACS staining

Intracellular expression of determined proteins was also assessed via flow cytometry. For cytokine detection cells were pre-treated with monensin (1 µl/ml, 4 hours). Cells were then harvested, centrifuged (1800 rpm/4 min) and resuspended into FACS buffer. For fixation, cells were incubated in a 4% paraformaldehyde solution for 15 min at 4°C. Subsequently, cells were permeabilized and blocked with saponin buffer (0.5% saponin in PBA buffer) and rabbit serum (1:200) respectively for 15 min at 4°C. Then, cells were stained for respective antibodies and their isotypes in saponin buffer and incubated for 30 min. Finally, cells were washed twice in FACS buffer and flow cytometric analysis was

performed on a flow cytometre (FACScalibur or Attune). Data was evaluated the analysis program Summit (Beckman Coulter, Version 4.3) or Attune analysis software.

2.2.7. Proliferation of $CD4^+$T cells in co-culture with adenosine-generating cells

"$CD4^+$T cell isolation kit II" was used to isolate $CD4^+$T cells from PBL. Non-target cells expressing either CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, or CD235a (Glycophorin A) were removed by immunomagnetic depletion with magnetic-bead labeled antibodies while passing through a magnetic column.

Directly after isolation, T cells were labeled with 2.5 µM CFDA-SE. To induce proliferation, anti-human CD3 (1 µg/ml) was immobilized on 96 well Maxisorp-plates by overnight-incubation in PBS. In each pre-coated well, 2×10^6T cells were then co-incubated with anti-human CD28 and with $5x10^5$ OAW-42 cells. CD39 and CD73 were blocked by either by addition of anti-human CD39 or anti-human CD73 antibody or by addition of small molecular inhibitors against CD39 and CD73 (ARL67156 and APCP respectively, 100 µM). Specificity was ensured by use of an isotype control antibody or by the A2A receptor antagonist SCH58261 (100 nM). All antibodies were used at 10 µg/ml. A FACScalibur flow cytometer (BD Biosciences) was used to measure proliferation on day 7. Calculation of proliferation rates in FACS analyses was performed: the absolute number of counts in each peak-region of the corresponding CFDA-SE histogram was divided by 2n (n standing for the number of the peak, being counted from right to left and beginning with 0) to calculate the number of cells "x" which originally divided into the cells of the peak. $\sum_{i=0}^{n-1}\left(x * 2^i\right)$ Is equal to the total number of cell division per peak n.

2.2.8. Adenosine Assay

A2AR-overexpressing HEK-293 cells were transiently transfected with the luciferase-encoding RIP1-CRE.luc^{+}cAMP-reporter plasmid. Transfection efficiency was normalized by assessment of co-transferred pRL-CMV. Binding of extracellular adenosine to A2A receptor activates adenylate cyclase and thus the inducible firefly luciferase signal. About 10^4 cells of interest were co-incubated with equal numbers of RIP1-CRE.luc- and pRL-CMV-transfected HEK-293 A2AR$^{+/-}$ cells for 4 h. Cells were lysed in passive lysis buffer, and the biophotonic signals were quantified in an Orion II Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany), using a non-commercial dual luciferase assay (Dyer, Ferrer, Klinedinst, & Rodriguez, 2000). All values were measured in triplicate and controlled for specificity by addition of the specific inhibitors ARL67156 for CD39 (100 µM) and APCP (100 µM). The corresponding adenosine concentrations were calculated using a determined standard curve ranging from 20 nM to 40µM adenosine.

2.2.9. Migration assay

Small molecular inhibitors against CD39 and CD73 (ARL67156 and APCP respectively) were added to OvCa cells and freshly isolated monocytes (100 µM) and the A2A receptor agonist SCH58261 was added to the monocytes (100nM). Inhibitors were incubated for 1 hour and then washed away with PBS. Thereafter, 200,000 cancer cells (OAW-42 or SK-OV-3) were seeded in the bottom plate of the transwell and freshly isolated monocytes (500,000) from a healthy donor's PBMC were placed in the upper insert of the transwell. Monocytes were allowed to migrate towards the OvCa cells for 4 hours in the incubator (37°C, 5% CO2 and 95% relative humidity). After migration, migrated monocytes were quantified by flow cytometry: a standard curve was created with different ratios of monocyte: cancer cells. Therefore, cancer cells were stained with EpCAM and different numbers of monocytes with CD11c. After migration, cells on the lower compartment of the transwell were collected and stained for EpCAM and CD11c and analyzed via FACS. Same numbers of cells were measured in every sample and the EpCAM: CD11c ratios were calculated and compared to the standard curve for relative quantification.

2.2.10. Macrophage polarization

Monocytes were isolated from healthy donors´ PBMC by double centrifugation (1st Biocoll, 2nd Easycoll) and maturated in Lumox® dishes during 7 days (RPMI+ 5% human AB serum). Mature macrophages were then polarized towards the M1 phenotype by addition of human recombinant IFN-γ (1 µg/ml) and LPS (10 µg/ml) for 48 hours. For M2 polarization, macrophages were co-cultured with OvCa cells (OAW-42) in transwell plates (pore size 1 µm) during 48h.

2.2.11. Arginase assay

The activity of arginase was determined from the urea production using a colorimetric assay. After each specified polarization, cells were washed with ice-cold PBS twice, scraped and centrifuged at 1,200 rpm for 5 min. Cell pellets were resuspended into 200 µl lysis buffer (50 mM tris(hydroxymethyl)aminomethane (Tris) · HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid, 1 mM dithiothreitol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates (50 µl) were added into 50 µl of Tris · HCl (50 mM; pH 7.5) containing 10 mM $MnCl_2$. Macrophage arginase was then activated by heating the mixture at 55–60°C for 10 min. The catalytic reaction of L-arginine by arginase was performed by incubating the lysates with 50 µl of L-arginine (0.5 M; pH 9.7) at 37°C for 1 h and stopped by adding 400 µl of the acid solution mixture (1 H_2SO_4:3 H_3PO_4:7 H_2O). Then, α-Isonitrosopropiophenone (25 µl, 9%; dissolved in 100% ethanol) was added to the mixture and heated at 100°C for 45 min. Once the samples were incubated in the dark for 10 min at RT, the urea concentration was determined with the help of a spectrophotometre by measuring the absorbance at 550 nm. The rate of urea production was used as an index for arginase activity.

2.2.12. CCL-64-Assay

To determine TGF-ß concentration after chemotherapeutic treatment the CCL64-Luc-bioassay was performed.

CCL-64 cells were seeded in 96-well plates at 10,000 cells/well and incubated for 24 hours. Then, TGF-ß standards (5.0, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, and 0 ng/ml TGF-β1) or samples (OvCa cell supernatants) were added to each well in triplicate. Samples were either acid activated (5 minutes at 85°C) to determine ‘‘total’’ TGF-ß activity, or were left untreated to determine ‘‘native’’ TGF-ß activity. Then, samples were added (50 µl/well) to the CCL-64 cells and were incubated for 48 hours at 37°C. After the incubation cells were washed two times with PBS and subsequently lysed with passive lysis buffer. Biophotonic signals originated from luciferase activity were quantified in an Orion II Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany) using a non-commercial luciferase assay (Dyer et al., 2000). The luciferase activity was recorded as relative light units (RLU). Finally, RLU values were converted to TFG-βactivity (pg/mL) using the TGF-βstandard curve.

2.2.13. Crystal violet assay

The crystal violet assay is used to quantify the cell number in monolayer cultures as a function of the absorbance of the dye taken up by the cells. However, this method can be used for a wide range of applications including determination of cytotoxicity or cell death produced by chemicals or to determine cell proliferation under different conditions. In brief; medium is removed from the plates and the crystal violet solution is added (0.5% crystal violet in 20% methanol). Then, the plates were incubated at RT for about 5 to 20 min. After incubation, the crystal violet solution was removed and plates were washed with H_2O and were left to dry at RT. Once the plates were dry, sodium citrate solution was added (0,1M sodium citrate in 50% ethanol). Finally, absorption was measured in the ELISA-reader at 550nm.

2.2.14. Isolation of RNA from tumor and immune cells

For isolation of RNA from immune cells or tumor cells, $5x10^6$ cells were centrifuged, 1 ml TriFast reagent was added and they were incubated at RT for 5 min. Then, for every 1ml TriFast 0.2 ml of chloroform were added and samples were shaken for 15 sec. Samples were further incubated (5 min at RT). The mixture was then centrifuged (15 min/12,000 g /4°C) and the upper aqueous phase containing the RNA was carefully transferred into a fresh vial. To precipitate the RNA, an equal amount of isopropanol was added to the aqueous phase and incubated for 10 min on ice, then centrifuged (15 min at 12,000 g at 4°C). The RNA pellet was carefully washed twice with 75% EtOH. Then, the RNA pellet was air-dried. Finally, the RNA pellet was dissolved in 30 µl of doubly deionized water (dd H_2O).

2.2.15. Determination of RNA concentration

RNA concentration was determined by a spectrophotometer. RNA samples were diluted 1:100 in (dd) H_2O and the absorbance was measured at 260 and 280 nm. The concentration was calculated using the following formula: C [µg / ml] = OD260 nm x dilution factor x 40. The quality of the isolated RNA was estimated by dividing the absorbance value obtained at 260 nm by the absorbance measured at 280 nm. Ideally, the ratio OD260 nm/OD280 nm should be 1.8.

2.2.16. Synthesis of cDNA from isolated RNA

iScriptTM cDNA Synthesis Kit was used for cDNA synthesis from isolated RNA. This kit consists of a modified MMLV (Mouse Moloney Leukemia Virus)-derived reverse transcriptase with RNase H endonuclease activity, an RNase inhibitor and a blend of oligo(dT) and random hexamer primers. Oligo(dT) primers bind to the polyA-tail contained in all cellular mRNAs while random hexamers can bind within any given RNA sequence. After primer binding at 25°C, reverse transcription occurred during 30 min at 42°C in a PCR thermocycler.

cDNA synthesis reaction:

Reagent	Volume
5xiscript reaction mix	4 µl
i-Script reverse transcriptase	1 µl
Template RNA	0.5µg
Nuclease free water	Up to 20 µl

cDNA synthesis program steps:

Temperature	Incubation time
25 °C	5 min
42°C	30 min
85 °C	5 min

2.2.17. Reverse transcriptase Polymerase Chain Reaction (PCR)

To qualitatively measure the expression level of mRNA transcripts, cDNA was used as template in PCR reactions. PCR reactions were performed using the Crimson longAmp Polymerase Kit and a PCR thermocycler.

Reagent	Volume
5x Crimson LongAmp Taq Buffer	5 µl
10 mM dNTP	0.75 µl
100 µM forward primer	1 µl
100 µM reverse primer	1 µl
cDNA	1 µl
Crimson LongAmp Taq Polymerase	0.5 µl
(dd) H_2O	Up to 25µl

PCR program:

Temperature	Time
94 °C	3 min
94 °C	30 sec
Primer-dependent annealing temperature	30 sec
72 °C	45 sec
72 °C	10 min
4 °C	hold

2.2.18. Semi-quantitative real time PCR

Relative quantification of mRNA transcript levels was performed by real time PCR (qRT-PCR). First, genes of interest were amplified from the synthesized cDNA using the ABsolute Blue QPCR SYBR Green low Rox mix kit for qRT-PCR. Purity of the PCR products was determined by the dissociation curve. All samples were measured in duplicate and they were only considered as valid when the deviation of C_t-values was ≤ 0.5 cycles. The C_t (cycle threshold) value is defined as the number of cycles required for the accumulating fluorescent signal to cross the threshold. The C_t value is inversely proportional to the amount of targeted nucleic acid in the sample. Therefore, a low C_t value corresponds to a relatively large amount of targeted nucleic acid). mRNA expression was quantified relative to the expression of the control 18S mRNA.

Reagent	Volume
2x Absolute blue qPCR SYBR green low rox mix	7.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Injection H_2O	0.5 µl
cDNA	5 µl

Temperature	Time
50 °C	2 min
95 °C	15 min
95 °C	15 sec
60 °C	1 min
95 °C	15 sec
60 °C	1 min
95 °C	15 sec

2.2.19. Preparation of cell lysates from tumor cells and tissues

Five million tumor cells were centrifuged (5 min /450 g/ 4°C) and the cell pellet was washed with 100 µl PBS containing protease and phosphates inhibitors (2 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml PMSF, 50 mM NaF, 200 µM NaV_2O_5 and phospho-stop). Then, lysis buffer-P (70 µl) also containing protease and phosphatase inhibitors was added to every sample and incubated on ice for 20 min. Once the cells were lysed, samples were centrifuged (15 min /13,000g / 4°C) and supernatant containing the protein lysates was transferred to clean microcentrifuge tubes.

2.2.20. Determination of the total amount of protein by the Bradford method

The Bradford assay is a colorimetric assay, based on the property of the CoomassieBrilliant Blue dye to form complexes with proteins and induce a photometrically detectable color shift. A 96 well-plate was filled with 50 µl/well distilled water and 1µl protein lysate or Protein standard (HSA) in triplicates. For the standard row, protein concentrations of 1, 2, 4, 6, 8, 10 and 12 µg/ml were used. Then, Rotiquant Bradford reagent was diluted (1:3.75) in distilled water. 200 µl of this solution were added to each well. After 20 min incubation at room temperature total protein concentration was determined in an ELISA reader (absorbance measured at 595 nm).

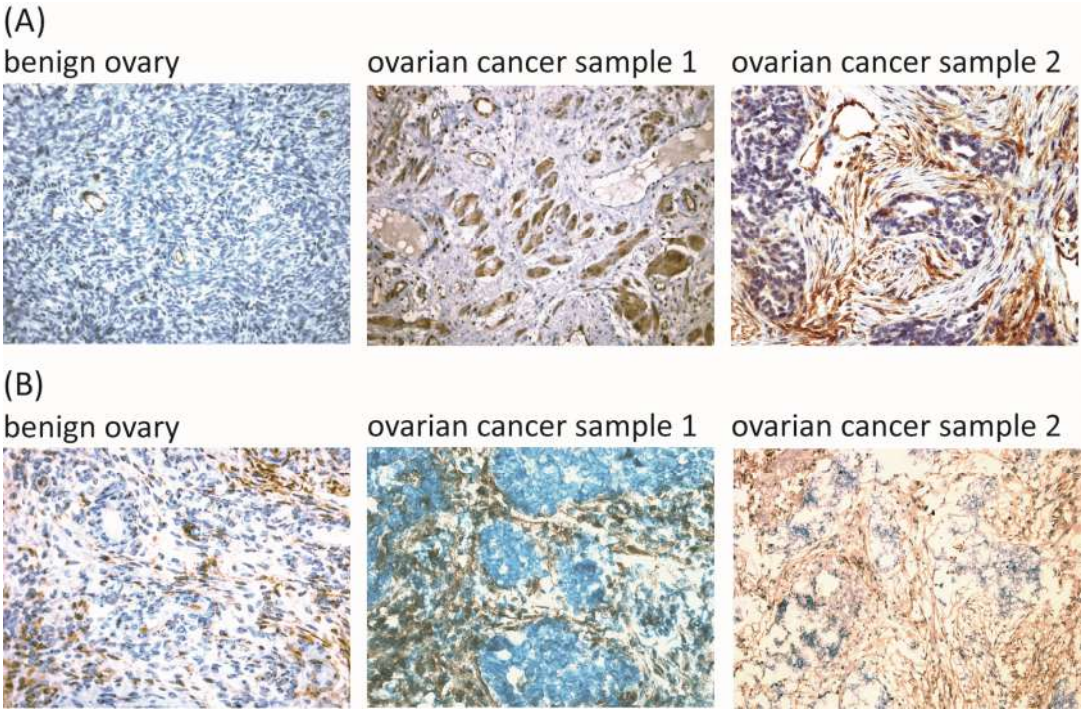
2.2.21. Immunoblotting

20 µg of total protein lysate were mixed with reducing protein loading buffer and incubated for 5 min at 95°C. Once proteins were denatured, samples were cooled on ice for 3 min. Samples were then shortly centrifuged and the supernatant was loaded into the pockets of a SDS- polyacrylamide gel. 8-10 µl of a pre-stained protein standard were used to determine the size of the other loaded proteins. The SDS-PAGE first run was performed at 90 V for 20 min to collect all the protein in the stacking gel. Thereafter, the voltage was increased to 130 V and it was allowed to run for approximately 100 min to separate the proteins in the running gel. Subsequently, proteins were electro- blotted on a PVDF membrane at 145 V for 85 min in transfer buffer. After blotting, unspecific binding of antibodies was minimized by incubating the membrane in blocking buffer for 2 h at RT. Thereafter, the primary antibody was included and incubated at 4°C overnight. After incubation with the primary antibody, the membrane was washed (3x 10 min with PBS –T) and then incubated for one hour with HRP-coupled secondary antibody diluted in the blocking buffer (1:5,000). After the incubation with the HRP-conjugated antibody the membrane was washed again with PBS-T (x3 for 10 min). Then, HRP activity was detected using a chemiluminescence detection system consisting of 1 ml solution A (50 mg Luminol in 200 ml 0,1 M Tris-HCl pH 6.8), 100 µl solution B (11 mg parahydroxycoumarinic acid in 10 ml of DMSO) and 0.3 µl solution C (H_2O_2). Solutions A, B and C were mixed and incubated on the membrane for 2 min. Chemiluminescence was detected using X-ray films, developed with developer solution (KODAK Developer) (3 min) and fixed (1 min) with fixative solution (KODAK Fixer). For re-probing, membranes were stripped with stripping buffer (0.2 M Glycin, 0,5 M NaCl, pH 2.8) for 10 min, followed by neutralization with neutralizing buffer (25 mM Tris 193 mM Glycin, 0,5% SDS pH 8.8) for 10 min. Finaly, the membrane was blocked again. The membrane it could be further incubated with a different antibody.

3. RESULTS

3.1. EXPRESSION OF CD39 AND CD73 IN HUMAN OVARIAN CANCER SPECIMENS AND CELL LINES

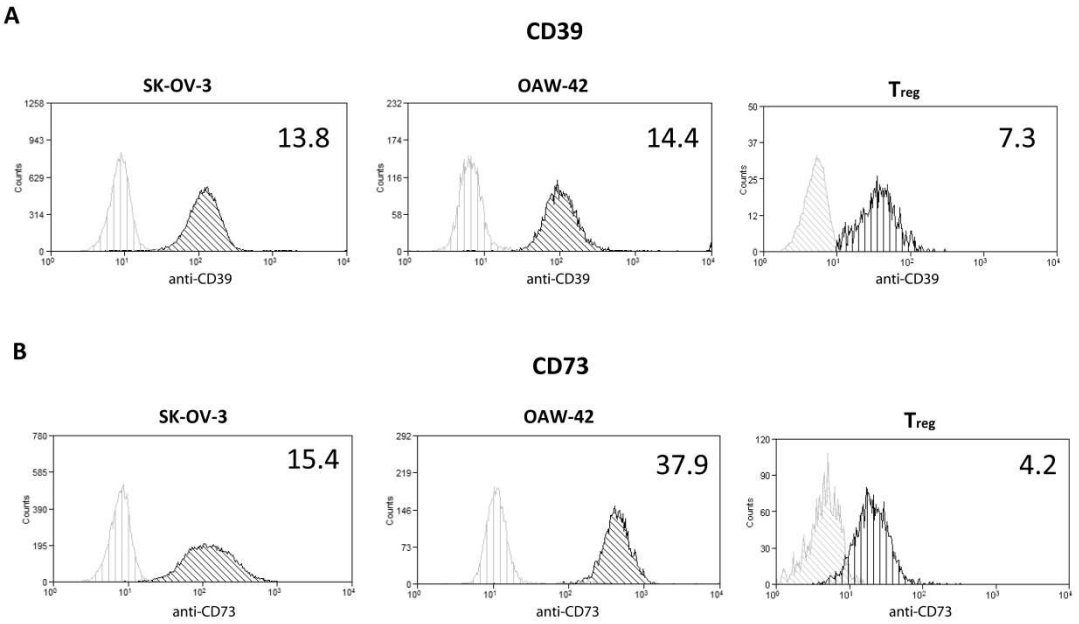
Expression of the ectonucleotidases CD39 and CD73 was evaluated in paraffin-embedded human ovarian cancer and in benign ovarian tissue samples. All malignant samples belonged to the most common serous-papillary subtype. Stainings with CD39- or CD73-specific antibodies showed that in benign ovaries (n=3) CD39 expression is restricted to endothelial vessels, which was used as an internal positive control. In ovarian cancer samples, however, 7 of 9 samples displayed moderate to strong expression of CD39 (fig. 7A). CD73, in contrast, was detected in 2 of 3 examined normal ovaries and in 6 of 9 ovarian cancer samples (fig. 7B). CD39- and CD73-positive cells were identified partly as epithelial tumor cells and partly as tumor stroma cells by morphological features.



(Häusler et al., 2011)

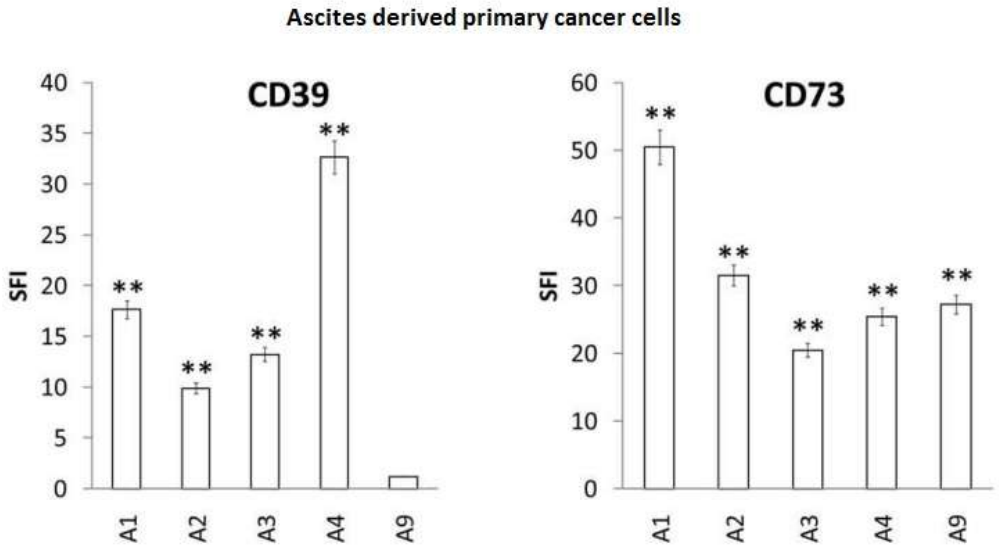
Figure 7. Immunohistochemical analysis of CD39 and CD73 expression in OvCa tissue. Immunhistochemical staining of serous-papillary epithelial ovarian cancer samples. Representative OvCa samples are shown in the middle and the right panel and benign ovary sample is shown in the right panel (n=3). **(A)** CD39 staining **(B)** CD73 staining. Experiment performed by Sebastian Häusler.

Corresponding to these *in vivo* data, the human ovarian cancer cell lines SK-OV-3 and OAW-42 were also found to strongly express CD39 and CD73 *in vitro* (fig. 8 A, B). $CD4^+CD25^+$ Treg showed a weaker expression of CD39. Likewise, CD73 expression was much higher on ovarian cancer cells than on Treg. These findings were confirmed as well in OvCa cells isolated from ascites of various patients (fig. 9).



(Häusler et al., 2011)

Figure 8. Analysis of CD39 and CD73 surface expression on OvCa cell lines and Treg. SK-OV-3, OAW-42 OvCa cell lines and freshly isolated human regulatory T cells (Treg) were stained for expression of CD39 **(A)** or CD73 **(B)** and analysed by flow cytometry. Representative histograms are shown. The indicated specific fluorescence indices (SFI values) were calculated by dividing the mean fluorescence obtained with the specific antibody (black profile) by the fluorescence intensity obtained with the corresponding isotype control (grey curve) (n=3). Experiment performed by Sebastian Häusler



(Häusler et al., 2011)

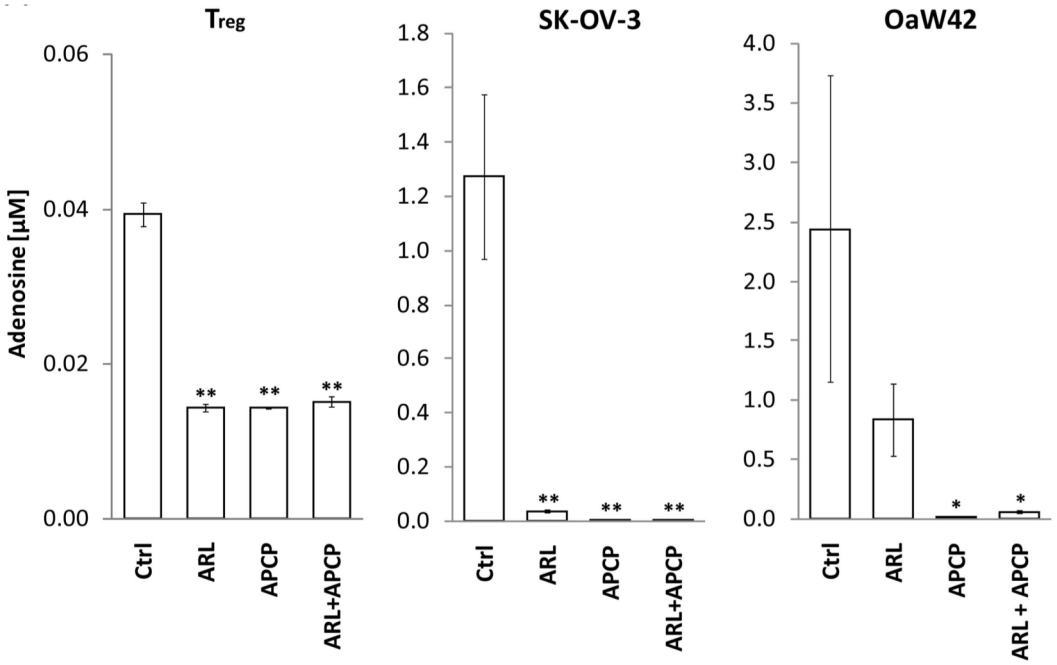
Figure 9. Analysis of CD39 and CD73 surface expression on primary OvCa cells. Magnetic beads were used to purify EpCAM-positive OvCa cells from ascites (n=15). SFI values for CD39 and CD73 expression were determined as described above and are shown for five representative primary tumor cell cultures. In flow cytometric assays at least 50,000 events were counted; two samples were considered to be significantly different (*) when they were separated by at least twice the sum of the standard deviations for the respective regions. A difference exceeding four times the sum of the respective standard deviations was considered as highly significant (**).

3.2. SK-OV-3 AND OAW-42 CELLS GENERATE ADENOSINE VIA CD39 AND CD73

RIP1-CRE-luc^{+} pRL-CMV ADORA2A^{+} HEK-293 sensor cells enable the detection of free extracellular adenosine within the cellular microenvironment (Hausler et al., 2010). Measured adenosine concentrations were 1.3 µM for SK-OV-3 and 2.4 µM for OAW-42 (fig. 10 A). When the specific inhibitors ARL67156 or APCP were added to block CD39 or CD73, respectively, the measured adenosine concentrations decreased to almost background values (fig. 10 A). When equal numbers of regulatory T cells were assayed in the same experiment, adenosine levels did not exceed 0.04 µM (fig. 10 A). Thus, ovarian cancer cells generate 30- to 60-fold more immunosuppressive adenosine than activated T_{reg} from healthy donors.

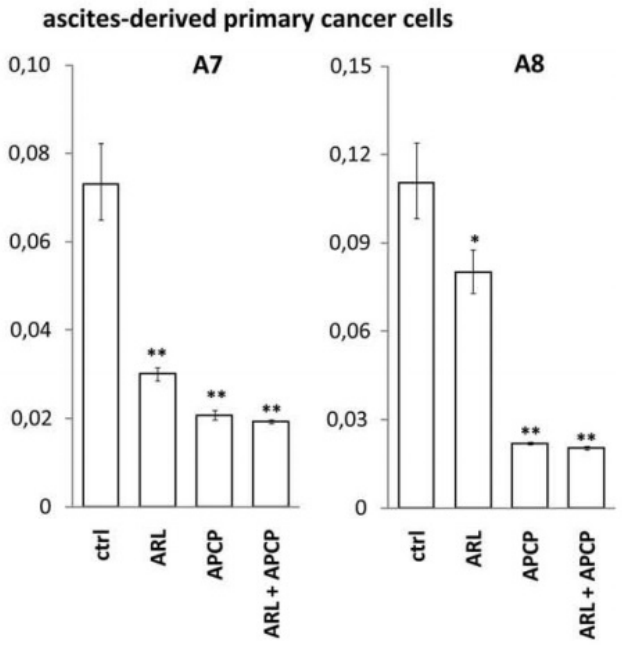
High levels of adenosine production were also measured in OvCa cells isolated from ascites of various patients (fig. 10 B).

A



(Hausler et al., 2011)

B



(Häusler et al., 2011)

Figure 10. Quantification of CD39- and CD73-dependent adenosine generation by Treg and ovarian cancer cells. Treg, SK-OV-3 and OAW-42 cells were co-incubated with RIP1-CRE-luc^{+} pRL-CMV^{+}ADORA2A HEK-293 reporter cells. Biologically active adenosine in the cellular microenvironment leads to increased cAMP levels and thus enhanced activity of the cAMP responsive RIP1-Cre-luc reporter. Firefly luciferase activity was normalized for co-transfected pRL-CMV activity and related to extracellular adenosine concentrations via a co-determined standard curve. Where indicated, the specific inhibitors of CD39 and CD73, ARL67156 and APCP were added (100 µM) (n=3). Significance levels were determined by Student's t-test. p-values < 0.05 were considered as significant (*), $p < 0.01$ as highly significant (**). Experiments performed by Sebastian Häusler.

3.3. ANTIBODIES A1 AND 7G2 BLOCK CATALYTIC ACTIVITY OF CD39 AND CD73

After confirmation of adenosine production via CD37 and CD39 in cancer cells, we wondered whether antibodies used for detection of CD39 and CD73, A1 and 7G2 respectively (fig. 8) could block the catalytic activity of ectonucleotidases and hence interfere with adenosine generation. Therefore, we used the already described luciferase-based reporter assay for the measurement of biologically active adenosine.

OAW-42 cells were co-incubated with the sensor cells (RIP1-CRE-luc^{+} pRLV-CMV ADORA2 HEK-293) and addition of A1 anti-CD39 antibody resulted in a >60 %-decrease of the measured adenosine concentration compared to the unspecific isotype control (fig. 11). Addition of 7G2 anti-CD73 mAb in the same setting reduced adenosine levels by 62 % (fig. 11), the combination of A1 and 7G2 by 64 % (fig. 11) confirming that these anti CD39 and anti CD73 antibodies inhibit enzymatic adenosine generation by ectonucleotidases. Of note, all antibodies used in these assays were azide-free.

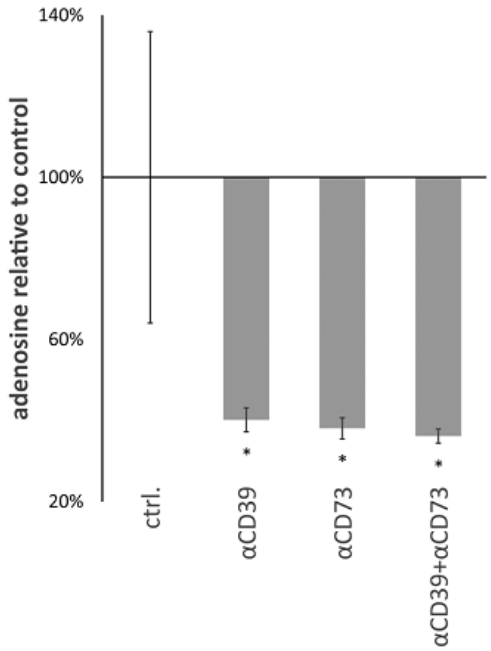
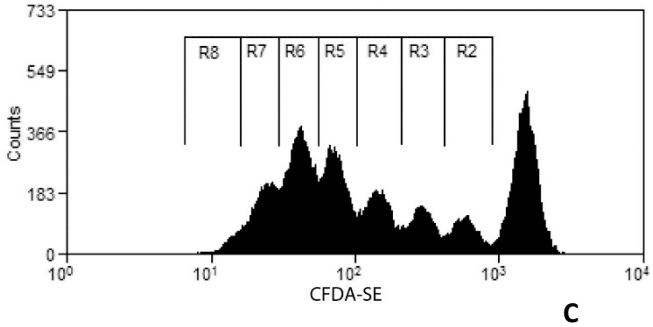


Figure 11. Antibodies A1 and 7G2 block adenosine production by CD39 and CD73. OAW-42 cells were co-incubated with RIP1-CRE-luc^{+}pRL-CMV^{+} ADORA2A $^{+}$HEK-293 reporter cells. Adenosine produced during the co-culture induces expression of firefly luciferase in the HEK-293 reporter cells. Samples containing an isotype control antibody (ctrl.) were compared with samples in which A1 (αCD39) or 7G2 (αCD73) or both antibodies (αCD39 + αCD73) were present. Adenosine levels are depicted relative to the controls (n=3). Significance levels were determined by Student's t-test. P-values < 0.05 were considered as significant (*).

3.4. ADENOSINE GENERATED VIA CD73 AND CD39 INHIBITS CD4$^+$ T CELL PROLIFERATION

To examine a possible suppression of CD4$^+$ T cell proliferation by ovarian cancer-derived adenosine, CFDA-SE$^+$ CD4$^+$ T cells were first activated with plate-bound antibodies against CD3 and CD28 and then co-incubated with irradiated SK-OV-3 or OAW-42 cells in the absence or presence of specific inhibitors for CD39, CD73 and A2A receptor. Results revealed that both OvCa cell lines reduced T cell proliferation by 65% (fig. 12). In co-culture with SK-OV-3 cells, inhibition of CD39, CD73 or A2A receptor significantly increased the proliferation of CFDA-SE$^+$ CD4$^+$ T cells (fig. 12B). With OAW-42 cells, similar but more pronounced effects were observed (fig. 12C). As expected, the effects of ARL67156 and APCP could be overruled by exogenous addition of 100µM adenosine analogue NECA (fig. 12 D, E).

A



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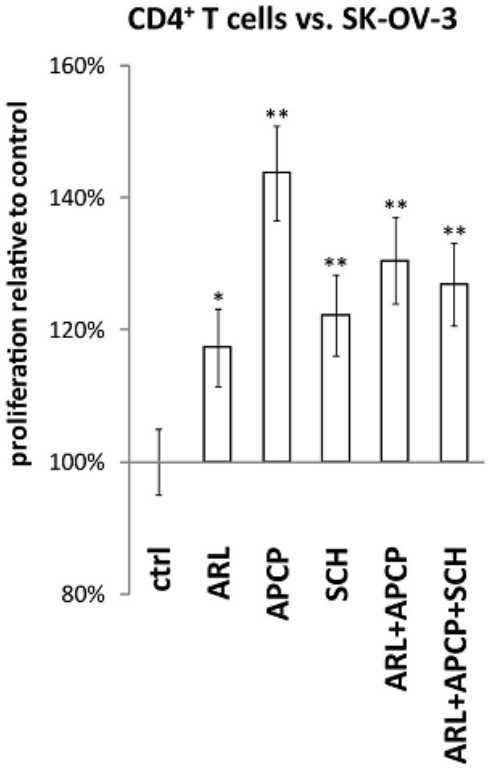

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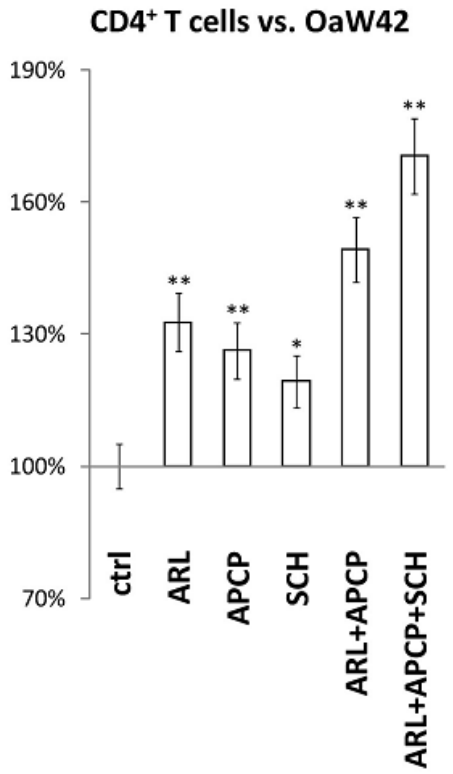
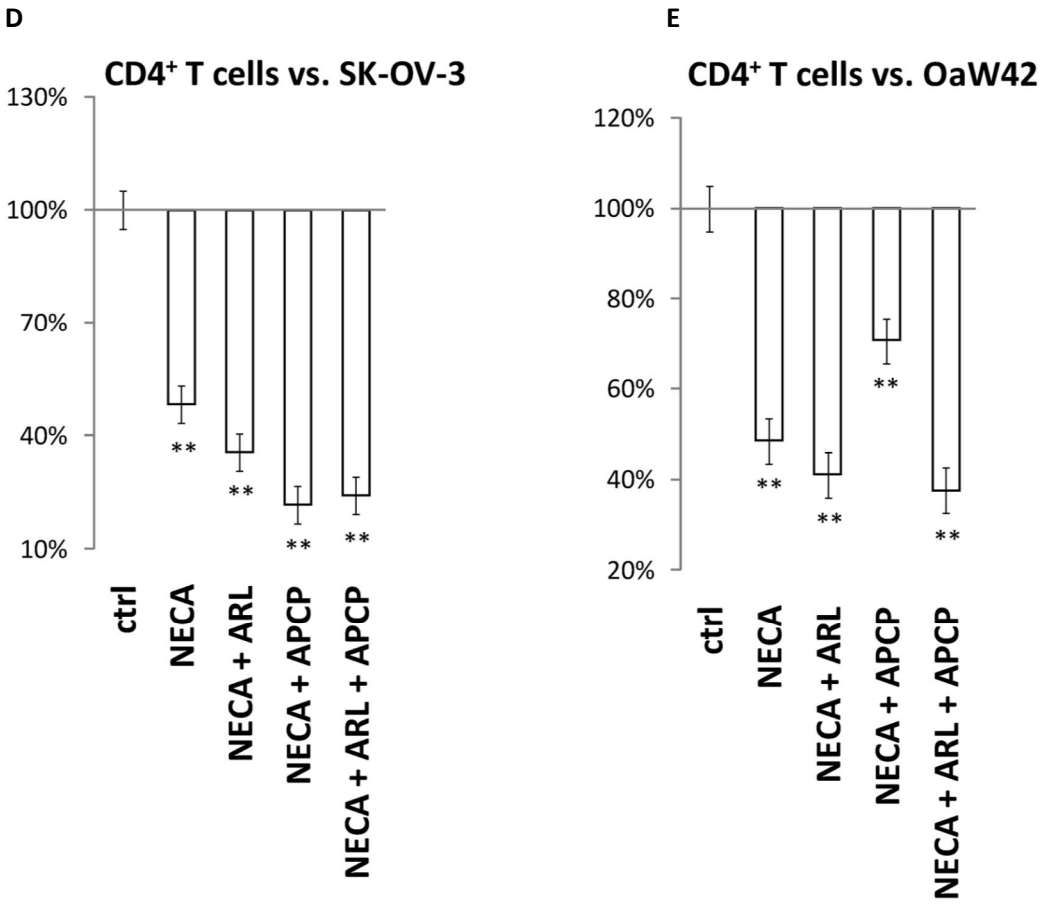




(Häusler et al., 2011)

Figure 12. Effect of CD39, CD73 or A2A adenosine receptor inhibition on the suppressive capacity of OvCa cells against proliferating activated CD4⁺ T cells. Treg and OvCa cells were co-cultured with CD4⁺ T cells that had been stained with CFDA-SE and stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. To inhibit ectonucleotidases, CD39 or CD73 inhibitors ARL67156 (ARL) and APCP were added (100µM each). A2A receptor was blocked using 100nM SCH58261. To activate adenosine receptors, 10µM adenosine-50-N-ethylcarboxamide (NECA) was used. **(A)** During each cell division, CFDA-SE is distributed equally between the daughter cells which enables the assessment of cell proliferation number by flow cytometry. In order to determine the total number of cell divisions, the number of counts in each region was divided by 2n (n=number of peak, from right to left, beginning with 0) to assess the number of cells 'x' which originally divided into the cytometrically determined cells of the peak. $\sum_{i=0}^{n-1}(x * 2^i)$ gives the total number of cell division per peak. **(B-E)** Representative and independent experiments are shown (n=3) for the co-culture of CD4⁺T cells with SK-OV-3 **(B)**, and OAW-42 **(C)** cells and with SK-OV-3 **(D)** and OAW-42 **(E)** cells in the presence of NECA±ARL67156 or APCP or both. Significance levels were determined by Student's t-test. p-values < 0.05 were considered as significant (*), p < 0.01 as highly significant (**). Experiment performed by Sebastian Häusler.

3.5. OVCA CELLS INCREASE MIGRATION OF MYELOID PRECURSOR CELLS BY GENERATION OF ADENOSINE VIA CD39 AND CD73

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy volunteers. To enrich for monocytes, a two-step gradient centrifugation protocol was used, starting with a standard Ficoll-based separation medium followed by a Percoll gradient. 500.000 monocytes per well were then placed in the upper inserts of 24-well Transwell plates (pore diameter 8 µm, membrane thickness 10 µm, cell growth area 0.33 cm^2) while 200.000 OAW-42 or SK-OV-3 cells were placed in the corresponding compartments at the bottom of the plate. All assays were conducted in RPMI 1640 medium with 5% human AB serum (500 µl per well). After an incubation period of 6h, all cells from the bottom plates were analysed by flow cytometry. Monocytes were identified with anti CD11c-FITC whereas OvCa cells were stained with EpCam-APC (both antibodies used at 1:100 dilution). Dead cells were excluded via co-staining with 7-aminoactinomycin D. To quantify the relative migration rate, a standard curve was generated from several samples containing $2x10^5$ cancer cells together with different numbers of monocytes (0 -10^6 cells). To block CD39 or, respectively, CD73 activity during the transwell coculture, ARL 67156 (250 µM), α,β-methyleneadenosine-5'-diphosphate ("APCP", 100 µM) or appropriate solvent controls were used (Crack et al., 1995; Krug et al., 1973). As positive control, the metabolically stable adenosine receptor agonist adenosine-5´-N-ethylcarboxamide (NECA) was employed at 100 nM.

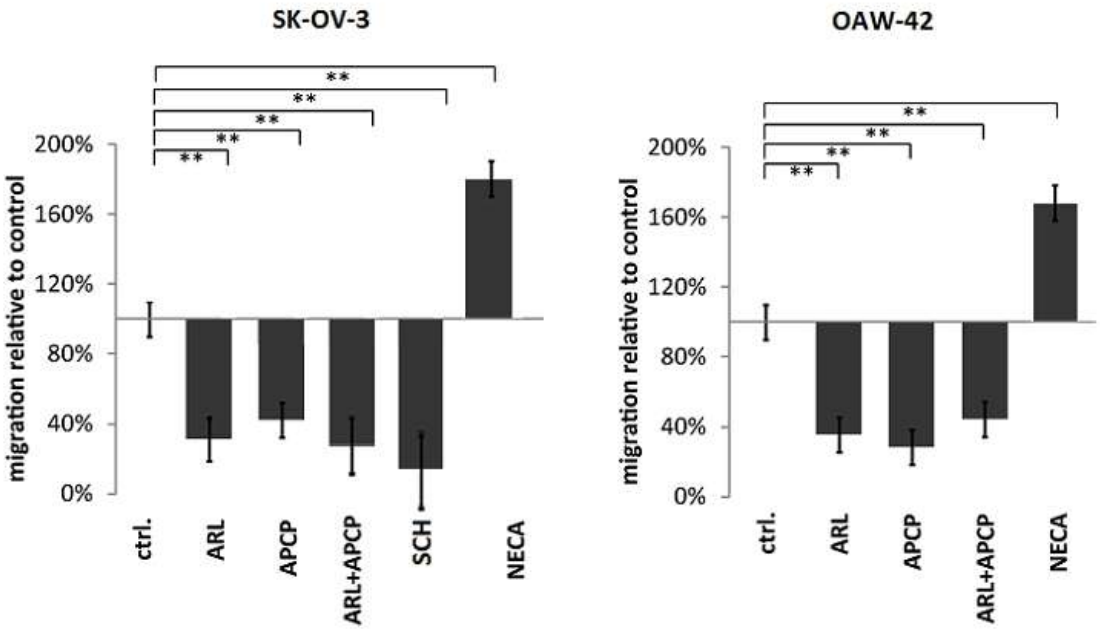


Figure 13. CD39 and CD73 activity on OvCA cells promote monocyte migration in transwell chambers. Primary human monocytes were placed in the upper inserts of transwell plates while SK-OV-3 (**A**) or OAW-42 (**B**) OvCA cells were seeded in the respective bottom compartments. To explore a potential influence of ectonucleotidases, CD39 activity in tumor cells was inhibited by 100 µM ARL67156 whereas CD73 was inhibited with 100 µM α,β-methyleneadenosine-5'-diphosphate (APCP). Equal amounts of solvent (DMSO) were added to the otherwise untreated controls. To exclude unwanted effects of the inhibitors on migrating monocytes, ovarian cancer cells were pre-incubated with the inhibitors for 30 min before being washed with PBS. To assess the effect of adenosine on monocyte migration directly, a positive control with the metabolically stable adenosine receptor agonist adenosine-5´-N-ethylcarboxamide (NECA) (used at 100 nM) was also included (n=3, * indicates $p<0.05$, ** denotes $p<0.01$, as assessed by unpaired Student's *t*-test). As additional control, A2A receptor signalling was blocked during the assay by SCH58261 (in **A**). Under all conditions, migration of monocytes through the 8 µm wide and 10 µm thick pores was analyzed after 4 h by staining transmigrated cells for CD11c followed by flow cytometric analysis. Tumor cells from the bottom compartment were identified by co-staining for EpCAM expression.

3.6. M2-MACROPHAGES POLARIZED BY CO-CULTURE WITH OVCA CELLS UP-REGULATE CD39 AND CD73 TO THE LEVELS OBSERVED IN TAMS

Immunosuppressive myeloid cells with a phenotype resembling tumor Associated Macrophages (TAMs) can be induced by co-incubation of monocytes with OvCa (Hagemann et al., 2006). Having found that OvCa cell-derived adenosine can attract monocytes towards the tumor cells, we wanted to confirm that these monocytes polarize to an M2 phenotype which resembles the TAMs found at the tumor microenvironment.

Therefore, mature macrophages from healthy donors were co-incubated with OAW-42 cells in a transwell setting where macrophage migration to the lower chamber was precluded by the narrow pore-diameter of the transwell membrane. After 48h of co-culture, macrophages were analysed by intracellular flow cytometry for expression of IL-10 and IL-12. As opposed to M1-polarized macrophages (generated from monocytes in the presence of IFN-γ and LPS), co-cultured macrophages displayed high levels of IL-10 and low levels of IL-12, which confirmed their M2-polarization (fig. 14).

Similar to recent reports on murine TAMs (Zanin et al., 2012) we also observed high levels of CD39 and CD73 on these *in vitro*-generated TAM-like cells, whereas CD73 surface expression was significantly lower on M1 macrophages (fig. 14).

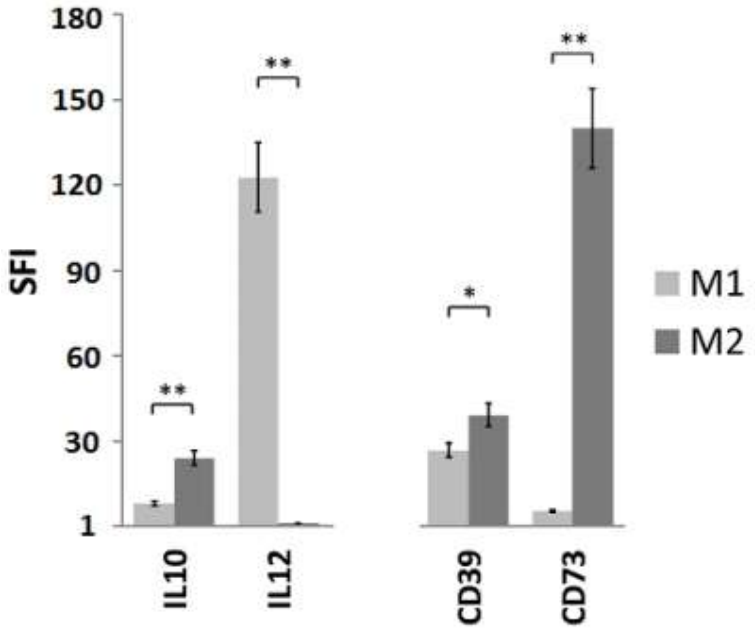


Figure 14. IL-10, IL-12, CD73 and CD39 expression by M1 and M2 macrophages. Macrophages were differentiated from healthy volunteers' monocytes and co-incubated with OAW-42 ovarian cancer cells. Polarized *in vitro*-TAMs where analysed by FACS for intracellular IL-10/IL-12 levels (left) and for CD39/CD73 surface expression (right). For control purposes macrophages were polarized to an M1-phenotype via treatment with LPS and IFN-γ and analysed in the same settings. The macrophages from the co-culture showed low expression of IL-12 and high intracellular levels of IL-10 confirming their M2-polarization (light grey, left) in comparison to M1-macrophages (dark grey, left). In M2 macrophages, CD39 and CD73-expression was also significantly increased (light grey, right) in comparison with M1 macrophages (dark grey, right). (n=3) In flow cytometric assays at least 50,000 events were counted; two samples were considered to be significantly different (*) when they were separated by at least twice the sum of the standard deviations for the respective regions. A difference exceeding four times the sum of the respective standard deviations was considered as highly significant (**).

Furthermore, CD73 and CD39 mRNA induction on macrophages after co-culture with OAW-42 was confirmed (fig. 15)

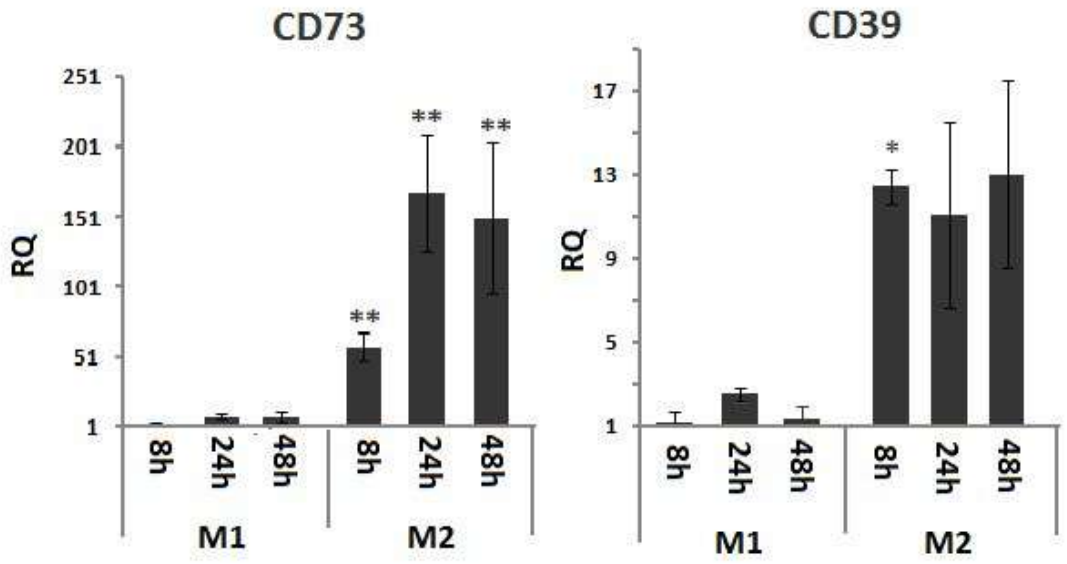


Figure 15. Relative CD73 and CD39 mRNA expression in M1 and M2 macrophages. RNA was isolated from i*n vitro* polarized M1 and M2 macrophages after 8, 24 and 48 hours and reverse-transcribed to cDNA. Transcriptional expression of CD73 and CD39 was determined by SybrGreen-based quantitative PCR. **(A)** Macrophages polarized towards M2 by co-culture with OAW-42 cells show significantly higher levels of CD73 mRNA after 8, 24 and 48h, whereas no induction of CD73 mRNA is observed in the M1 macrophages. **(B)** CD39 mRNA levels are significantly increased in M2 macrophages 8 hours after co-culture with OAW-42 cells. No significant increase of CD39 mRNA can be observed in M1 macrophages. Significance levels were determined by Student's t-test. p-values <0.05 were considered as significant (*), p <0.01 as highly significant (**).

To correlate this finding with the *in vivo* situation, TAMs were isolated from fresh ascites samples of OvCa patients. These *ex-situ*-TAMs likewise expressed high levels of ectonucleotidases (fig. 16). For control purposes macrophages and monocytes from healthy donors were analysed and showed negligible expression of CD39 or CD73 (fig 16).

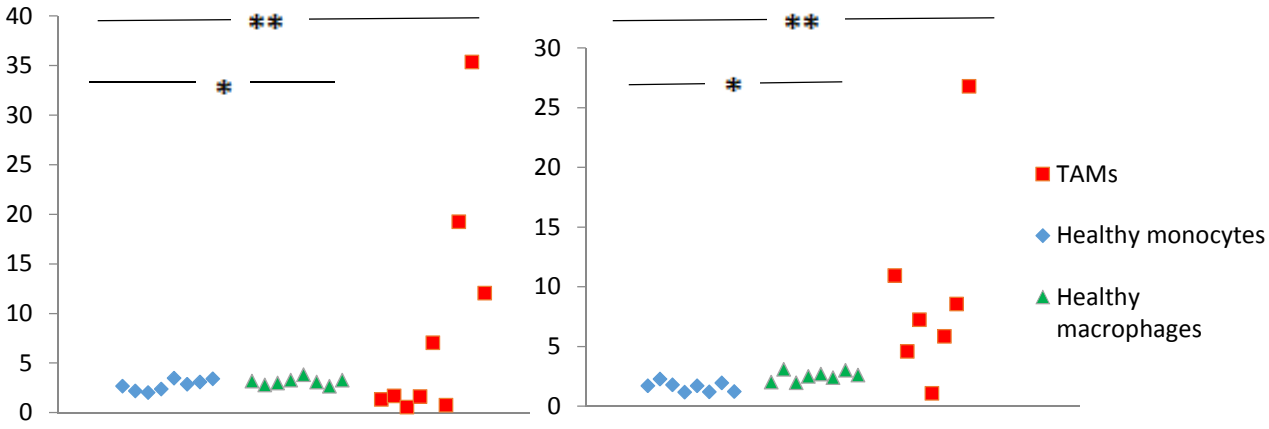


Figure 16. CD39 and CD73 levels in healthy monocytes, mature macrophages and tumor associated macrophages (TAMs). Monocytes were isolated from healthy donors by gradient centrifugation and subsequent adherence depletion. Monocytes were then matured in Lumox® dishes for 9 days to obtain mature macrophages. Tumor-associated macrophages were isolated from ascites of OvCa patients. Graphic representation shows CD39 and CD73 SFI of the three populations. Significance levels were determined by Student's t-test. p-values <0.05 were considered as significant (*), p <0.01 as highly significant (**).

Further metabolic characterization of macrophages after co-culture was performed by measuring the activity of arginase. As described by Chiung-I Chang et al., high arginase activity is related to a macrophage phenotype which promotes tumor growth (Chiung-I Chang et al., 2001). To examine the activity of the aforementioned enzyme a colorimetric assay was performed (see methods arginase assay). This assay confirmed a higher enzymatic activity in the M2 polarized macrophages as well as in TAMs isolated from fresh ascites of OvCA patients (fig.17).

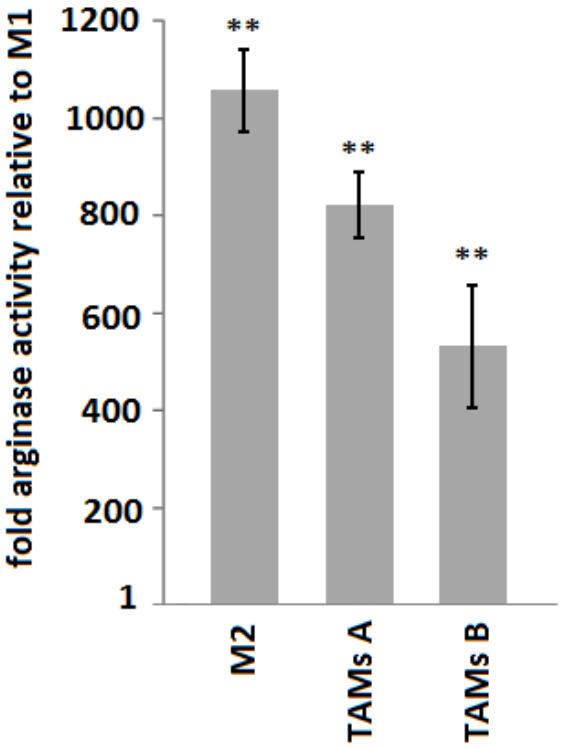


Figure 17. **Arginase activity of M1, M2 and tumor associated macrophages**. Mature macrophages from a healthy donor were polarized towards an M1 or M2 phenotype as described in the methods section. Tumor associated macrophages were isolated from malignant tumor tissue. The concentration of urea (the final product of arginase activity) was measured using a colorimetric assay (see methods section, arginase assay). M2 macrophages and TAMs from tumor 40 and 42 show significantly higher arginase activity compared to M1 macrophages. Significance levels were determined by Student's t-test. p-values <0.01 were considered as highly significant (**).

3.7. OVER-EXPRESSION OF CD39 AND CD73 ON *IN VITRO* GENERATED TAM-LIKE MACROPHAGES RESULTS IN HIGHER LEVELS OF BIOLOGICALLY ACTIVE ADENOSINE

To elucidate whether the *in vitro* polarized macrophages could also produce higher levels of biologically active adenosine due to the up-regulation of CD39 and CD73, a luciferase-based adenosine reporter assay was performed as previously described (Hausler et al., 2010). The assay confirmed that pro-inflammatory M1 macrophages produced lower levels of adenosine (0.27 µM ± 0.18 µM) while TAM-like or M2 polarized macrophages generated significantly higher amounts of adenosine (on average 3.8 µM, range: 1.6-5.4 µM) (fig. 18). As predicted, in the presence of specific inhibitors for CD39 (ARL 67156) and CD73 (APCP) adenosine levels were drastically decreased (to 0.13 µM ± 0.03 µM with ARL 67156 and to 0.10 µM ± 0.01 µM with APCP; Figure 18)

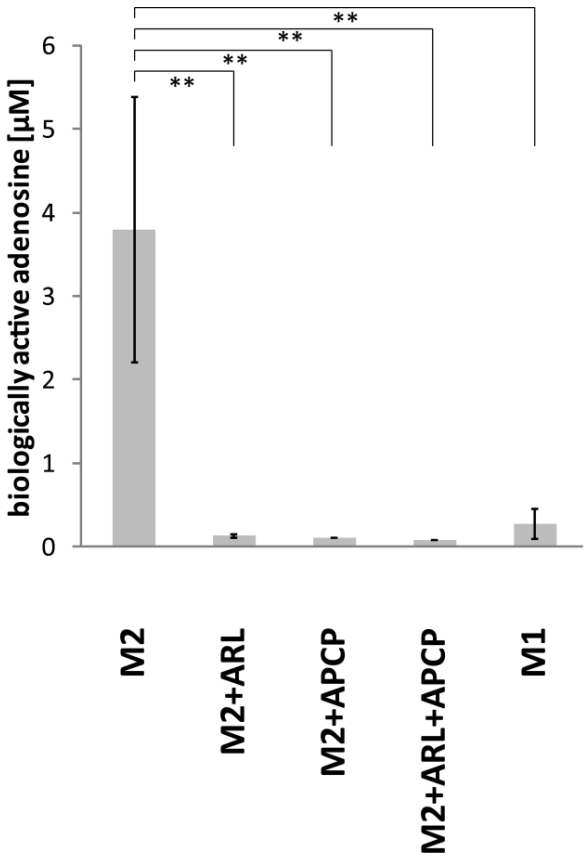


Figure 18. Adenosine production by macrophages. M2-polarized macrophages were generated in co-culture settings with OAW-42 OvCa cells. For control purposes M1-macrophages were induced by addition of LPS and IFN-γ. A luciferase-dependent reporter assay was performed to determine the amount of the produced biologically active adenosine (Hausler et al., 2010). During the measurement CD39 was blocked with ARL67156 ("M2+ARL"), CD73 by APCP ("M2+ APCP") and both ectonucleotidases by combining ARL67156 and APCP ("M2+ARL+APCP"). For control purposes only the solvents were applied (n=3). Significance levels were determined by Student's t-test. p-values <0.01 were considered as highly significant (**).

3.8. *IN VITRO* POLARIZED M2 MACROPHAGES SUPPRESS CD4$^+$ T CELL PROLIFERATION VIA ADENOSINE PRODUCTION

Functional relevance of increased adenosine generation by M2 macrophages was tested by measuring their effect on CD4$^+$ T cell proliferation, using the already mentioned proliferation assay. *In vitro* polarized macrophages were co-cultured with T cells stimulated with agonistic antibodies against CD3 and CD28. In this setting, TAM-like macrophages decreased CD4$^+$ T cell proliferation by more than 50% when compared to M1 macrophages (fig. 19). Interestingly, when the ectonucleotidase CD39 activity on M2 macrophages was specifically blocked by ARL67156, CD4$^+$ T cell proliferation was increased about 3.5 fold compared to solvent controls. Inhibition of CD73 by APCP enhanced T cell proliferation by a factor of 2.7.

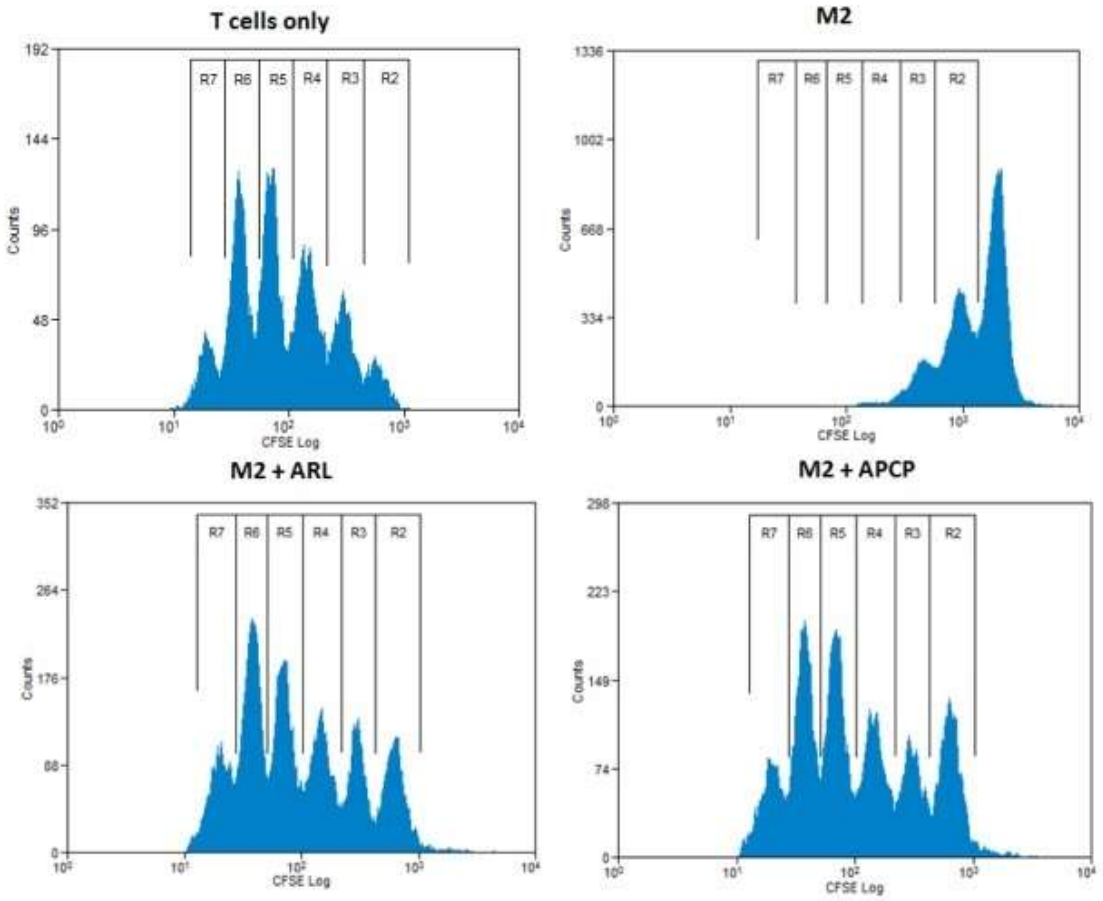


Figure 19. Adenosine from M2 macrophages suppresses CD4$^+$ T cell proliferation. M2-polarized macrophages were generated in co-culture settings with OAW-42. For control purposes M1-macrophages were induced by application of LPS and IFN-γ. CD4$^+$ T cells were isolated from healthy volunteers' PBMC and stained with CFSE before activation with anti-CD3 and anti-CD28-antibodies. The CFSE$^+$ CD4$^+$ T cells were co-incubated with M2 macrophages in the presence or absence of CD39-/CD73-inhibitors ARL67156 ("T+M2+ARL"), or, respectively, APCP ("T+M2+APCP"), or a solvent control ("T+M2"). Proliferation was determined via flow cytometry as described previously (n=3). Significance levels were determined by Student's t-test. P values <0.01 were considered as highly significant (**).

3.9. UP-REGULATION OF ECTOENZYME EXPRESSION AFTER THERAPEUTIC TREATMENT

OAW-42 cells were treated with chemotherapeutics commonly used in the clinics for treating relapsed ovarian cancer, namely with doxorubicin and cisplatin. These drugs were applied to OvCa cells at high concentrations (15µM doxorubicin and 20µM cisplatin) for 30 minutes and were then washed off with PBS. A third population of OAW-42 cells was irradiated (3Gy). Ectoenzyme levels were measured 48h after treatment. The effect of therapeutic treatment on the ectoenzyme expression was measured by flow cytometry. As shown in figure 20, all treatments significantly enhanced expression of both CD73 and CD39.

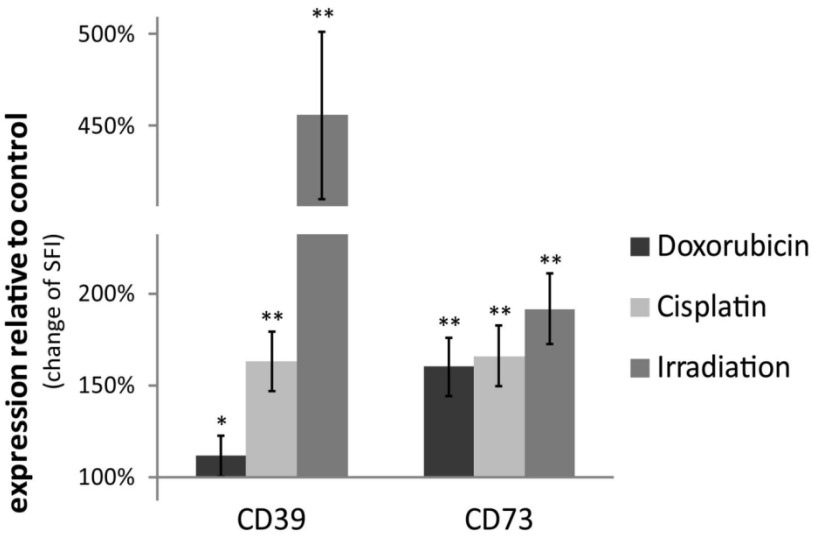


Figure 20. Expression of CD39 and CD73 after chemotherapeutic treatment and irradiation. OAW-42 cells were irradiated with 3Gy or incubated for 30 minutes with 15µM Doxorubicin or 20µM Cisplatin. After incubation and irradiation, the cells were washed thoroughly with PBS. 24 hours after treatment, SFI values for CD39 and CD73 were determined by flow cytometry (n=3). In flow cytometric assays at least 50,000 events were counted; two samples were considered to be significantly different (*) when they were separated by at least twice the sum of the standard deviations for the respective regions. A difference exceeding four times the sum of the respective standard deviations was considered as highly significant (**).

Transcript levels of CD39 and CD73 mRNA were also shown to be up-regulated after the aforementioned doxorubicin treatment (fig. 21). The directly pulse-treated cells show the highest CD73 transcript expression 8 hours after the treatment, with a reduction after 24 hours. Untreated cells in co-culture with treated cells also show a significant increase of CD73 mRNA 8 hours after co-culture. Values for CD39 transcription were not significantly altered although they showed the same tendency as CD73.

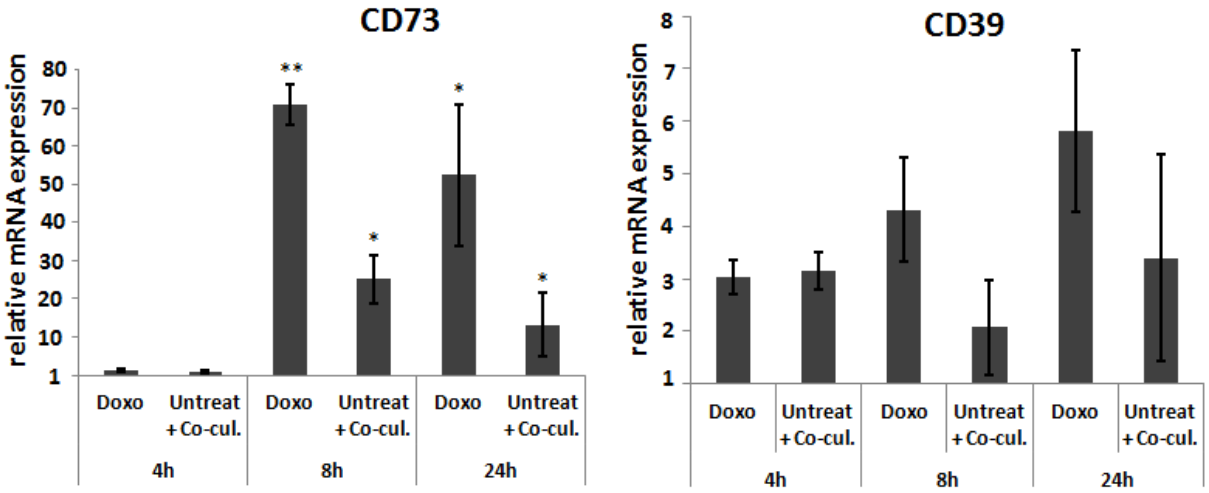


Figure 21. Relative CD73 and CD39 mRNA expression in Doxorubicin treated OAW-42 cells. OAW-42 cells were given a pulse of 15µM Doxorubicin for 30 minutes and were then washed with PBS (Doxo). In a parallel experiment, untreated OAW-42 cells were put in a transwell and co-culture with already doxorubicin (15µM) pulsed OAW-42 cells (Untreat C/C). RNA was isolated from OAW-42 cells and reverse-transcribed to cDNA 4, 8, and 24 hours after treatment. Transcriptional expression of CD73 and CD39 was determined by SybrGreen-based quantitative PCR. Significance levels were determined by Student's t-test. p-values <0.05 were considered as significant (*), p <0.01 as highly significant (**).

We hypothesized that this ectoenzyme up-regulation could be due to an induction of cell death. Adenosine production could then constitute a mechanism to evade an ATP-dependent immune response after treatment. To elucidate whether dying cells were responsible for the enhancement of ectonucleotidase expression, OvCa cells were treated with classical inducers of apoptosis such as staurosporin and fas ligand (fig. 22). 24h after treatment ectoenzyme levels were measured by flow cytometry.

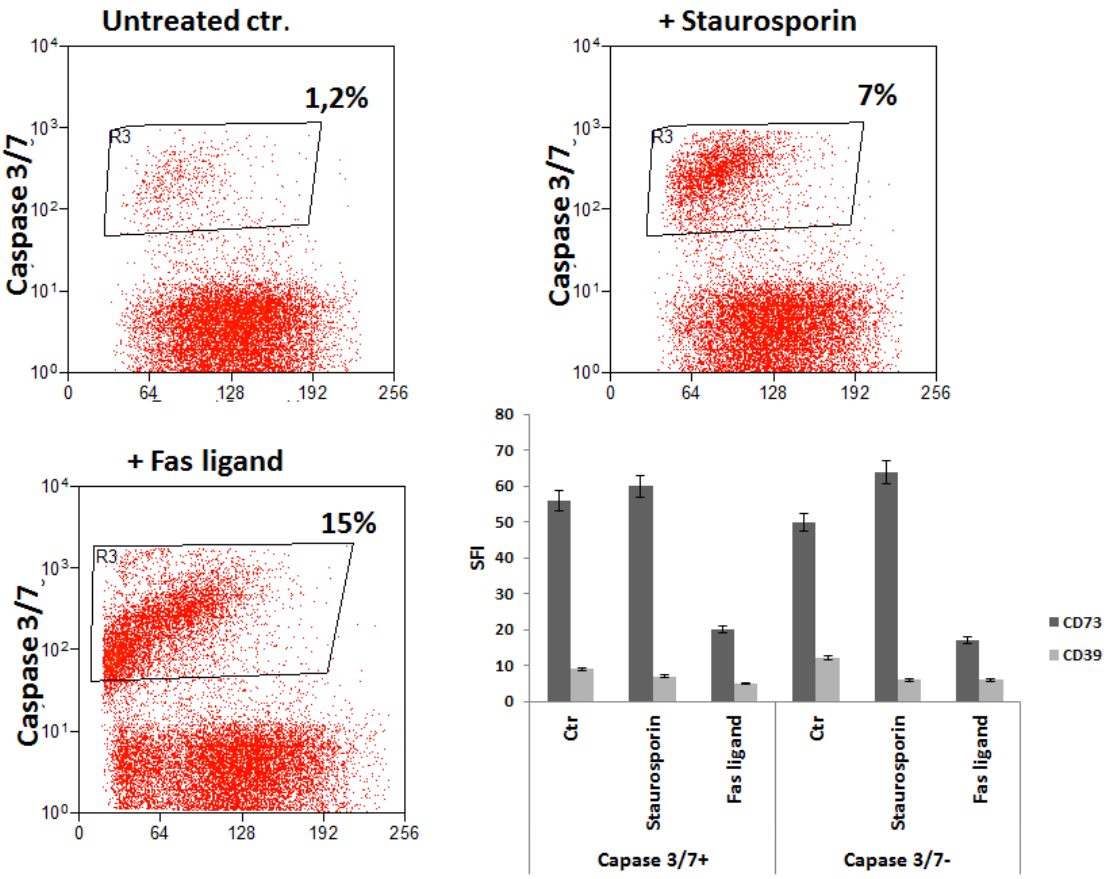


Figure 22. Ectoenzyme CD39 and CD73 expression after induction of apoptosis. OAW-42 cells were treated with staurosporin 1µM or Fas-ligand 10nM and stained with CellEvent® Caspase 3/7 which enables the detection of caspase activity by flow cytometry. The apoptotic or, respectively, the non-apoptotic population was gated and their SFIs for CD73 and CD39 were measured. None of the apoptosis inducers caused any induction of the ectoenzymes.

No significant enhancement of ectonucleotidases could be observed after induction of apoptosis. This result contradicted the above mentioned hypothesis, leading us to the idea that the up-regulation might occur in living cells as a response to a stress stimuli.

3.10. ECTOENZYME UP-REGULATION UPON THERAPEUTIC REGIMEN IS MEDIATED BY A SECONDARY MESSENGER

Having found that OvCa cells respond to certain stress stimuli by over-expressing CD73 and CD39, we set out to investigate whether it is an effect caused by the treatment itself, or whether upon treatment cells secrete a second messenger inducing the afore mentioned up-regulation. Therefore, OAW-42 cells were pulse-treated with doxorubicin, washed thoroughly and brought together with untreated OAW-42 cells stained with cell proliferation dye eFluor670®. In FACS analyses the SFI for CD73 was determined for eFluor670® positive and negative cancer cells. In both subpopulations CD73 levels were highly increased which means that also cells that had not been in contact with doxorubicin showed a strong induction of CD73 (fig. 23).

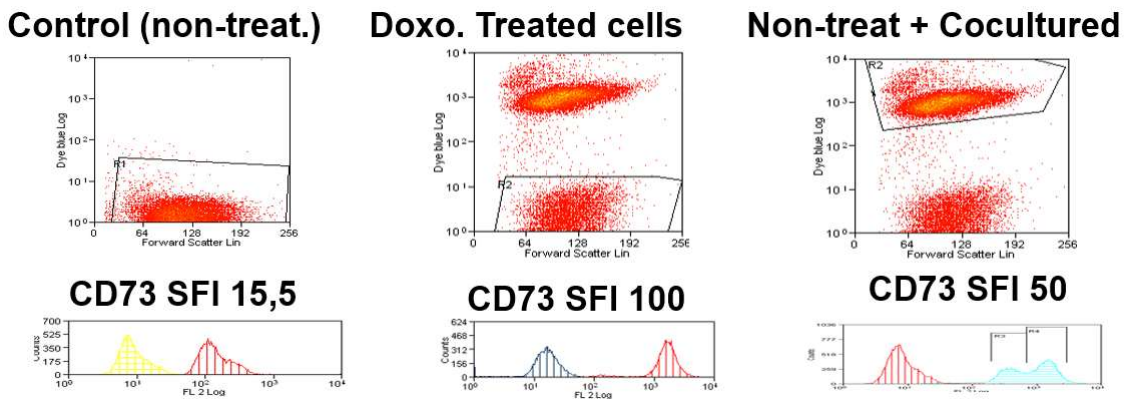


Figure 23. Expression of CD73 after direct doxorubicin treatment or co-culture with doxorubicin-treated OvCa cells. OAW-42 cells were pulse treated with 15µM doxorubicin for 30 minutes and washed with PBS. After treatment, the treated cells were placed in the upper compartment of a transwell plate together with untreated OAW-42 cells which had been stained with eFluor670® (lower insert). After 24 hours of transwell culture, SFI values for CD73 were assessed by flow cytometry. Even though the distinct populations had no direct contact with each other, CD73 was also found to be up-regulated in the untreated cell population.

3.11. DRUG-TREATED OVCA CELLS PRODUCE HIGHER LEVELS OF ADENOSINE

To investigate whether the treatment-induced ectonucleotidase up-regulation also enhances the immunmodulatory potential of the cancer cells, adenosine production was analyzed in the already mentioned luciferase-dependent adenosine assay. Compared to untreated control-OAW-42, stressed cells showed significantly higher adenosine production (fig. 24). Addition of small molecular inhibitors ARL and APCP reduced the concentration of the produced adenosine, proving that the measured adenosine is a result of the catalytic activity of CD39 and CD73.

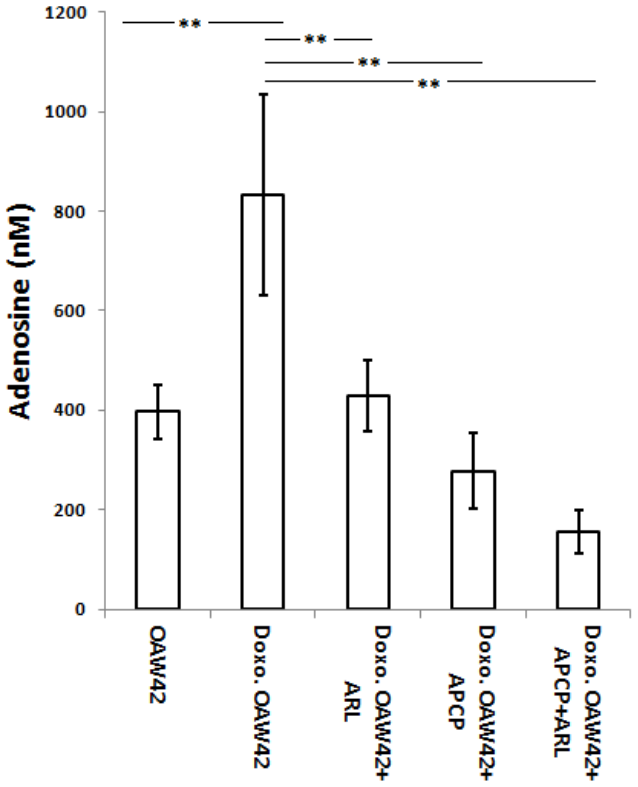


Figure 24. Adenosine production of doxorubicin-treated OvCa cells. OvCa cells were treated with a pulse of doxorubicin (15µM) for 30 minutes; subsequently cells were washed with PBS and incubated for 24 hours. The already mentioned luciferase-based adenosine assay enabled the measurement of adenosine production from untreated and treated OAW-42 cells in the absence or presence of ectonucleotidase inhibitors.

3.12. DRUG-TREATED OVCA CELLS HAVE ENHANCED IMMUNOSUPPRESSIVE ACTIVITY DUE TO THEIR HIGHER ECTOENZYME EXPRESSION

To test the functional properties of the increased adenosine production in the doxorubicin-treated cells, co-culture experiments with CFSE-labeled $CD4^+$ T cells were performed. In comparison to OAW-42 cells which had not been in contact with doxorubicin before, we detected significantly stronger proliferation inhibition after co-incubation with OAW-42 cells that had been exposed to doxorubicin before. This inhibition of T cell proliferation was overruled when small molecule inhibitors for CD39 or CD73 (ARL67156 and APCP) were applied, which confirms the CD39/CD73-dependency of the determined effect.

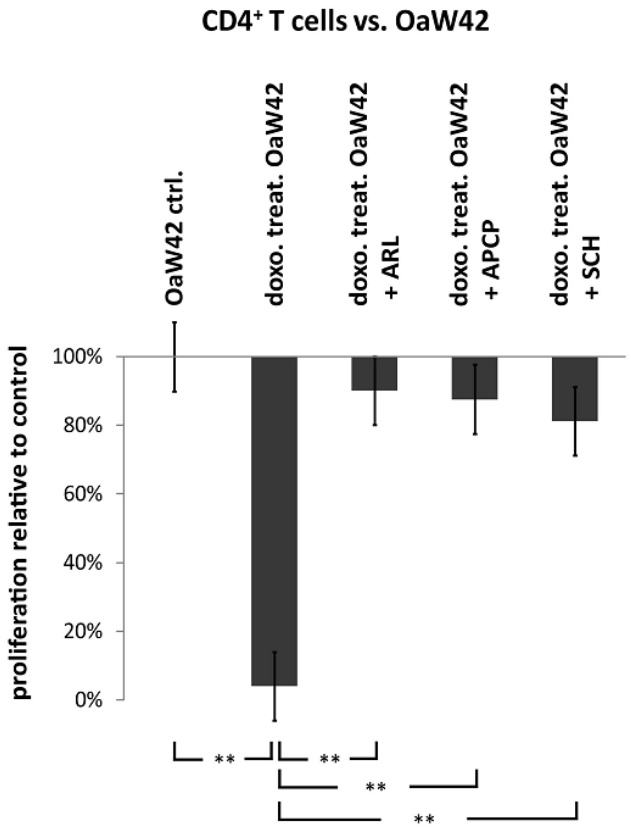


Figure 25. Effect of CD39, CD73 or A2A adenosine receptor inhibition on the suppressive capacity of doxorubicin treated OAW-42 cells against proliferating activated $CD4^+$T cells. Doxorubicin treated (15µM, 30 minutes) OAW-42 cells were co-cultured with $CD4^+$T cells that had been stained with CFDA-SE and stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. To inhibit ectonucleotidases, CD39 or CD73 inhibitors ARL67156 (ARL) and APCP were added (100µM each). $ADENOSINER_{A2A}$ ADORA2A was blocked using 100nM SCH58261. Proliferation was assessed as described before (See figure 12 legend).

3.13. DIRECT TREATMENT OF MACROPHAGES WITH CHEMOTHERAPEUTIC DRUGS DOES NOT INCREASE ECTOENZYME EXPRESSION, WHEREAS CO-CULTURE WITH TREATED OVCA CELLS DOES

Chemotherapeutics are non-targeted drugs which do not only affect cancer cells but also all other cells present at the tumor microenvironment. Therefore, we next proceeded to investigate their effect on another stromal cell population, the macrophages. Direct doxorubicin (15µM, 30 min) treatment of mature macrophages or *in vitro* polarized M1 or M2 macrophages did not cause any apparent ectoenzyme up-regulation. However, *in vitro* polarized M2 macrophages did show an over-expression of CD73 after co-culture with doxorubicin- treated OvCa cells (fig. 26). These data support the idea of the released second-messenger after stress induction in cancer cells.

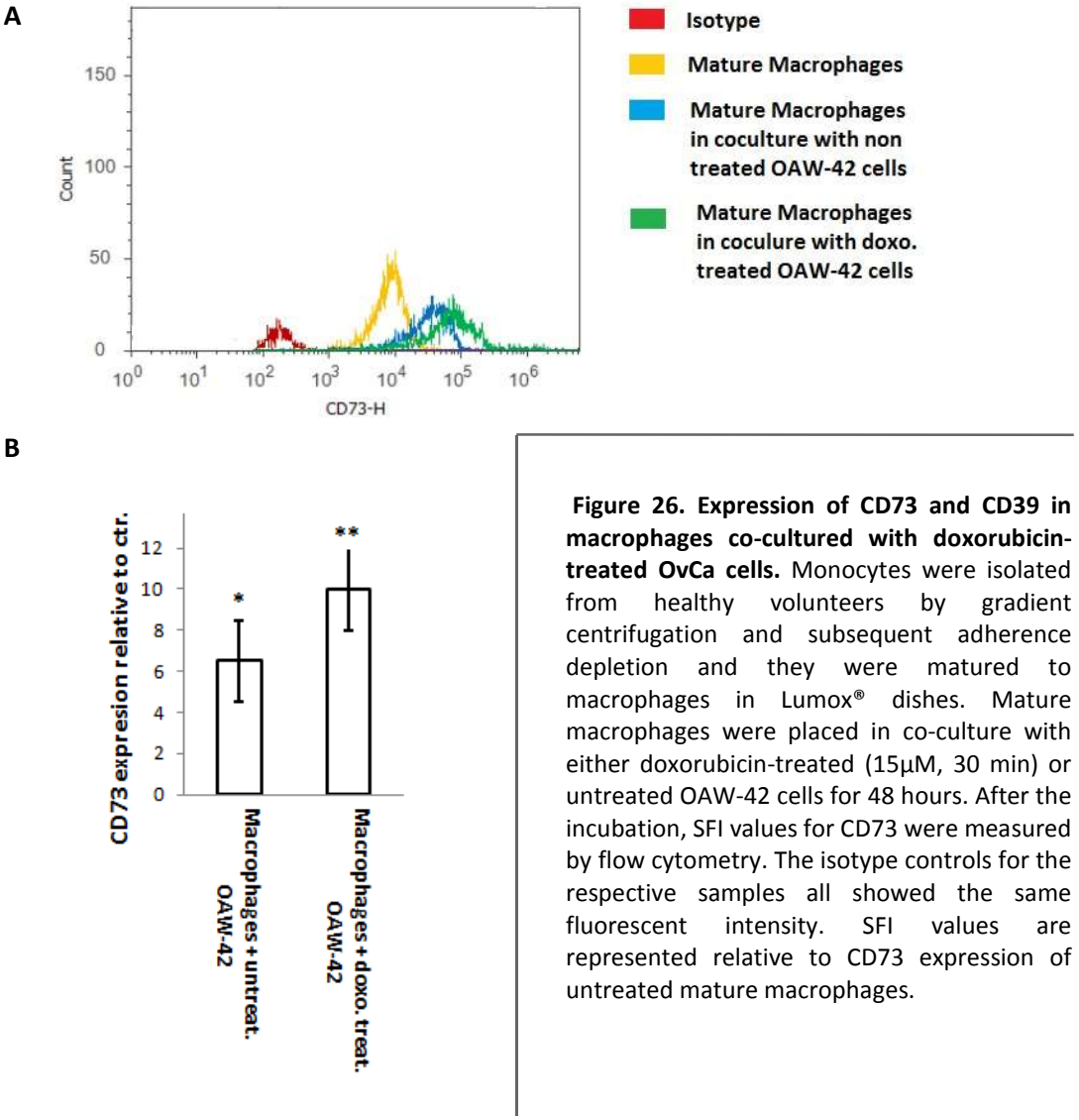


Figure 26. Expression of CD73 and CD39 in macrophages co-cultured with doxorubicin-treated OvCa cells. Monocytes were isolated from healthy volunteers by gradient centrifugation and subsequent adherence depletion and they were matured to macrophages in Lumox® dishes. Mature macrophages were placed in co-culture with either doxorubicin-treated (15µM, 30 min) or untreated OAW-42 cells for 48 hours. After the incubation, SFI values for CD73 were measured by flow cytometry. The isotype controls for the respective samples all showed the same fluorescent intensity. SFI values are represented relative to CD73 expression of untreated mature macrophages.

3.14. DIRECT TREATMENT OF OVCA CELLS WITH ATP DOES NOT ENHANCE ECTOENZYME EXPRESSION

It is already known that ATP is actively released in the extracellular environment in response to tissue damage and cellular stress, acting as a danger signal which may attract immune cells. Therefore, we first aimed at reproducing the effects of therapeutic treatment on ectoenzyme up-regulation by adding exogenous ATP to OvCa cultures. As shown in figure 27, after direct ATP treatment neither CD73 nor CD39 up-regulation could be seen.

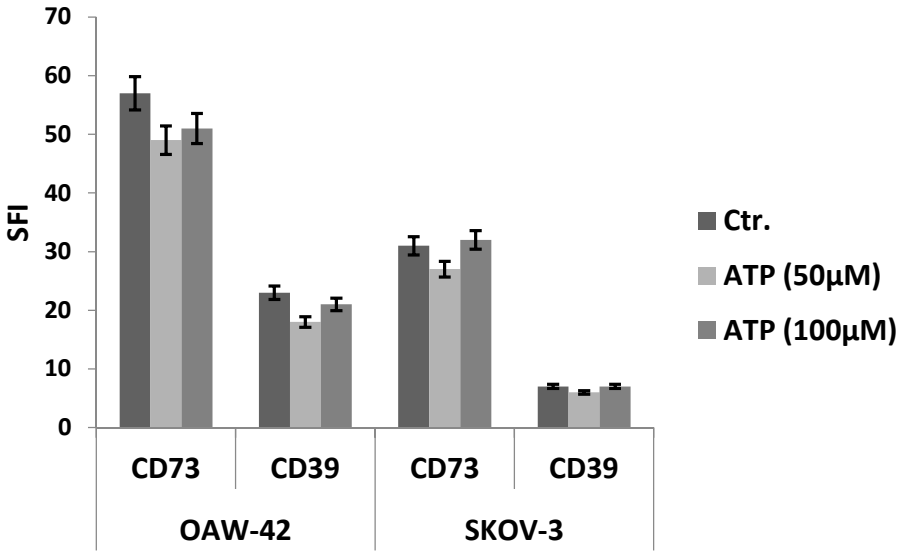
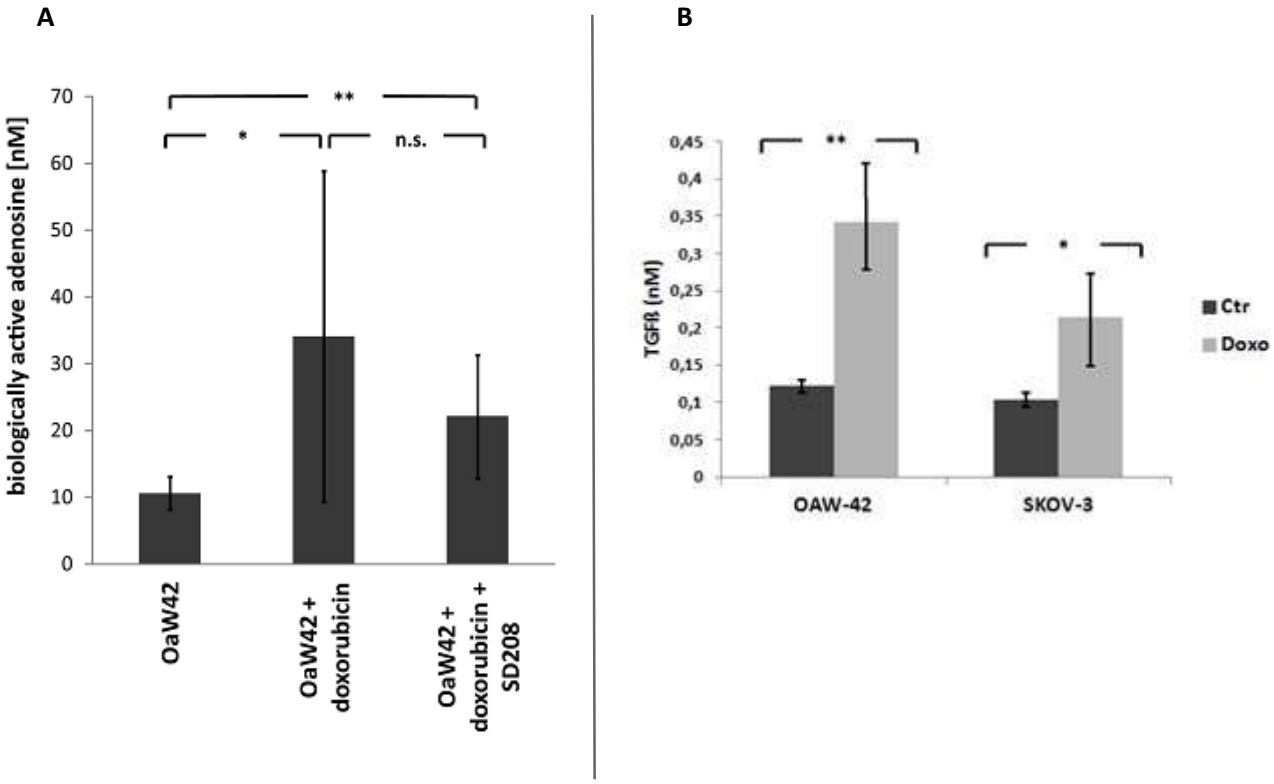


Figure 27. CD73 and CD39 expression on OvCa cells upon ATP treatment. OAW-42 and SK-OV-3 cells were treated with ATP 50µM (light grey) or ATP 100µM (dark grey). After 24 hours of treatment SFIs of CD39 and CD73 were measured by flow cytometry.

3.15. TGF-ß PLAYS ONLY A MINOR ROLE FOR ECTOENZYME UP-REGULATION

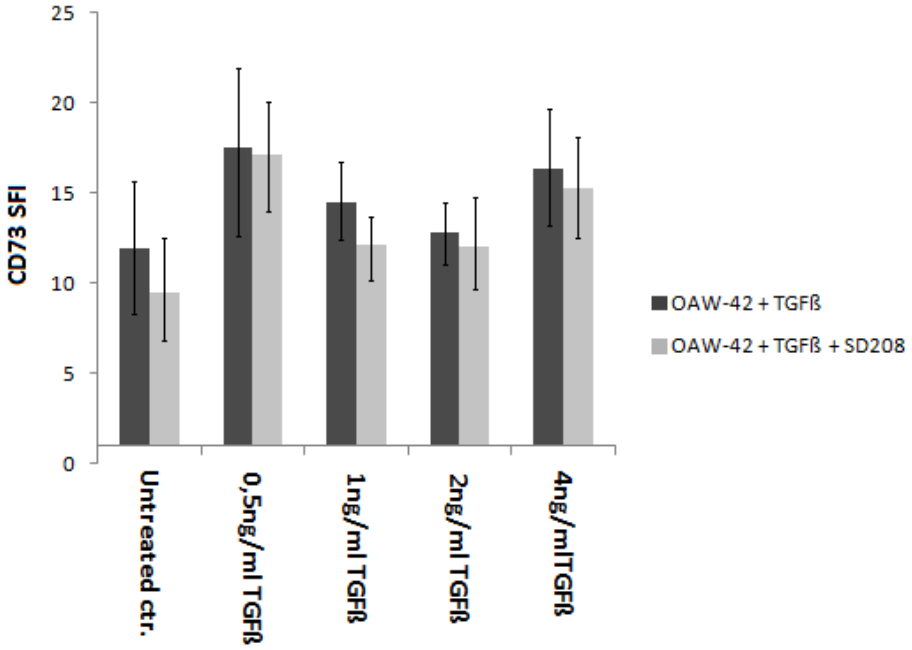
TGF-β has been reported to induce CD73 (Regateiro et al., 2011); thus, we tested doxorubicin-triggered induction of CD73 on OvCa cells in the presence of the selective TGF-β receptor I kinase inhibitor SD208 (fig.28). While a TGF-β activity assay (Mazzieri et al., 2000) with CCL-64 mink lung cells showed a highly significant release of TGF-β upon treatment with doxorubicin (fig. 28B), induction of CD73 and adenosine production were only partly attenuated by addition of SD208 (fig. 28 A ,C). This shows that another, yet-to-be discovered soluble factor contributes to the CD73-inducing signal. Recombinant TGF-ß and SD208 activity were measured by SMAD phosphorylation (fig. 28 D).



C

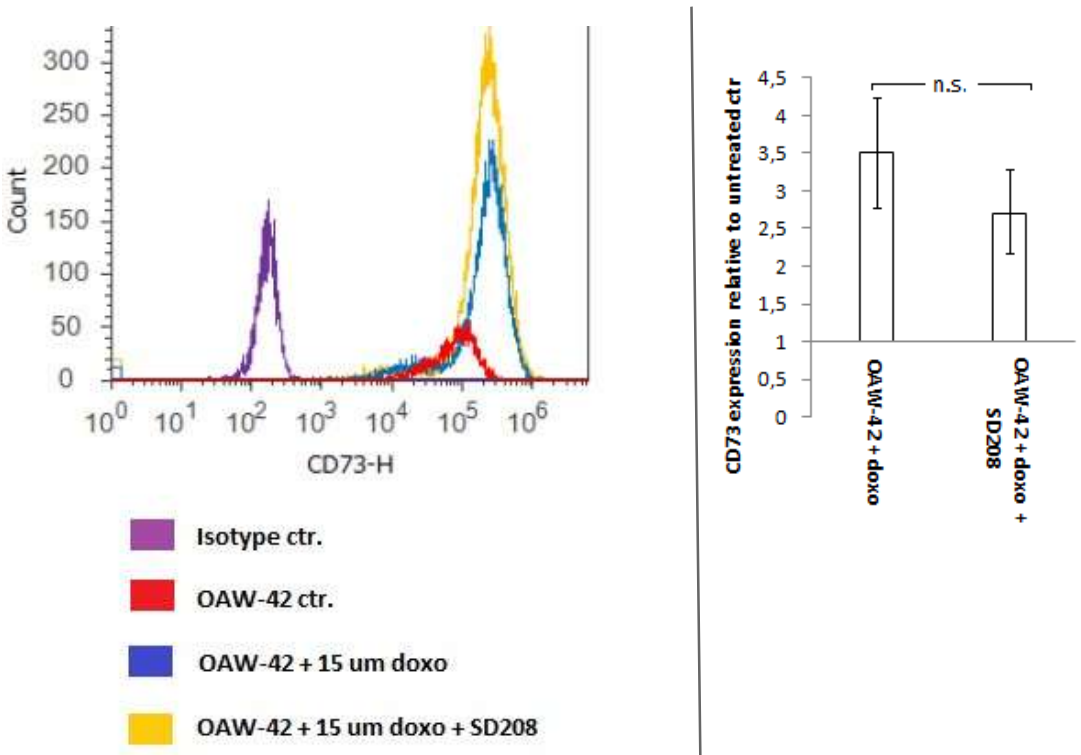
(1)

OAW-42 + TGF-ß + SD208



(2)

OAW-42 + Doxo. + SD208



D

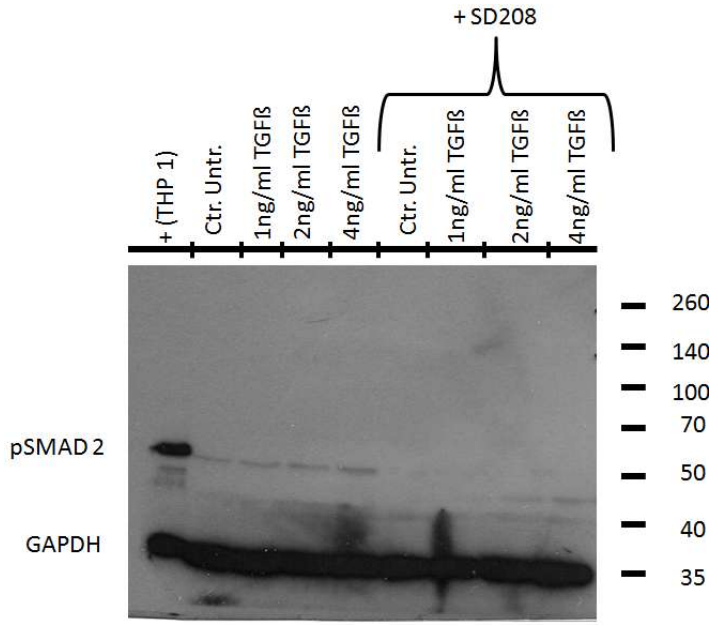


Figure 28. Ectoenzyme expression upon TGF-β treatment. (A) Adenosine production of doxorubicin treated (15µM, 30 min) and doxo treated OAW-42 cells plus SD208 (1µM) was measured using the already described luciferase-based method (Hausler et al., 2010). Reduction of adenosine production after addition of SD208 was not significant. **(B)** TGFβ production of doxo-treated (15µM, 30 min) SK-OV-3 cells and OAW-42 cells was measured using the CCL-64 mink lung assay (See methods). **(C)** CD73 expression was measured on OAW-42 cells after addition of different concentrations of recombinant TGFβ (0.5, 1, 2 and 4 ng/ml) plus SD-208 (1) and 15 µM doxorubicin plus SD-208 (). **(D)** Bioactivity of recombinant TGF-ß1 was assessed in a western blot for phosphorylated SMAD2. GAPDH was measured as loading control.

4. DISCUSSION

Ovarian cancer patients often exhibit immunosuppression and therefore diminished immune recognition of the tumor (Rongcun et al., 1999). Tumors can defeat the immune system by different approaches; such as inhibition of T helper 1 cell (TH1 cell) cytokine production, deregulation of mononuclear phagocyte cell differentiation and maturation, and suppression of effector T cells (Hasko & Pacher, 2012; Longhi, Robson, Bernstein, Serra, & Deaglio, 2013), secretion of tumor derived factors (i.e. immunosuppressive cytokines), expression of membrane associated molecules and serine proteases and downregulation of molecules required for antigen presentation and processing. These and further immunosuppressive mechanisms, together with the genetic instability of cancer cells, facilitate immune escape (Rongcun et al., 1999).

Accumulating evidence has revealed that interactions with non-transformed cells are key factors for manipulating tumor activity and tumor responses to anticancer therapies (Grivennikov et al., 2010; McMillin, Negri, & Mitsiades, 2013). Infiltration with cytotoxic or regulatory T cells (Treg) significantly influences the outcome (Curiel, Cheng, et al., 2004; L. Zhang et al., 2003) of patients with OvCa. Several publications suggest that Treg can perform immunosuppression by generating adenosine from extracellular ATP via CD39 and CD73 (Borsellino et al., 2007; Deaglio et al., 2007). Dendritic cells (DC) in particular express the purinergic receptor $P2X_7$ (Ghiringhelli et al., 2009). When this receptor is engaged by ATP, the NLRP3 inflammasome gets activated which in turn stimulates the priming of antigen-specific $CD8^+$ T cells (Ghiringhelli et al., 2009) and thus enhances the immune system's anti-tumor capacity.

Another type of immune cells with antigen-presenting properties are macrophages which can assume two major phenotypes. M1 macrophages produce high levels of IL-12, TNF-α or IL-6 and have mainly a pro-inflammatory function (Sica & Bronte, 2007). Macrophages of the M2 subtype, in contrast, secrete high levels of immunosuppressive cytokines such as IL-10 and TGF-β (Sica & Bronte, 2007). They are thought to fulfil physiological roles in the regulation of wound healing and the coordination of inflammatory processes (Pollard, 2004). The Macrophages in the tumor tissue (so-called "Tumor Associated Macrophages") were shown to polarize to this M2 subtype (Pollard, 2004). They have been shown to promote tumor growth by suppressing anti-tumoral

immune responses and also by inducing angiogenesis, releasing growth factors and remodelling the tumor matrix (Sica & Bronte, 2007). In ovarian cancer, infiltration of TAMs is a common phenomenon (Kawamura, Komohara, Takaishi, Katabuchi, & Takeya, 2009). Kawamura et al. found out that in OvCa TAMs are associated with the malignancy of serous and mucinous epithelial ovarian cancers (Kawamura et al., 2009), revealing that TAMs play a crucial role in the immune evasion of ovarian cancer and are correlated with a poor prognosis (Lan et al., 2013).

There is growing awareness that tumor cells build up a "self-advantageous" microenvironment that decreases effectiveness of anti-tumor immune responses (Albini & Sporn, 2007). Depending on the balance between tumor-derived immunosuppressive factors and host-derived immunoactivating factors, the microenvironment can be favourable or unfavourable for tumor growth. In fact, the need for a supportive microenvironment limits metastasis in many cases. The complicated network of pro- and anti-inflammatory factors might possibly be modulated by the targeting of specific pathways. In this context, hypoxic conditions that characterize tumor tissue may be a key player on tumor protection. Hypoxic conditions activate hypoxia-inducible factor 1 α (HIF-1α) and promote accumulation of extracellular adenosine. Both factors support tumor growth; as HIF-1α controls angiogenesis and adenosine exerts a profound immunosuppressive activity. Studies have shown that solid tumors have a gradient of adenosine concentration from the centre to the periphery, higher than the surrounding healthy tissue (Ohta et al., 2006).

Mounting evidence supports the relevance of adenosine in the tumor microenvironment as an important autocrine and paracrine regulatory factor (Ohta et al., 2006). The concentration of adenosine which is kept low under normal physiological conditions can rise rapidly in response to a pathophysiological stimuli such as inflammation, trauma, hypoxia or ischemia (Hasko et al., 2008). This nucleoside will then bind to specific adenosine receptors on target cells and activate several cellular responses with the objective of restoring tissue homeostasis (Antonioli et al., 2008; Hasko et al., 2008). Indeed, this increase in adenosine concentration during injury has been shown to protect the tissue from an excessive inflammatory response (Csoka et al., 2008; Hasko & Cronstein, 2004; Hasko et al., 2008; Longhi et al., 2013). However, in

spite of its protective effects, prolonged adenosine high concentrations can turn harmful for the tissue by triggering immune suppression or by activating an unremitting wound-healing process (Chan & Cronstein, 2010; Karmouty-Quintana, Xia, & Blackburn, 2013). Moreover, accumulation of adenosine in the TME and stroma has been linked with tumor progression (M. V. Sitkovsky, Kjaergaard, Lukashev, & Ohta, 2008).

The extracellular concentration of adenosine is mainly increased by the action of membrane-bound ectoenzymes which produce adenosine by phosphohydrolysis of ATP and ADP released from damaged cells (Borsellino et al., 2007; Deaglio et al., 2007).

In the last years it has become clear that dying cells release ATP in a passive or active manner (Ayna et al., 2012; Chekeni et al., 2010; Garg et al., 2012; Krysko et al., 2011; Martins et al., 2009; Michaud et al., 2011). Different mechanisms of ATP secretion have been described in dying, dead or stressed cells (Chekeni et al., 2010; Garg et al., 2012; Krysko et al., 2011; Martins et al., 2009; Michaud et al., 2011). The mechanism for ATP secretion in cells undergoing immunogenic cell death (ICD) depends on the cell death stimuli, type of stress or on the apoptotic stage (Krysko et al., 2012).

For example, the ATP released during ICD, as a result of therapeutic treatment with anthracyclines, activates the immune system. However, as a negative-feedback mechanism, the extracellular ATP is converted to immunosuppressive adenosine. This conversion of ATP into AMP is mainly catalysed by CD39, and further conversion of AMP into adenosine is catalysed by CD73 (Beavis, Stagg, Darcy, & Smyth, 2012; Krysko et al., 2012).

Furthermore, different tumors over-express enzymes involved in the catabolism of extracellular nucleotides and in the generation of adenosine (Buffon et al., 2007). Accumulation of adenosine into the tumor microenvironment does not only protect tumor cells from the immune response, but may also exert a trophic effect on the tumor itself by stimulating endothelial cell proliferation and angiogenesis (Adair, 2005; Fischer, Sharma, Karliczek, & Schaper, 1995).

Moreover, ATP in the tumor microenvironment is important not only as a source of adenosine but also for its intrinsic activity. In fact, ATP can modulate inflammation by triggering IL-1 maturation and release, dendritic cell differentiation by inducing a Th2-

skewing phenotype and cell proliferation or cell death, depending on the concentrations and activation of individual P2 receptors (Di Virgilio, 2007). Furthermore, ATP causes shedding of metalloproteases (MMP9) (Gu & Wiley, 2006) and expression of indoleamine oxygenase (Marteau et al., 2005). While metalloproteases facilitate tumor invasion, andindoleamine oxygenase has immunosuppressive activity, creating an ideal scenario for tumor growth. Nevertheless, these may be part of negative feedback mechanisms in response to the strong pro-inflammatory effects of ATP. Moreover, many vaccines incorporate antigens and adjuvants that boost immune stimulation by releasing ATP at the inoculation site (Aimanianda, Haensler, Lacroix-Desmazes, Kaveri, & Bayry, 2009; Elliott et al., 2009).

Tregs have been shown to express CD39 and CD73 (Borsellino et al., 2007; Deaglio et al., 2007). As already mentioned, CD39 catalyses extracellular ATP and ADP to AMP (Deaglio et al., 2007) which is subsequently degraded to adenosine by CD73 (Resta et al., 1998). The produced adenosine suppresses TH1, TH2 (Csoka et al., 2008), CTL, and NK cells (Elliott et al., 2009). Furthermore, extracellular ATP is degraded preventing this way immune activation.

Of note, CD39 has been found to be abnormaly expressed in several malignancies such as, pancreatic cancer (Kunzli et al., 2007), melanoma (Dzhandzhugazyan, Kirkin, thor Straten, & Zeuthen, 1998) and small cell lung carcinoma (X. J. Shi & Knowles, 1994).

Morrone et al., showed that glioblastoma growth was reduced by apyrase activity (a calcium activated enzyme which degrades ATP to AMP and inorganic phosphate) in a rat glioma model. In this study, microvascular proliferation was reduced in tumors treated with apyrase, with a subsequent reduction in the blood flow in tumor areas with high cell proliferation. Concluding that insufficient vascular supply is a delimiting aspect for tumor expansion (Morrone et al., 2006).

On the other hand, the activity of CD73 and the resulting extracellular adenosine have been shown to promote tumor cell proliferation (Zhou et al., 2007), adhesion, chemotaxis and metastasis (Stagg et al., 2010; L. Wang et al., 2008).

Moreover, a study conducted by a Jin et al. revealed a major role for CD73-generated adenosine in tumors. This study shows that adenosine can prevent tumor eradication from antitumor T cells and suggest new approaches to overcome this tumor-induced immunosuppression: genetic removal or pharmacologic inhibition of CD73 activity and targeting the A2A receptor pathway. These findings prompted us to investigate the expression and putative immunosuppressive role of CD39 and CD73 in human OvCa specimens *ex vivo* and cell lines *in vitro.*

In this work, we have shown that OvCa cells express CD39 and CD73 both *in vitro* (fig. 9) and *in vivo* (fig.7), enabeling them to generate immunosuppressive extracellular adenosine, as already described for Treg (Deaglio et al., 2007). Conversely, benign ovarian tissues show expression of CD73 but barely no CD39 (fig.7).

The expression of both ectonucleotidases is essential for an efficient catalysis of extracellular ATP into adenosine. Although the enzymatic activity of CD73 and CD39 leads to a high production of extracellular adenosine, the ubiquitous adenosine deaminase degrades it (Havre et al., 2008) or nucleoside carriers internalise it (Eltzschig et al., 2005). Hence, overall adenosine level in the cell culture supernatant may be low and could not be detected by standard methods such as high-performance liquid chromatography (HPLC) (Häusler et al. 2011). Nonetheless, a reporter gene assay utilizing A2A-transfected HEK-293 'sensor' cells that indicate the presence of adenosine by activation of a downstream luciferase plasmid (Hausler et al., 2010), showed that OvCa cell lines SK-OV-3 and OAW-42 generate 30 – 60 fold more adenosine than resting or activated Treg (fig.10, A)(Häusler et al. 2011).

The low levels of adenosine production observed in Tregs can be explained by suboptimal activation of Treg. However, Tregs perform their biological function in a highly controlled and localised manner and therefore the observed low concentration of adenosine may be enough to have a biological influence. On the other hand, a high concentration of adenosine could create an extended immunosuppressive environment an thus, be beneficial for tumor cells.

In both cases, OvCa cells and Tregs, the addition of specific small molecular inhibitors against CD73 and CD39, APCP and ARL respectively, reduced adenosine concentration

significantly, revealing the blocking effect that these small molecular inhibitors exert in the ectoenzyme activity (fig. 10 A) (Häusler et al. 2011).

Adenosine production was also measured in primary OvCa cells isolated from fresh ascites (fig. 10, B). Addition of ARL and APCP drasticaly reduced the adenosine concentration measured in primary OvCa cells, demonstrating that the detected adenosine comes from the enzymatic activity of the ectoenzymes CD39 and CD73 (Häusler et al., 2011).

In a proliferation assay we could also show the immunosuppressive effect of adenosine in $CD4^+$ T cells (fig. 12). The adenosine generated by the ectoenzymes in OvCa cells that were in co-culture with activated $CD4^+$ T cells, significantly reduced the proliferation rate of $CD4^+$ T cells. When APCP, ARL and/or SCH58261 were added to the co-culture, the proliferation rate of the lymphocytes was restored to the control values.

Hence, these *in vitro* experiments prove the existence of an immunosuppressive mechanism which depends on the enzymatic activity of CD39 and CD73 as well as on the A2A receptor (Häusler et al. 2011).

Of note is the addition of serum containing adenosine deaminases to these assays. Adenosine deaminase is a ubiquitous enzyme *in vivo* and it rapidly degrades adenosine. In spite of the presence of adenosine deaminases, the half-life of free adenosine is enough to exert its immunosuppressive effects both *in vivo* and *in vitro*.

As already mentioned, macrophages are abundant in the tumor microenvironment and their presence is linked to a poor prognosis since they enhance tumor progression and metastasis (Deaglio et al., 2007; Resta et al., 1998). In the present study we wanted to elucidate the immunosuppressive relevance of adenosine produced by CD73 and CD39 with regard to these antigen presenters. Thus, we observed that adenosine generated by OvCa cells via CD39 and CD73 attracts myeloid precursor cells such as monocytes (fig. 13). Inhibition of adenosine production or signaling by addition of ARL67156 or APCP to the OvCa cells or by treatment of the monocytes with SCH58261 reduced the number of migrated monocytes. Thus, we could demonstrate that migration of monocytes towards OAW-42 cells is adenosine dependent.

Within the tumor microenvironment monocytes mature and differentiate towards an M2-phenotype (Hagemann et al., 2006) which expresses high levels of the adenosine-generating ectonucleotidases CD39 and CD73. This was verified in TAMs isolated from OvCa-ascites (fig. 16). We could simulate this polarization *in vitro* by co-culturing matured macrophages (from healthy volunteers) with OvCa cells for 48 hours in a transwell setting. After co-culture, the macrophage phenotype was confirmed by cytokine production and arginase activity. Thus, while the classically activated M1 macrophages (incubated with IFNγ and LPS) expressed high levels of IL-12 and low levels of IL-10, the *in vitro* modulated M2 macrophages showed the opposite cytokine profile, as well as significantly higher levels of CD73 and CD39 (fig. 14). Up-regulation of ectonucleotidases was confirmed at mRNA level (fig. 15). These findings were also proven to correlate with the *in vivo* situation (fig. 16). TAMs isolated from ascites of patients with OvCa revealed higher levels of ectoenzyme expression compared to those of monocytes and macrophages from healthy donors (fig. 16).

Further phenotypic characterization was done by measuring arginase activity. Macrophages use L-arginine to synthesize nitric oxide (NO) and polyamines through the inducible NO synthase (iNOS) and arginase, respectively. The released NO contributes to the tumoricidal activity of macrophages, whereas polyamines may promote the growth of tumor cells (Chang, Liao, & Kuo, 2001). In a colorimetric assay that measures the concentration of urea (final product of arginase activity) the *in vitro* polarized M2 macrophages revealed a significantly higher arginase activity compared to the M1 macrophages (fig. 17).

Once the M2 phenotype of the *in vitro* polarized macrophages was confirmed, their ability to produce adenosine was measured by using the already mentioned luciferase assay (fig. 18). The measured amounts of biologically active adenosine generated by the TAMs are considerably higher than the levels produced by OvCa cells (fig. 24). Furthermore, we could confirm their immunosuppressive effect on $CD4^+$ T cell proliferation (fig. 19). Proliferation levels of the lymphocytes were restored when CD39 or CD73 were blocked by ARL67156 or APCP respectively, confirming the immunosuppressive potential of the generated adenosine.

Taken together, we have shown that myeloid precursor cells developing into TAMs in the milieu of OvCa cells can be attracted by adenosine produced via CD39 and CD73 expressed on the cancer cells. Subsequently, TAMs upregulate their own CD39/CD73 expression with corresponding functional consequences (as shown by their suppression of $CD4^+$ T cell proliferation). We postulate that adenosine-dependent infiltration of OvCa cells with TAMs represents a positive feedback mechanism multiplying the adenosine-mediated immune escape of ovarian cancer. As TAMs are critical for the poor prognosis of ovarian cancer this could offer a chance to intefere.

Supporting this idea recent publications show that myeloid derived suppressor cells (MDSC) use CD73-mediated generation of adenosine as effector mechanism (Young et al., 2014). Of note, there is no clear distinction between MDSC and TAMs: some authors regard TAMs as differentiation product of MDSCs (Buira et al., 2010), while others claim that TAMs should not be considered as subset of MDSCs (Neufeld, Towner, & Pace, 1975). CD39, which is needed as first step in the ectonucleotidase-dependent conversion of ATP to adenosine seems to be expressed in a more tumor-specific pattern than CD73. Additionally, ATP (substrate for CD39) is an immunological danger signal responsible for an inflammatory activation of immune cell subtypes like dendritic cells or macrophages (Crack et al., 1995). Hence, only the co-expression of CD39 and CD73 enables the TAMs to transform the immunostimulatory ATP into the suppressive adenosine.

Taken together, the data obtained in our *in vitro* model system and from primary tumor tissues, strongly suggest that ovarian cancer cells are able to suppress the activity of various immune cell populations by modulating the adenosine concentration in the tumor microenvironment via the two ectonucleotidases CD39 and CD73, identifying them as a possible targets for novel immunomodulatory approaches.

In addition, we have demonstrated that CD73- and CD39-mediated immunosuppression may be promoted by chemotherapy. It is well known that the outcome of chemotherapy can be influenced by the host immune system (Zitvogel, Kepp, & Kroemer, 2011). Chemotherapeutics can kill cancer cells by stimulating a direct immune attack, or by increasing their sensitivity to an immune response. Furthermore, chemotherapeutics might have a direct effect on immune effectors or stimulate immunosuppressive

mechanisms. Apart from intrinsic factors that protect cancer cells against the cytotoxicity of chemotherapeutic compounds, the functional state of the host immune system needs to be taken into account; since it has a higher predictive value on the response of the patient to the given drug (Zitvogel et al., 2011). In fact, in a considerable range of tumors immunological parameters seem to be the strongest predictors of outcome (Galon et al., 2006.).

Lately, new light has been shed on the mechanism of action of anthracyclines. This may be helpful to clarify the mechanisms by which cancer cells gain resistance against chemotherapeutics. Several studies have revealed that anthracyclines can mediate their anti-tumor effect by direct cytotoxic action and also through activation of adaptative immune responses (Korkola et al., 2007) and thus, they induce an immunogenic cell death (Sorlie et al., 2003; Sotiriou et al., 2003).

One of the hallmarks of immunogenic cell death is the secretion of ATP (Ghiringhelli et al., 2009). This ATP release requires activation of caspases as well as the *pre-mortem* activation of autophagy. Thus, blockade of caspase activation or inhibition of autophagy prevents ATP release; however, caspase activation alone or stimulation of autophagy without cell death does not lead to extracellular ATP release. Presumably, both processes must occur sequentially (first autophagy and following caspase activation) to allow ATP to be released into the extracellular milieu of dying tumor cells (Martins et al., 2009; Michaud et al., 2012). Released ATP then activates the $P2X_7$ receptors on DCs and initiates a cascade of events which culminates in the production of IFN-γ–generating $CD8^+$ T cells. Due to its proinflammatory effects, extracellular levels of ATP are tightly controlled by CD73 and CD39 (Pawitan et al., 2005).

Interestingly, we observed that current therapeutic strategies, including doxorubicin, cisplatin or irradiation lead to an up-regulation of both CD39 and CD73 in OAW-42 cells (fig. 20). As we could confirm at mRNA level, the ectoenzymes were *de novo* synthetized, ruling out the possibility of an intracellular storage release (fig. 21). These findings are supported by a study conducted by Stagg et al., where they showed that anthracyclines like doxorubicin significantly up-regulate CD39 and CD73 in human breast cancer, melanoma and leukemia cells (Allard et al., 2014).

We hypothesized that this ectoenzyme up-regulation could be due to an induction of cell death with adenosine production as a mechanism to evade a subsequent immune response after treatment. To elucidate whether cell death was responsible for the enhancement of ectonucleotidase expression, OvCa cells were treated with classical inducers of apoptosis (fig. 22) for 24h. No significant up-regulation of the ectoenzymes was measured after treatment with staurosporin or Fas-ligand, thereby discarding the hypothesis of an apoptosis linked up-regulation, and leading us to the idea that the up-regulation occurs in living cells as a response a stress stimuli.

To determine whether the treated OvCa cells up-regulate ectonucleotidase expression autonomously in response to the cytotoxic insult, we pulse-treated OAW-42 cells with doxorubicin, washed them thoroughly with PBS and brought them together with untreated and stained (cell proliferation dye eFluor670®) OAW-42 cells. In FACS analyses the expression of CD73 was determined for eFluor670®-positive and -negative cancer cells. In both subpopulations CD73 levels were highly increased, which means that also cells which had not been in contact with doxorubicin strongly induced CD73. We hence conclude that the treated (stressed) cells send out “danger”-signals to the microenvironment where CD73 expression is enhanced (fig. 23).

To investigate if the treatment-induced ectonucleotidase up-regulation also enhances the immunmodulatory potential of the cancer cells, we analyzed adenosine production in the already mentioned luciferase-dependent adenosine assay. Compared to untreated control-OAW-42, stressed cells showed significantly higher adenosine production (fig. 24).

To test the functional properties of the increased adenosine production we again performed co-culture experiments with CFSE-labeled $CD4^+$ T cells. In comparison to OAW-42 cells which had not been exposed to doxorubicin before, we detected significantly stronger proliferation inhibition in co-incubation with OAW-42 cells that had been exposed to doxorubicin before. This inhibition of T cell proliferation was overruled when small molecule inhibitors for CD39 or CD73 (ARL67156 and APCP) were applied, which confirms the dependence of the determined effect on CD39/CD73 (fig. 25).

Interestingly, when macrophages isolated from healthy donors were pulse-treated with doxorubicin no up-regulation of the ectoenzymes was observed (fig. 26), whereas co-culture with pulse-treated OvCa cells could induce ectonucleotidase expression on healthy macrophages. This observation is in line with the soluble factor hypothesis and suggests that mainly cancer cells can secrete it upon treatment.

As already mentioned, cancer cells release ATP as a response to stress and thereby stimulate an immune response. It occurred to us that OvCa cells might respond to the treatment by secreting ATP which could in turn be degraded to adenosine by the activity of CD39 and CD73. Thus, an increased ATP concentration in the microenvironment would require a higher enzymatic activity, and therefore OvCa cells may overcome this situation by up-regulating the ectoenzymes. However, this does not seem to be the right mechanism since direct treatment of OvCa cells with ATP did not induce an up-regulation of ectoenzyme expression (fig. 27).

TGF-β has been reported to induce CD73 expression in lymphocytes (Regateiro et al., 2011) and therefore we wondered whether doxorubicin-triggered induction of CD73 on OvCa cells was due to TGF-β release. While a TGF-β activity assay with CCL-64 mink lung cells (Mazzieri et al., 2000) showed a highly significant release of TGF-β upon treatment with doxorubicin (fig. 28), treatment with recombinant TGF-β did not significantly enhance CD73 expression. Still, adenosine production was partly attenuated by addition of the TGF-β receptor inhibitor SD-208 (fig. 28). This result suggests that upon treatment with doxorubicin an unkown factor enhances ectoenzyme expression together with TGF-β.

Further research needs to be done to identify the soluble factor responsible for ectoenzyme up-regulation upon chemotherapeutic treatment. First step towards identification of that factor could be size-exclusion centrifugation; using different pore size filters during centrifugation, the fraction containing the factor could be identified. Subsequently, assuming that the mentioned factor is a protein, a two dimensional SDS-page should be done. Once the proteins are properly separated in the gel, a comparison between the spots in the gel coming from treated cells with the non-treated cells should reduce the number of possible candidates. Finally, applying recombinant proteins of the identified spots would reveal the identity of the molecule.

We considered the possibility that the factor which is released by treated cancer cells could reside in exosomes. Exosomes are nanovesicles (30–120 nm) of endocytic origin produced and released by many different cell types, such as dendritic cells (DC), B cells, T cells, mast cells, epithelial cells and tumor cells among others (Escola et al., 1998; Johnstone, Mathew, Mason, & Teng, 1991; Morelli et al., 2004). These vesicles have been detected in different body fluids such as peripheral blood, urine, malignant effusions and bronchoalveolar lavage fluids. In addition, exosomes have been shown to be involved in signal transduction, antigen presentation to T cells and tolerance development (Azmi, Bao, & Sarkar, 2013; Mathivanan, Ji, & Simpson, 2010; Vlassov, Magdaleno, Setterquist, & Conrad, 2012).

In an approach to elucidate whether the unknown factor responsible for the ectoenzyme up-regulation upon doxorubicin treatment is released in exosomes by OvCa cells, we isolated exosomes from serum-free supernantant of doxorubicin- treated tumor cells. Different techniques were used in this attempt: ultracentrifugation, dynabead® conjugation and exoquick® exosome isolation system. After isolation the purity of the exosome content was verified by the presence of several exosome markers (CD63, CD81). Unfortunately, no pure exosome sample was obtained making further results inconclusive. New isolation protocols would need to be established for obtaining pure exosome pellets.

The results obtained in this work should also be confirmed in *vivo*. Our lab has already established a fully immunocompetent OvCa mouse model (Dinulescu et al., 2005). For tumor induction, an adenoviral Cre-recombinase is injected in the ovarian bursa of 129 S4/SvJae-C57BL/6J loxP-*Stop*-loxP-K-ras$^{G12D/+}$ Pten$^{loxP/loxP}$ mice which inactivates the tumor suppressor protein PTEN and activates a latent oncogenic K-ras. This results in the clinical development of endometrioid ovarian cancer within 7-8 weeks. Ascites formation, localization of metastases and even histomorphological aspects like infiltration with immune cells or molecular findings are highly similar to the human counterpart. The presence of a functional immune system makes this model particularly suitable to investigate immunological characteristics of ovarian cancer that cannot be recapitulated in athymic nude mice.

Recent studies conducted by Stagg et al. in an immune competent breast cancel model, revealed that CD73 is a critical factor in tumorigenesis. Immunological depletion experiments showed that therapeutic potential of CD73 depletion was dependent on $CD8^+$ T cells.

Moreover, Jin et al. showed that CD73-generated adenosine can prevent tumor eradication by suppressing antitumor immunity. Their experiments revealed that a combination of tumor CD73 knockdown and tumor-specific T-cell transfer healed all tumor-bearing mice, whereas, mice bearing tumors without CD73 knockdown, did not show any therapeutical improvement after the adoptive T-cell immunotherapy. Furthermore, inhibition of the A2A receptor with SCH58261 increased the efficacy of adoptive T-cell therapy.

This results corroborate the relevance of CD73 activity in an immune-competent mouse model and reveal that the functional implication of the ectoenzymes CD39 and CD73 cannot be determined in immune-deficient xenograft models.

We could confirm *in vitro* the effect of CD73 expressed in OvCa cells. Moreover, our experiments revealed that CD39 shows a more tumor-specific pattern than CD73 (Fig. 7) (Mandapathil et al., 2010; Mandapathil, Lang, Gorelik, & Whiteside, 2009), and therefore the targeting of CD39 or both ectonucleotidases might be beneficial. Inhbition of CD39 may detain extracellular ATP degradation and therefore, ATP accumulation could act as an immunological danger signal preceding to an inflammatory response, recruiting antigen presenting cells and (Elliott et al., 2009) and inducing their maturation. In addition, the hypoxic conditions in the tumor microenvironment promote ATP release from necrotic cells (Shin et al. 2009) as well as induce expression of CD39 and CD73 (Eltzschig et al., 2003). Thus, anti-tumoral immune responses could be considerably enhanced by inhibition of CD39 (Häusler et al 2011).

In addition, we have now shown that current therapeutic treatments against OvCa enhance expression of the ectoenzymes by the release of a yet to be discovered soluble factor. Thus, administration of CD73 and CD39 inhibitors as adjuvants to chemotherapeutics could substantially improve the immune-response against the tumor.

In concordance with our results, studies conducted by Guido Kroemer revealed that CD39 expression can abolish the immune-dependent anticancer effects of anthracyclines *in vivo*. They attributed this immunosuppressive effect of CD39 to its ATP-degrading enzymatic activity, since intratumoral injection of the pharmacological ecto-ATPase inhibitor, ARL67156, re-established the efficacy of anthracycline therapy (Mickaël et al., 2012).

Of note is that the small molecule inhibitors used in our experiments are available and have been shown to be fully functional *in vivo* (see table 1, pg. 50) being able to inhibit the function of CD39 (Enjyoji et al., 2008), CD73 (Synnestvedt et al., 2002) and the A2A receptor (Ohta et al., 2006). Furthermore, Tanganelli et al. clinically tested the inhibitor SCH58261 in Parkinson's disease (Tanganelli et al., 2004). Hence, application of the small molecular inhibitors in OvCa patients may no imply major difficulties, since they already shown their therapeutical efficacy in different OvCa models *in vivo* (Häusler et al., 2011). Moreover, we have shown that specific antibodies against CD39 (clone A1) or CD73 (clone 7G2) could be an alternative to the small molecule inhibitors (fig. 11). Therapeutic antibodies offer several advantages such as, good biosafety profile and facilitate furthere ffector mechanisms like antibody dependent cellular cytotoxicity (ADCC) (Hellstrom, Garrigues, Lavie, & Hellstrom, 1988). ADCC derived from the use of antibodies against CD39 and/or CD73 may imply important advantages *in vivo,* as it happened with Trastuzumab in the treatment of HER2-positive breast cancer (Baselga et al., 1996; Gampenrieder, Rinnerthaler, & Greil, 2013; Reim et al., 2009). Furthermore, it has been already shown that antibodies against CD73 can activate immune responses against breast cancer cells (Stagg et al., 2010). In adition, experiments performed by Terp et al., have rececently revealed that an anti-human CD73 monoclonal antibody can inhibit metastasis formation in human breast cancer by inducing clustering and internalization of CD73 (Terp et al., 2013). Since the effect of the mentioned antibody is associated with internalization, ingeniering an antibody drug conjugate may be a very interesting and specific therapeutic approach (Häusler et al., 2014). Besides, thanks to the new antibody engineering techniques, further functionalities can be added to antibodies, such as enhancement of ADCC or improvement of anti-cancer specific targeting (Häusler et al., 2014). Inconveniences for the use of antibodies instead of the small molecular inhibitors are on one hand the high value and presumably a limited

tissue penetration. Despite the fact that CD73 and CD39 are not tumor specific, until now, no *in vivo* studies have reported side effects for the use of antibodies against the mentioned targets (Häusler et al., 2014). In addition, the multiple immune regulatory effects of adenosine (Koshiba et al., 1997) were reported to be associated with the suppressive function of Treg cells (Borsellino et al., 2007; Deaglio et al., 2007).

Regarding that OvCa recruits Tregs and that penetration of these cells has been strongly linked with a poor prognosis (Curiel, Coukos, et al., 2004), targeting of Treg with antibodies against CD39 and CD73 may lead to a favourable outcome (Häusler et al., 2014). Indeed, new CD39 based strategies with the objective of funtionally inhibiting Treg are being developed (Bastid et al., 2013). Furthermore, a growing number of targeted anti-cancer therapies is starting to offer multiple possibilities for rational combinations. Interestingly, different studies reveal that blockade of CD39 and CD73 improve the survival rates of cells in hypoxic areas: the release of ATP from necrotic cells act as an immunological danger signal but can also exert direct cytotoxic effects on tumor cells (Mukhopadhyay & Datta, 2004; J. Shi, Wan, & Di, 2008). Thus, inhibition of ATP hydrolysis by blocade of CD39 and CD73 may increment hypoxic stress on cancer cells. In hypoxic conditions OvCa cells have been reported to secret pro-angiogenic cytokine vascular endothelial growth factor (VEGF) (Olson, Mohanraj, Carson, & Ramakrishnan, 1994). Furthermore, four different phase III trials ICON7 (Perren et al., 2011), GOG-0128 (Burger et al., 2011), OCEANS (Aghajanian et al., 2012) and AURELIA (Pujade-Lauraine et al., 2014) have proven that a therapy with Bevacizumab (a humanized antibody that blokcs VEGF), is beneficial both for the adjuvant as well as for the palliative treatment of ovarian cancer. Thus, the combination of Bevacizumab with antibodies that block the function of CD39 and CD73 may lead to synergistic effects by reducing tumor vascularisation and by lowering the capacity of tumor cells to bear hypoxic conditions (Häusler et al., 2014).

Our study provides strong evidence that immunosuppression mediated by CD73 and CD39 suppresses anthracycline efficacy. Ectoenzyme blockade may act synergistically with anthracyclines; furthermore, we have proven that this effect is not specific to anthrayclines as observed with other chemotherapeutic agents (fig. 20). Taking these data together, we propose that CD73 and CD39 together with their downstream effector

A2A adenosine receptor could represent unique therapeutic targets in patients with ovarian carcinoma, currently featured by an absence of specific treatments.

In addition, the still unknown soluble factor responsible for ectoenzyme up-regulation upon chemotherapeutic treatment, might represent a further target. Administering an inhibitor of this factor as an adjuvant to conventional chemotherapeutic regimes could enhance an anti-tumor immune response. The suitability of this approach can, however, only be determined once the identity and hence the tumor specificity of the respective factor has been identified.

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6. ABBREVIATIONS AND ACRONYMS

°C	Celsius
µg	microgram
µl	microlitre
µM	Micromolar
A1R	A1 Adenosine receptor
A2AR	Adenosine receptor A2A
A2BR	Adenosine receptor A2B
A3R	A3 Adenosine receptor
aa	amino acid
ADA	Adenosine deaminase
ADCC	Antibody-dependent cellular cytotoxicity
AdCre	Adenovirus encoded Cre-recombinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	base pair
BSA	Bovines serum albumin
CA 125	Cancer antigen 125
CA-12	Cancer antigen 12
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
CECR1	Cat eye syndrome critical region protein 1
cm^2	square centimeters
CMP	Citosine monophosphate
CNS	Central nervous system
CO_2	Carbon dioxide

CoA	Coenzyme A
CT	Computed axial tomography
C_T	Cycle threshold
CTL	Cytotoxic T cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCR 6	Chemokine receptor 6
DC	Dendritic cell
ddH_2O	Double-distilled water
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
Doxo	Doxorubicine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EOC	Epithelial Ovarian Cancer
EpCAM	Epithelial cell adhesion molecule
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FAD	Flavine adenine dinucleotide
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
FLuc	Firefly luciferase
Foxp3	Forkhead box P3
GMP	Guanosine monophosphate
GPCR	G protein coupled receptor
Gy	Gray
h	Hour
Her2/neu	Receptor tyrosine-protein kinase erbB-2
HIF1-α	Hipoxia inducible factor 1 α
HLA	Human leukocyte antigen

HRP	Horseradish peroxidase
HSA	Human serum albumin
hTERT	Telomerase reverse transcriptase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IRF-1	Interferon regulatory factor 1
kDA	kiloDalton
KIR	Killer cell immunoglobulin-like receptor
LAK	Lymphokine-activated killer
LPS	Lipopolysaccharides
MACS	Magnetic-activated cell sorting
MAGE	Melanoma antigen E
MDSC	Myeloid-derived suppressor cells
mg	Milligram
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence A
min	Minute
MIP-1	Macrophage inflammatory protein 1
ml	Milliliter
MMLV	Mouse moloney leukemia virus
mRNA	Messenger RNA
MUC1	Mucin 1
NADH	Nicotinamide adenine dinucleotide
NECA	5’-N-ethylcarboxamidoadenosine
NK cells	Natural killer cells
NKG2D	Natural killer group 2D
nm	nanometer
nM	nanomolar

NTPDase	Ectonucleoside triphosphate diphosphohydrolase
NY-ESO-1	Cancer-testis antigen
OA3	Ovarian antigen 3
OvCa	Ovarian cancer
PBL	Peripheral blood lymphocytes
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered Saline
PBS-T	Phosphate-buffered saline with 0.05% Tween20
PCR	Polymerase chain reaction
PD-L1	Programmed death-ligand 1
(R-)PE	R-phycoerythrin
PFA	Paraformaldehyde
PFS	Progression free survival
PLC	Phospholipase C
PVDF	Polyvinylidene Fluoride Membrane
qRT-PCR	(semi-)quantitative real-time PCR
RanBPM	Ran-binding protein M
rpm	Revolutions per minute
RPMI1640	Roswell Park Memorial Institute cell culture medium 1640
RQ	Relative quantification
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SCID	Severe combined immunodeficiency Syndrome
SDS-PAGE	Sodium dodecylsulfate (SDS) - Polyacrylamide Gel Electrophoresis
SFI	Specific fluorescence intensity
STIC	Serous tubal intraepithelial carcinoma
TAG-72	Tumor-associated glycoprotein 72

TAM	Tumor-associated macrophages
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
Th	Helper T cells
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T cells
UMP	Uridinmonophosphate
UV	Ultraviolet
V	Volt
VCAM-1	Vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor
ZAP-70	Zeta-chain-associated protein kinase 70