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ATP, HMGB1, and S100A4 promote immunosuppressive mesenchymal stromal cells by enhancing their kynurenine production. Impact of necrosis on tumor-associated MSCs

Dissertation

Submitted to the Medical Faculty of the University of Ulm to obtain the degree of

Doctor of Medicine

Presented by

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2017

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Day of Grant of Doctorate:

February 15th, 2019

I dedicate this piece of work
to my loving parents, who,
by selfless support in all possible ways,
granted me to take on every chance in my life.
Thank you.

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List of Abbreviations

α-MEM	alpha- modified minimal essential medium
hABS	human AB-serum
ATP	adenosine triphosphate
CFSE	carboxyflourescein succinimidyl ester
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sufoxide
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FSC	forward scatter
F/T	freezing with subsequent thawing
HCl	hydrochloric acid
HCT-116	human colon tumorline 116
HMGB1	high mobility box 1
IC	isotype control
IDO	indolamine 2,3-dioxygenase
IgG	immunoglobulin G
IFN-γ	interferone gamma
MFI	mean fluorescence intensity
MSC	mesenchymal stromal cell
NaCl	sodium chloride
NaOH	sodium hydroxide
PB(M)C	peripheral blood (mononuclear) cells
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PL	platelet lysate
RAGE	receptor for advanced glycation end products
TCA	trichloroacetic acid
TLR	toll-like receptor
WST	water-soluble tetrazolium

1 Introduction

1.1 Tumor Microenvironment

A hallmark, intrinsically tied to solid tumors is their specific microenvironment (Pietras & Östmann, 2010), characterized by chronic inflammation and necrosis (Vakkila & Lotze, 2004; Lotfi et al., 2007; Lotfi et al., 2009).

Reaching a critical point in cell mass, the tumor's nutrient supply becomes insufficient. Following cell disruption, starting from the tumor mass's core, necrotic material is released into the extracellular matrix. Furthermore host's cytotoxic immune response to the tumor and down regulation of programmed (apoptotic) cell death by the tumor itself (Hirst, 1986) lead to further perpetuation of necrotic cell death. These liberated intracellular factors, acting as danger signals to ambient tissue and immune cells (Chung et al., 2003; Medzhitov & Janeway, 2000) are also referred to as damage associated molecular patterns (DAMPs).

In addition to DAMPs, communication in tumor microenvironment is performed via cytokines, chemokines, and soluble receptor ligands (Mantovani et al., 2008). Embedded in extracellular matrix, cell populations from different site of origin are found alongside tumor tissue. There are healthy residential tissue cells like various populations of stromal cells (e.g. fibroblasts, mesenchyme stromal cells (MSCs), and tissue based leukocytes (e.g. macrophages and dendritic cells), but also migrated leukocytes, fibroblasts, and MSCs (Grivennikov et al., 2010) responding to local signals such as chemokines, cytokines, and DAMPs, which induce their chemotaxis to the tumor site. The prevalent tumor milieu activates the wound healing mechanisms - like angiogenesis (Thijssen et al., 2004) and immunosuppression (Mollen et al., 2006). Thus neoplastic cells harness the regenerative capacities of their host for their own survival, proliferation, malignant transformation and metastasis. Immunosuppression protects the tumor from being attacked by immune cells and promotes its spreading on the one hand, but renders the tumor totally dependent on its microenvironment (Vakkila & Lotze, 2004; Pietras & Östmann, 2010), on the other hand. A tumor's reliance on its microenvironment *in vivo* can be furthermore observed when considering the difficulty to cultivate tumor cells *ex vivo*, even though presumably optimal conditions are provided *in vitro* (Whitescarver, 1974). Moreover *in vivo* assays of chemotherapeutics poorly recapitulate *ex vivo* effectiveness

(O Trédan, 2007), thus emphasizing the importance of detailed knowledge about tumor microenvironment. Of note, this complex tumor microenvironment is not static, but alters during solid tumor's progression in growth (Sung et al., 2008). Bioactive factors responsible for these effects and for tumor survival need to be better understood. Therefore this field opens new therapeutic options aiming to target and modulate this microenvironment.

1.2 Damage Associated Molecular Patterns (DAMPs)

Primary intracellular factors (cytosolic or nuclear) released into extracellular space due to necrotic cell disruption are referred to as DAMPs (Lotze et al., 2007). Some of their representatives occur to be sensitive to oxidation and hence prone to alter their bioactivity (Lotfi et al., 2011).

1.2.1 S100 A4

S100 A4 is a DAMP member belonging to the family of S100-calcium-binding proteins, comprising at least 21 different S100 molecules. S100 A4 is localized in cytoplasm and nuclei of a wide range of cells. It takes a major part in tumor progression by the stimulation of angiogenesis (Semov et al., 2005) and promotion of metastasis (Garret et al., 2006). It furthermore perpetuates stromal and mesenchymal cell migration (Lotfi et al., 2011). Besides conditions in cancer, elevated levels of S100-proteins can be detected during various inflammatory conditions and trauma (Schneider et al., 2008).

Being sensitive to oxidation, its biological activity depends on reducing conditions – as found within tumor microenvironment (Eisenbacher et al., 2014). Various intra- and extracellular interactions have been reported to be associated with S100-proteins binding to the receptor for advanced glycation end products (RAGE) and Toll-Like-Receptors (TLR) (Fatatis, 2012; Yammani et al., 2006).

1.2.2 HMGB1

High mobility group box 1 protein (HMGB1) is found mainly in the cell nucleus, where it physiologically interacts with nucleosomes, transcription factors and histones (Agresti & Bianchi, 2003). It was also shown that it is secreted by activated monocytes and macrophages during inflammatory processes (Wang et al., 1999). As a prototypic DAMP member, HMGB1 is released upon necrotic cell death (Scafidi et al., 2002). High levels of HMGB1 are found in diverse autoimmune diseases, like systemic lupus erythematous or rheumatoid arthritis (Pistesky et al., 2008).

To maintain its biologic activity, reducing conditions are required, as HMGB1 is known to be sensitive to oxidation (Lotfi et al., 2011). Relevant receptors to carry out it function as an intercellular messenger are notably RAGE (Kokkola et al., 2005), TLR-2 and TLR-4 (Sung Park et al., 2004).

1.2.3 ATP

ATP is well known for its role as universal and direct deliverer of chemically bound energy to the cell, but this coenzyme can also act as signaling molecule as recognized by purinergic receptors (Wiley et al., 2011). A large increase in extracellular levels implicates necrotic cell death (Trautmann, 2009). Thus, about thousand times higher ATP concentrations (e.g. 100 μM) are found within tumor microenvironment when compared to normal tissue (Pellegatti et al., 2008; Falzoni et al., 2013). Like most immunomodulating agents ATP can either act as an immunosuppressive or an immunostimulatory factor, depending on the cytokine microenvironment and the type of cell receptor.

Of note the P2X7 receptor, which is a ligand gated ion channel mainly responding to ATP, is highly expressed on immune cells (Wiley et al., 2011). It has emerged to be a central player in the pathophysiology of several skeletal diseases such as osteoporosis, osteoarthritis and rheumatoid arthritis (Jørgensen et al., 2013). The P2X7 receptor is also overexpressed in most malignant tumors (Di Virgilio et al., 2009).

1.2.4 Uric Acid

In humans uric acid is the physiological final breakdown product of purine metabolism, excreted in urine. Katabolic metabolism schemes and high cell turnover come along with elevated concentrations of uric acid in serum. Local accumulations can also be found following cell injury and proliferation or neoplastic transformation, thus functioning as DAMP (Shi et al., 2003).

Compared to other potential non-enzymatic antioxidants, its plasma level is rather high within humans, constituting around 30-60% of the peroxyl radical-scavenging capacity (Becker, 1992). Operating as antioxidant uric acid possibly protects instable DAMPs from oxidation and thus inactivation, prolonging their biologically active state.

1.3 Mesenchymal Stromal Cells (MSCs)

1.3.1 Regenerative Potential of MSCs

MSCs reside in several tissues, being most common in connective tissue and bone marrow, but also identified in umbilical cord blood, amniotic fluid, placenta and parenchymal organs. They are known for their multipotential capacity, namely being able to differentiate into bone, cartilage, adipose tissue, tendon, and muscle *in vitro* (Bernardo et al., 2009).

Tissue injury not only activates local MSCs, but also induces trafficking of MSCs from bone marrow to the site of injury (Yagi et al., 2010). To provide conditions necessary for a regenerative microenvironment, MSCs secrete a broad spectrum of bioactive molecules (Caplan, 2007) that suppress the local immune response, fibrosis and apoptosis. Concurrently MSC-released factors enhance angiogenesis, and stimulate fibroblast mitosis and differentiation. With such abilities they play a crucial role in the field of tissue engineering and regenerative medicine.

1.3.2 Immunosuppressive Capacities of MSCs

Another important aspect concerning MSCs encompasses their ability to modulate immune response. As mentioned above, immunosuppression can be carried out via release of soluble factors. Tissue growth factor β (TGF-β), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), indoleamine-2,3-dioxygenase (IDO), nitric oxide, and interleukin (IL)-10 are ascribed to inhibit T-Cell proliferation (Bernardo et al., 2009). There is also evidence for B-Lymphocyte (Corcione et. al., 2006), dendritic-cell (Jiang et al., 2005) and natural-killer-cell suppression by MSCs (Sotiropoulou et al., 2006).

1.3.3 Interactions in Tumor Milieu

MSCs are found within solid tumors (Yagi et al., 2010; Hall et al., 2006) and contribute to cancer progression and metastasis (Spaeth et al., 2009; Karnoub et al., 2007), probably due to their contribution to reduced immune response. RAGE, TLR-4 (Lotfi et al., 2011, Kume et al., 2005; Yagi et al., 2010) and P2X7 (Ferrari et al., 2011) are expressed on MSCs surface, thus rendering them sensitive to many DAMPs within the tumor's surrounding. Previous studies of ours show DAMPs prompting migration and proliferation of MCSs (Lotfi et al., 2011).

1.4 Indolamine 2,3-Dioxygenase (IDO)

Indoleamine-2,3-dioxygenase (IDO) converts the essential amino acid tryptophan to kynurenine. The depletion of tryptophan and subsequent production of kynurenine by MSCs inhibits T-cell proliferation and activation (Li et al., 2009; Di Nicola et al., 2002). Yet in the 1950s, multiple investigators published observations pointing at an altered tryptophan metabolism (production of kynurenine) in pathologic states of the human body associated with inflammation (Spacek, 1955; Musajo et al., 1956).
MSCs constitutively express IDO; however, when stimulated with IFN-γ, which is generally found within inflammatory tissue, IDO expression is significantly enhanced (Meisel et al., 2004; Krampera et al., 2006).

1.5 Aims

MSCs play a major role in tumor milieu evolution, resulting in poor prognosis due to metastasis and cancer survival (Spaeth et al., 2009; Karnoub et al., 2007). It is important to learn how MSC realize their role as immune modulators.

A promising candidate mechanism of action in this concern is the tryptophan breakdown via IDO, as this enzyme is upregulated in MSCs within inflammatory milieu (Meisel et al., 2004). Furthermore the ratio of tryptophan/ kynurenine crucially inhibits T-cell proliferation (Di Nicola et al., 2002).

This study shall characterize some members of the DAMP family, which are capable of influencing the levels of kynurenine and IDO in MSCs.

Besides S100A4, HMGB1 and uric acid, which we have already been demonstrated to influence MSC migration and proliferation (Lotfi et al., 2011), we examine ATP, because of reported involvement of the P2X7-receptor in migration of MSCs to the site of necrotic cell death (Ferrari et al., 2011).

2 Materials and Methods

2.1 Materials

2.1.1 Devices

Heraeus Laminair	Haeraeus, Hanau, Germany
Biofuge A Haeraeus™ Sepatech	Haeraeus, Hanau, Germany
Eppendorf® Centrifuge 5810R	Eppendorf, Hamburg, Germany
FACScan™	Becton Dickinson, Heidelberg, Germany
POLARstar® Omega	BMG Labtech, Ortenberg, Germany
Incubator for cell cultures	Binder, Tuttlingen, Germany
Incubator (water bath)	Köttermann Labortechnik, Uetze, Germany
Microscope Carl Zeiss 4654140	Carl Zeiss, Oberkochen, Germany
Vortex Genie® 2	Scientific Industries, Bohemia, New York

2.1.2 Consumables

Falcon® 15ml, 50ml	Becton Dickinson, Heidelberg, Germany
Falcon® 5ml polystyrene round bottom tubes	Becton Dickinson, Heidelberg, Germany
Haemacytometer (Neubauer chamber)	Precicolor HBG, Gießen, Germany
Eppendorf T.I.P.S. Reload 50-1000µl, 20-300µl	Eppendorf, Hamburg, Germany
Pipette Tips 10µl, 100µl, 1250µl	Biotix, San Diego, USA
Nunclon Delta Surface 96-Well U-Bottom	Nunc, Wiesbaden, Germany
Cell culture flasks T175	Thermo Fisher Scientific, Dreieich, Germany

Cellstar Tubes 50ml, 10ml	Greiner Bio-One, Frickenhausen, Germany
Cellstar 96-Well F-Bottom	Greiner Bio-One, Frickenhausen, Germany
Minisart single use filter 0,2µm	Sartorius Stedim Biotech, Göttingen, Germany

2.1.3 Reagents

Acetic Acid 99%	Sigma-Aldrich Chemie, Sleeze, Germany
Adenosine 5'-triphosphate disodium salt	Sigma-Aldrich Chemie, Sleeze, Germany
alpha-modified minimal essential medium	Lonza, Cologne, Germany
Ampuwa™ distilled sterile water	Fresenius Kabi, Bad Homburg, Germany
Anti-(HLA-DR, DP, DQ)-FITC	Becton Dickinson, Heidelberg, Germany
Anti-(HLA-A, B, C)-PE	Becton Dickinson, Heidelberg, Germany
Anti-human CD3-PerCP	Becton Dickinson, Heidelberg, Germany
Anti-human CD34-PE	Becton Dickinson, Heidelberg, Germany
Anti-human CD45-PerCP	Becton Dickinson, Heidelberg, Germany
Anti-human CD73-PE	Becton Dickinson, Heidelberg, Germany
Anti-human CD90-FITC	Becton Dickinson, Heidelberg, Germany
Anti-human CD105-FITC	Becton Dickinson, Heidelberg, Germany
Anti-human IDO-AF 488	R&D Systems, Wiesbaden, Germany
Anti-human IgG-FITC	Becton Dickinson, Heidelberg, Germany
Anti-human IgG-PE	Becton Dickinson, Heidelberg, Germany
Anti-human IgG-PerCP	Becton Dickinson, Heidelberg, Germany

Anti-human IgG1-AF 488 Mouse	R&D Systems, Wiesbaden, Germany
Mouse IgG1 Fluorescein Conjugated	R&D Systems, Wiesbaden, Germany
Apyrase	Sigma-Aldrich Chemie, Sleeze, Germany
Biocoll™ Separating Solution	Biochrom, Berlin, Germany
CellTrace™ (CFSE)	Life Technologies, Darmstadt, Germany
CyQuant™	Life Technologies, Darmstadt, Germany
Cytoperm/Cytofix™	BD Bioscience, Heidelberg, Germany
4-(Dimethylamino)benzaldehyde 99+%	Acros Organics, Farmstadt, Germany
Dimethyl Sufoxide	Wak-Chemie, Steinbach, Germany
Dulbecco's Phosphate Buffered Saline (DPBS) w/o Ca2+ & w/o Mg2+	Life Technologies, Darmstadt, Germany
Dulbecco's Modified Eagle Medium	Life Technologies, Darmstadt, Germany
DynabeadsTM Human T-Activator CD3/CD28	Thermo Fischer Scientific, Karlsruhe, Germany
EDTA	Sigma-Aldrich Chemie, Sleeze, Germany
HCT-116	DSMZ, Braunschweig, Germany
Human Albumin Solution 20	German Red Cross Blood Service Baden-Wuerttemberg, Germany
Human Serum, Blood Group AB	German Red Cross Blood Service Baden-Wuerttemberg, Germany
Hydrochloric Acid	AppliChem, Darmstadt, Germany
Interferone-γ	R&D Systems, Wiesbaden, USA
Kynurenine	Sigma-Aldrich Chemie, Sleeze, Germany
Perm Wash™	BD Bioscience, Heidelberg, Germany

Phosphate Buffered Saline	Lonza, Cologne, Germany
Platelet Lysate	German Red Cross Blood Service Baden-Wuerttemberg, Germany
Recombinant Human HMGB1	R&D Systems, Wiesbaden, Germany
Recombinant Human S100A4	R&D Systems, Wiesbaden, Germany
Saline (NaCl)	Sigma-Aldrich Chemie, Sleeze, Germany
Sodium Hydroxide (NaOH)	Sigma-Aldrich Chemie, Sleeze, Germany
Trichloroacetic Acid	Sigma-Aldrich Chemie, Sleeze, Germany
L-Tryptophan, non-animal source	Sigma-Aldrich Chemie, Sleeze, Germany
Trypan Blue Solution	Sigma-Aldrich Chemie, Sleeze, Germany
Trypsin (2.5%)	Life Technologies, Darmstadt, Germany
Uric Acid	Sigma-Aldrich Chemie, Sleeze, Germany
WST-1	Roche Diagnositics, Mannheim, Germany

2.1.4 Software

CellQuest™ 3.1 Software	Data acquisition (FACScan™)
MARS™ data analysis software (BMG Labtech)	Data analysis (POLARstar™ Omega)
Microsoft™ Office Excel 2003 and Microsoft™ Office Excel for MAC 2011	Graphics
Microsoft™ Office Word for MAC 2011	Transcription
Summit™ v4.2	Data analysis (FACS data analysis)

2.2 Methods

2.2.1 Experimental Assembly

2.2.1.1 Isolation and Characterization of MSCs

Heparinized bone marrow was taken from healthy volunteer donors after informed consent and was cultured in alpha-modified minimal essential media (α-MEM) supplemented with 10% human platelet lysate (PL) and 100 U/ml penicillin, and 100 µg/ml streptomycin according to published protocols (Kocaoemer und et. al. 2007), (Bernardo, et al. 2009). Only MSCs from passage 1 to 3 were used. MSCs were characterized based on surface antigen expression analyzed by flow cytometry using FACScan with CellQuest 3.1 software. MSCs ($1x10^5$ cells) were stained in phosphate buffered saline (PBS) for 15 minutes at room temperature applying the following antibodies according to the manufacturer's recommendations: CD3-PerCP, CD34-PE, CD45-PerCP, CD73-PE, CD90-FITC, CD105-FITC, (HLA- DR, DP, DQ)-FITC, (HLA-A, B, C)-PE and IgG conjugated with FITC, PE, or PerCP, respectively. Bone marrow-derived adherent cells which were positive for expression of CD73, CD90, CD105 and (HLA-A, B, C) and negative for expression of CD3, CD34, CD45 and (HLA-DR, DP, DQ) were considered as MSCs.

2.2.1.2 Standard Cell Culture

Media used for isolation and primary expansion of MSCs (see above) was replaced by our standard cell culturing media consisting of glucose rich DMEM containing 10% human AB-Serum (hABS). Before starting the experiments, MSCs were cultured for at least 5 days in the standard cell culturing media to acclimatize their metabolism to the conditions with no significant levels of DAMPs, given the high DAMP concentrations in media containing PL. With confluence reaching not more than 80%, adherent cells were harvested and passaged by applying trypsin and EDTA (ratio 10:1). Only MSCs from passage 1 to 3 were used. For all experimental settings, MSC were planted in 96-well plates ($15x10^3$ MSCs per well) and cultured for 4 days in tryptophan-supplemented medium of 100 µg/ml. To trigger IDO expression IFN-γ was added at (100 ng/ml). With the application of different DAMP-members the various experimental settings were created.

2.2.1.3 Preparation of Reagents and Cells

2.2.1.3.1 Obtaining Tumor Cell Lysate

The human colorectal tumor cell line HCT-116 was cultured according to our standard culturing conditions in DMEM plus hABS (10%). For further treatment, cells were harvested by applying EDTA-trypsin composition. Cells were washed and resuspended in sterile PBS at concentrations of 10^7 to 10^8 cells/ml and lysed by 4 cycles of freeze with subsequent thawing (F/T). The viability following F/T treatment was assessed using trypan blue exclusion and was always below 0.1%.

2.2.1.3.2 Preparation of PBMCs and PBLs

Following informed consent, whole blood, drawn from healthy donors was diluted in equal parts with PBS. About 15ml Biocoll™ Separating Solution was overlayed with 30ml of diluted whole blood. After spinning for 20 min with 1200 x g without break, peripheral blood mononuclear cells (PBMCs) could be obtained by extracting the buffy layer. After assimilation and washing with PBS, the PBMCs were resuspended in 30ml medium (DMEM + 10% hABs), and cultured in plastic flasks at 37 °C with 5% CO2. Non-adhering peripheral blood lymphocytes (PBLs) were harvested after 45 min. PBL concentration was estimated using Neubauer chamber. Proliferation of PBL was induced by adding anti-CD3/anti-CD28-beads (Dynabeads™) to the cell suspension of 10^5 PBLs/ml with a ratio of 1 bead per 62.5 PBLs.

2.2.1.3.3 Preparation of Uric Acid

Uric acid was dissolved in 1 M sodium hydroxide (NaOH) by heating at 60 °C for 1 hour to obtain a stock solution of 30mg/ml. Working concentration was obtained by a further 1:10 dilution in tryptophan rich DMEM-medium (3 mg/ml). The pH adjustment to 7.4 was performed by gradually adding 1 M hydrochloric acid (HCl). Sodium chloride (NaCl) was used instead of uric acid in osmolarity and pH controls.

2.2.2 Methods of Measurement

2.2.2.1 Kynurenine-Assay

To each well of a 96 well plate (U-bottom) 30µl of sample from supernatants of stimulated MSCs and 60 µl trichloric acid (TCA) was added. As a reference standard, serial dilution was created, starting off with 500 µmol kynurenine in 30 µl standard media. The plate was incubated for 30 min at 50 °C in a water bath to induce precipitation of proteins within the sample. After reaching room temperature, one part freshly prepared Ehrlich Reagent (2% p-dimethylaminobenzaldehyde in glacial acetic acid) was added to one part sample. After the incubation for 5min at room temperature the samples were spun down hard (5 min at 3716 x g). The extracted supernatants were transferred into a flat bottom 96 well plate and absorption at a wavelength of 492 nm was measured using the POLARstar Omega microplate reader.

2.2.2.2 Metabolism-Assay

Metabolism was quantified by applying the colorimetric WST-1 reagent, which stands for 'water soluble tetrazolium'. The tetrazolium salt is cleaved by mitochondrial dehydrogenases to formazan. The so caused increase in dye directly correlates with the metabolic activity of the cells. A final dilution of 1:10 was made with standard media. After 4 days of MSC culturing with certain stimuli, this original culturing media was completely replaced by the dilution made of WST-1 reagent. Absorbance was measured at intervals of 10 min for a total of 4 hours, using the appropriate weave length (492 nm). The measure of transmittance was displayed as optical density (OD). The optical density equals the absorbance in a logarithmic ln (10) scale.

2.2.2.3 Proliferation-Assay

MSCs were cultured in a sterile 96-well plate (200 MSCs/well) in our standard culturing media (see above) containing IFN-y (100 ng/ml) plus recombinant human S100A4, recombinant HMGB1, ATP or uric acid as stimulus at indicated concentrations. After 4 days adherent MSCs were washed with PBS and the cell staining for desoxyribonucleic acid (DNA) with CyQuant™ was applied according to manufacturer's guidelines. A micro plate reader was used to measure fluorescence. Fluorescence emission of the dye nucleic-complexes correlates with the DNA content within each well. Excitation detection was adjusted at 480 nm and emission detection at 580 nm.

2.2.2.4 Assessing PBL proliferation by FACS-Analysis

PBLs were stained with carboxyflourescein succinimidyl ester (CFSE) according to the manufacturer's recommendations. In brief, PBLs were stained for 10 min (37°C) with CFSE, then washed and with PBS in order to remove remaining dye. Afterwards PBLs were incubated for further 30 min in standard media to undergo acetate hydrolysis. Thus stained PBLs were cultured in the presence of DynaBeads™ for 4 days in conditioned media obtained from stimulated MSCs. PBL concentration was adjusted to the concentration of 0.5x106 PBL/ml. Proliferation was assessed by the method of fluorescence-activated cell sorting (FACS).

2.3 Ethics Statement and Statistics

This study was conducted according to the principles expressed in the Declaration of Helsinki. All bone marrow and blood donors provided written informed consent for the collection of samples for the generation of MSCs and the isolation of peripheral blood mononuclear cells (PBMCs), as well as for subsequent analyses. The study was approved by the Institutional Review Board (Ethical Committee of the University of Ulm). It was catalogued under the reference number 172/13-Fa/bal.

Student's t test for means (paired two samples) was used for calculating significance, and *p*-values equal or below 0.05 were considered significant and indicated by asterisk in the figures.

3. Results

3.1 HCT-Lysate reduces Kynurenineproduction while enhancing IDO-expression in MSCs

Kynurenine – the catabolite of Indolamine-2,3-dioxygenase (IDO) - is known to have immune modulatory capabilities. Therefore, we measured its concentration in the supernatants of MSCs stimulated with tumor lysate in the presence or absence of the inflammation-associated cytokine IFN-γ (100 ng/ml). The tumor cell lysate was obtained from a human colorectal tumor cell line (HCT-116). Kynurenine levels were assessed by the absorptive kynurenine assay.

As expected IFN-γ by itself induced the production of kynurenine in MSCs but interestingly this effect was inhibited by tumor lysate in a dose-dependent manner (Fig. 1). The Application of 10^6 lysed HCT cells/ml nearly abolished the stimulatory effect of IFN-γ on MSCs' kynurenine production, while a concentration of 10^4 cells/ml had no influence.

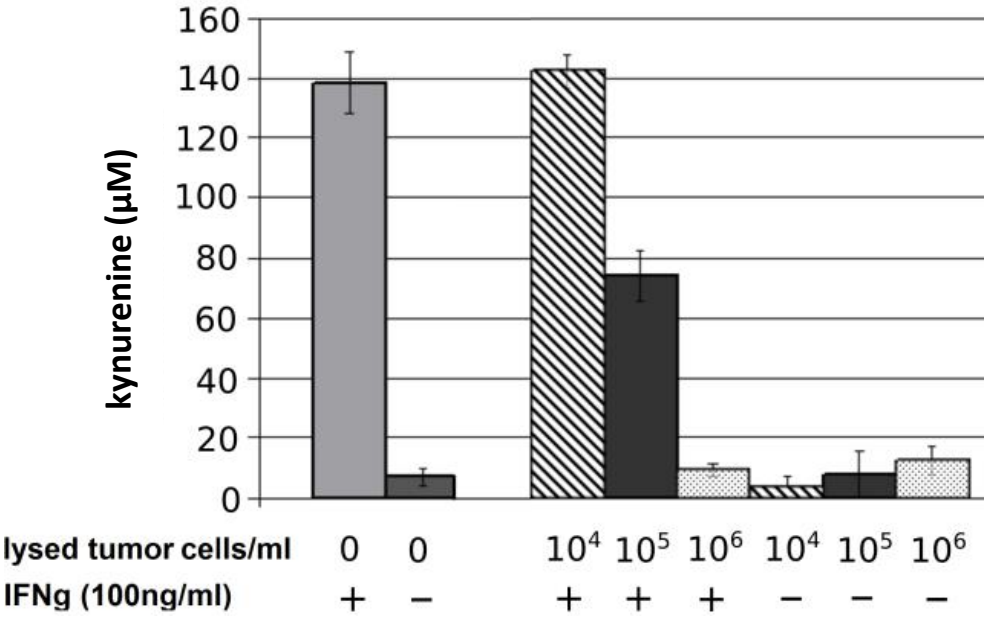


Figure 1. Human colorectal tumor-116 lysate (HCT-116) reduces kynurenine production in interferon-γ (IFN-γ)-stimulated mesenchymal stromal cells (MSCs).

MSCs were stimulated with indicated concentrations of tumor lysates +/-IFN-γ (100 ng/ml) and cultured for 4 days in tryptophan containing media (100 µg/ml). Supernatants were measured for kynurenine. Tumor lysate inhibits dose-dependently IFN-γ induced kynurenine production in MSCs. Shown are mean kynurenine concentrations in µM - ± standard deviation (SD) as error bars - from 1 representative out of 3 independent experiments.

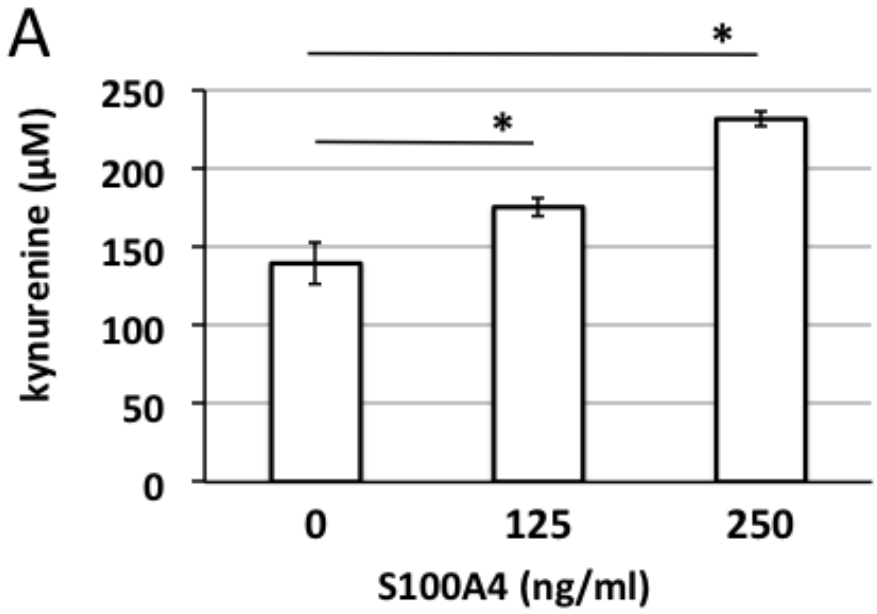
3.2 Impact of single DAMP-Representatives on Kynurenine Production in MSCs

3.2.1 S100A4 enhances Kynurenine Production in MSCs

We were interested in the single effect of DAMPS such as S100A4 on MSCs' kynurenine production. Supernatants of MSCs, which underwent IFN-γ stimulation and the exposition to rising concentrations of S100A4, were analyzed according to their kynurenine levels by the absorption assay after 4 days of incubation. To evaluate any changes in kynurenine amounts, we also looked at the metabolic activity of the MSCs after the 4-day treatment using the WST-assay. Prior data to this work had already shown that S100A4 inhibits the MSC proliferation.

Adding graded concentrations of S100A4 to the standard media resulted in a dose dependent kynurenine increase within the supernatants compared to the IFN-γ triggered basal kynurenine production within 4 days of incubation. Kynurenine production was increased by 25% when adding 125 ng S100A4/ml and 64% for 250 ng S100A4/ml respectively, compared to the column showing sole treatment with IFN-γ (Fig. 2A). MSC overall metabolism rate was not affected by the treatments with S100A4 and IFN-γ (Fig. 2B).

Providing the same culturing conditions, but in co-cultured with PBMCs, MSCs produced a larger amount of kynurenine (Fig. 2C, grey columns), compared to MSC monoculture (Fig. 2C, white columns). It was an increase of nearly 50% using 125 ng S100A4/ml and 25% with stimulations of 250 ng S100A4/ml. PBMCs themselves did not raise kynurenine concentrations as the dark columns show.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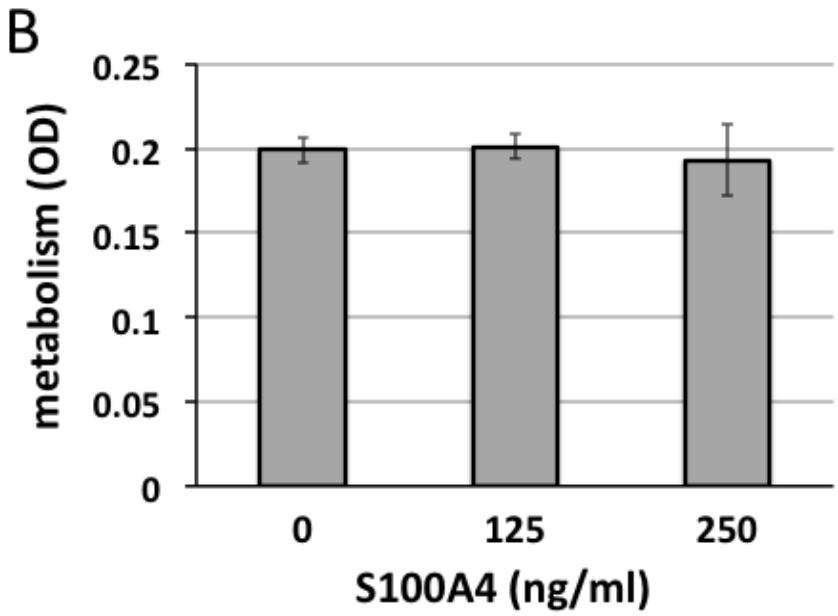
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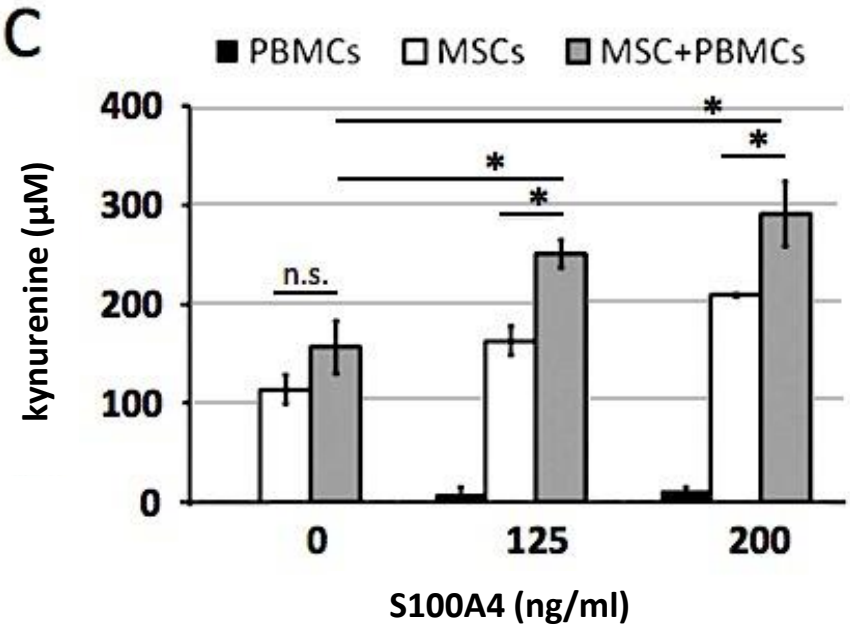


Figure 2. S100A4 enhances kynurenine production in interferon-γ (IFN-γ)-stimulated mesenchymal stromal cells (MSCs), with an enhanced effect in MSC co-culture with peripheral blood mononuclear cell (PBMC). No effect can be observed on MSC metabolism.

(A) S100A4 increased dose dependently kynurenine levels in MSC culture. In the presence of IFN-γ (100 ng/ml), MSCs ($15x10^3$ cells/cm^2) were stimulated with indicated concentrations of S100A4 for 4 days within a media saturated with tryptophan (100 µg/ml). Kynurenine concentration within the supernatant was assessed by the colorimetric kynurenine assay. Shown are mean values with error bars exhibiting SD from o1 of 6 individual experiments. Asterisks indicate significance $p \leq 0.05$.

(B) No significant effect on metabolism rate could be detected by exposing IFN-γ (100 ng/ml) stimulated MSCs to different concentrations of S100A4. WST-1 reagent was added to MSCs cultured as described in section A and optical density (OD) was measured as surrogate for metabolism. Shown are means of 1 out of 3 individual experiments with the according error bars.

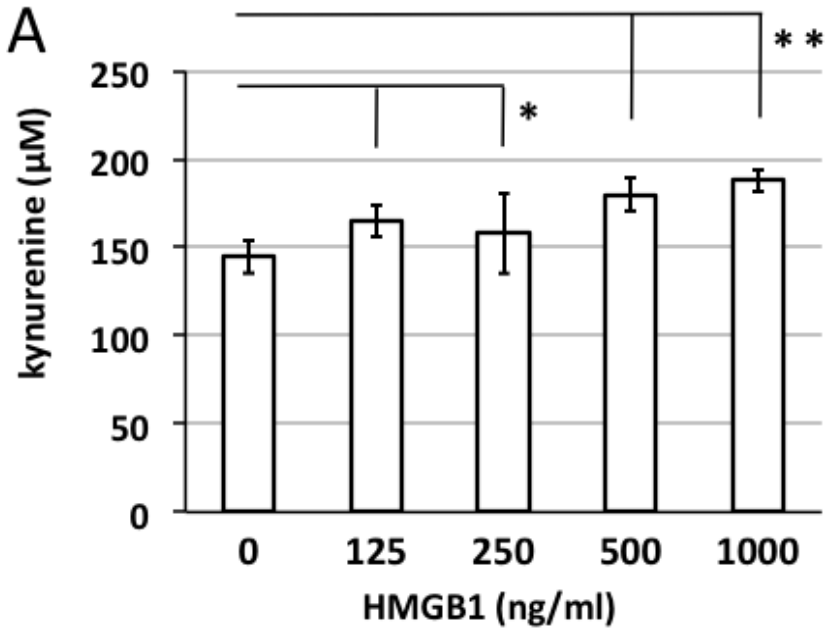
(C) In the presence of indicated concentrations of S100A4 and anti-CD3/CD28 beads, PBMCs and MSCs were cultured alone or co-cultured at 1:1 ratio in tryptophan-saturated culture media (100 µg/ml) and the stimulation with IFN-γ (100 ng/ml). After 4 day kynurenine was measured within the supernatants. An additional effect in kynurenine production can be seen in MSC co-culture with PBMCs (grey bar). Shown are mean kynurenine concentrations from 1 of 3 individual experiments with standard deviations as error bars. Asterisks indicate * $p \leq .05$. The abbreviation *n.s.* stands for *no significans*.

3.2.2 HMGB1 enhances Kynurenine Production in MSCs

To gain further insight at the single effect of DAMP members on the kynurenine production by MSCs, HMGB1 of the DAMP member family was chosen next to be looked at. Again, to classify the results we measured MSC metabolism and proliferation concurrently.

IFN-γ stimulated MSCs were incubated for 4 days in the presence of HMGB1 at graded concentrations. Kynurenine levels were again detected by the kynurenine absorption assay, metabolic activity by WST-1 assay. Proliferation was determined by cell count present at the end of the 4-day experiment using the fluorescent CyQuant™ assay.

Similar to S100A4, HMGB1 also led to a dose dependent elevation of kynurenine levels (Fig. 3A). This was highly significant at concentrations of 500 ng HMGB1/ml with an increase of 24.5% and 30% using the doubled concentration (1000 ng HMGB1/ml) compared to non-stimulated state. But even stimulations with 125 ng/ml and 250 ng/ml led to significant results. HMGB1 did neither have an impact on MSCs' metabolic activity (Fig. 3B) nor on their proliferation rate (Fig. 3C).



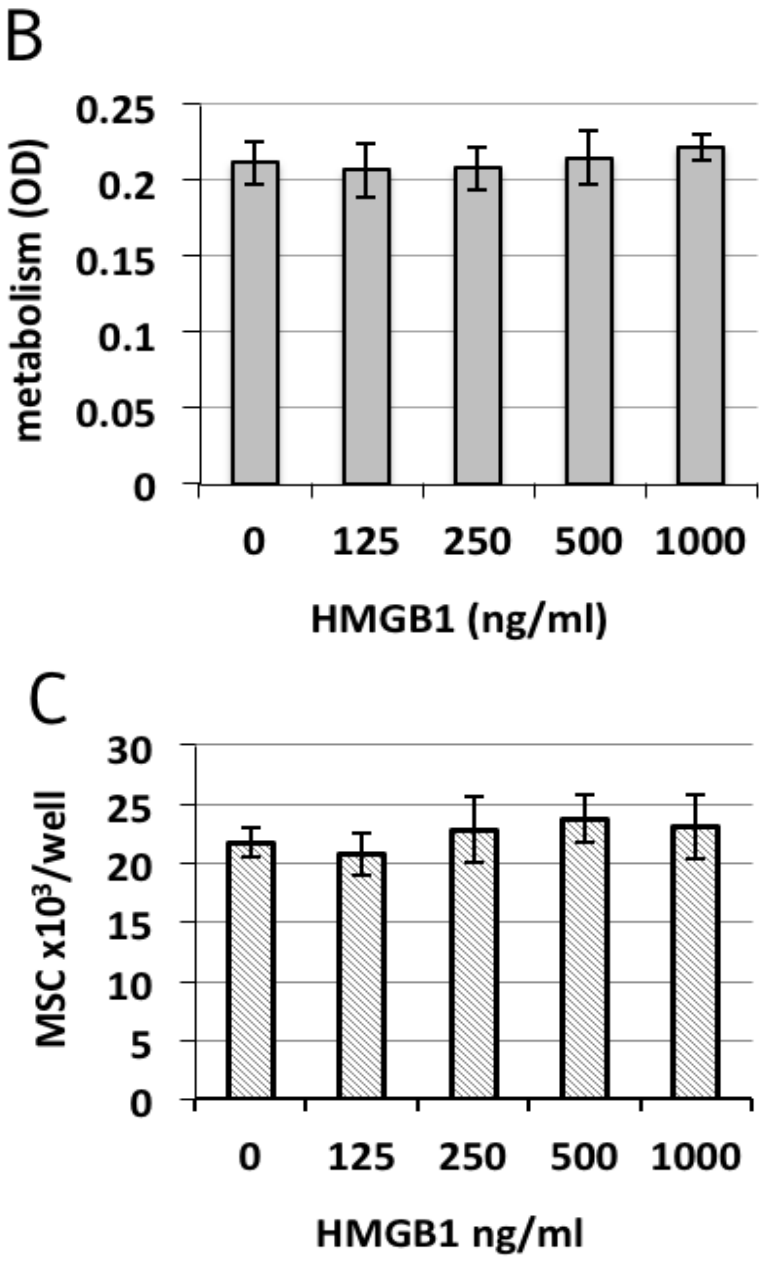


Figure 3. High mobility box group 1 (HMGB1) enhances kynurenine production by Interferon-γ (IFN-γ) stimulated mesenchymal stromal cells (MSCs), without an effect on metabolism and proliferation rate.

(A) The application of the displayed HMGB1 concentrations increased kynurenine levels in the MSC culture dose dependently. Supernatants derived from IFN-γ (100 ng/ml) stimulated MSCs after 4-day culture in tryptophan-saturated media (100 μg/ml) were measured. One representative experiment was taken from 9 holding equal results; SD is displayed as error bars. Asterisks indicate significance with * = $p \leq .05$ and * * = $p \leq .005$

(B) No significant effect on MSCs metabolism rate, displayed as optical density (OD), could be detected applying HMGB1 to the standard media. Data derived from 1 out of 5 representative experiments is displayed (±SD).

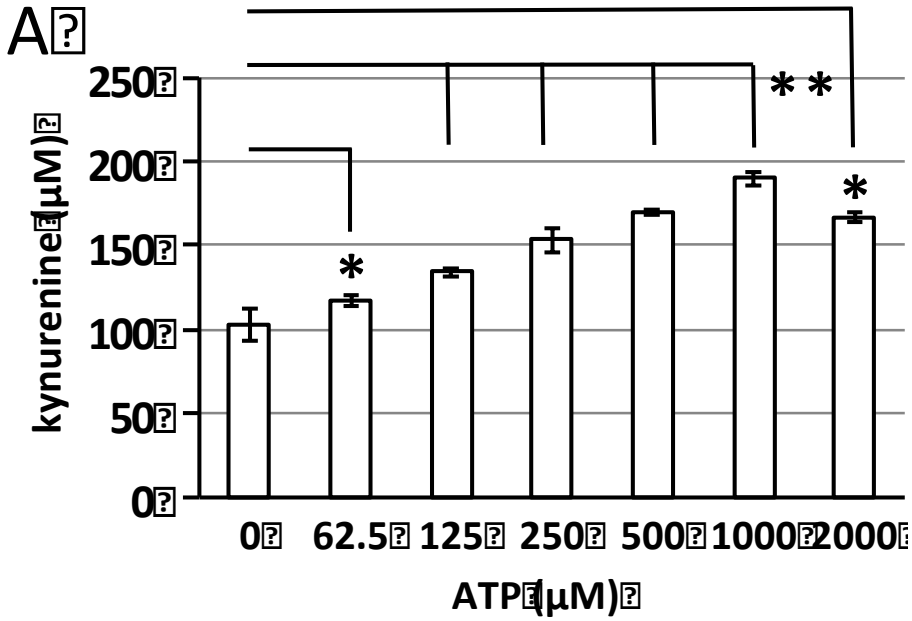
(C) In the presence of indicated HMGB1 concentrations, no change in MSC proliferation could be observed. Shown is data from 1 (±SD) out of 5 experiments.

3.2.3. ATP enhances Kynurenine Production

Alongside the influence on kynurenine concentrations in the sample, MSC metabolism and proliferation was assed in this standard media setting examining another DAMP member, namely ATP.

ATP had a dose dependent effect, strongly augmenting kynurenine concentration in MSC supernatants (Fig. 4A). Even at small amounts of 62,5 µM ATP, kynurenine concentration climbed about 14,8% compared to non-stimulated MSCs. The optial ATP concentrations ranged between 500 and 1000 µM, showing increases to 65,7% and 85,7%, respectively. Beyond these concentrations kynurenine levels stayed identical or even tended to decrease again.

ATP enhanced MSCs' metabolic activity (Fig. 4B). Consistent with the measured kynurenine increase a maximum metabolic rate could be detected, when applying ATP at concentrations between 500 µM and 1000 µM. MSC proliferation however remained uninfluenced (Fig. 4C).



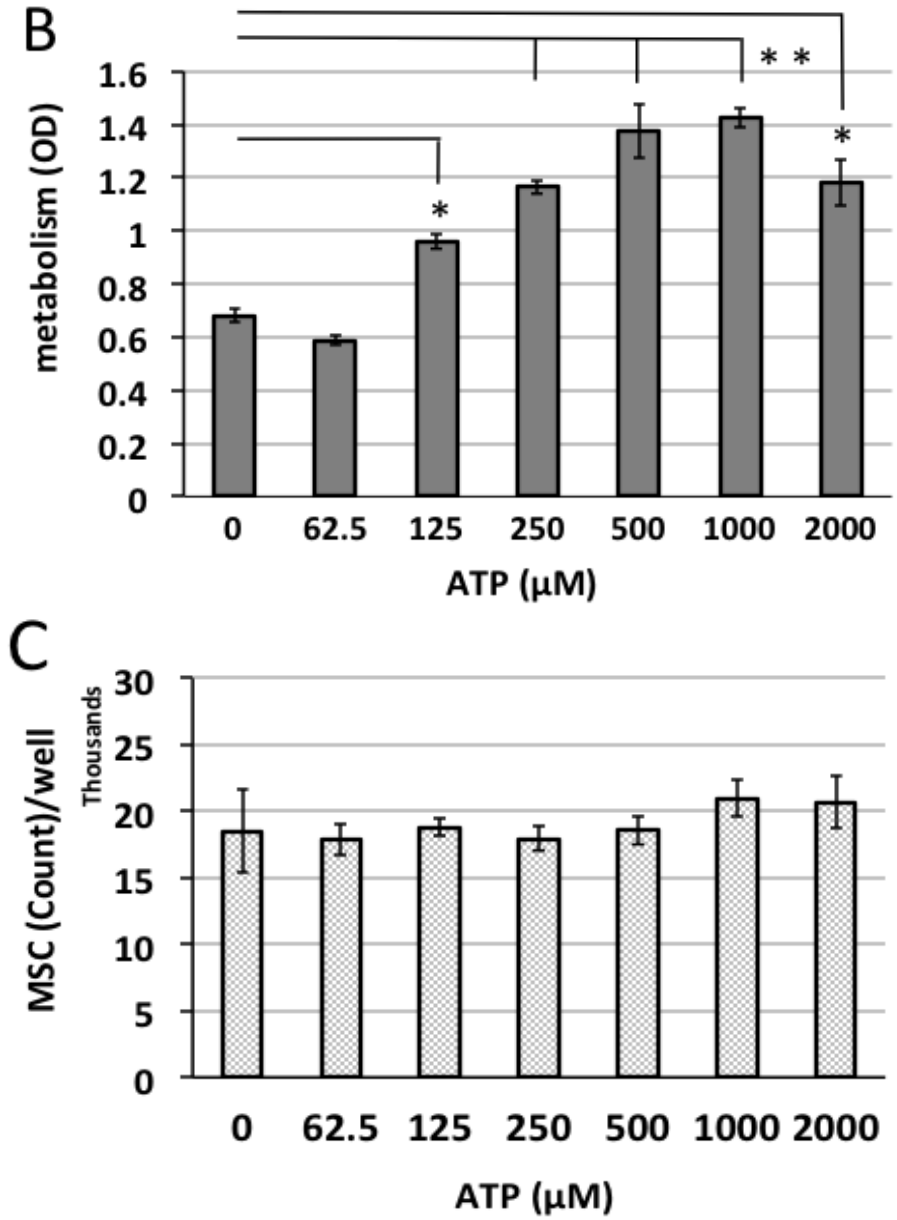


Figure 4. Adenosine tri phosphate (ATP) dose-dependently enhances kynurenine production and metabolism by interferon-γ (IFN-γ) stimulated mesenchymal stromal cells (MSCs), without any proliferative effects on MSCs.

(A) In the presence of 100ng IFN-γ/ml culturing media, containing 100µg tryptophan/ml, bone marrow derived MSCs ($15x10^3$ cells/cm^2) were stimulated with indicated concentrations of ATP for 4 days. Kynurenine concentration was assessed within the supernatant by a colorimetric assay. Shown is 1 out of 8 independent experiments with mean values and error bars indicating SD. Significance is shown as asterisks * $p \leq .05$; * * $p \leq .005$

(B) The representative data out of 6 experiments shows the increase in metabolic rate (±SD) of ATP stimulated MSCs as optical density (OD) in the WST-assay. * $p \leq .05$; * * $p \leq .005$

(C) MSC proliferation in standard medium was not influenced after applying ATP at different concentrations for 4 days. Panel shows data from 1 out of 6 trials (±SD).

To demonstrate the specificity of ATP effect on MSCs, we repeated the kynurenine experiments using 500 µM ATP in the presence of graded concentration of ATP-degrading enzyme apyrase, abolishing the stimulatory effect of ATP on MSCs (Fig. 5). While apyrase alone did not influence kynurenine production in IFN-γ stimulated MSCs, the enzyme abrogated the effect of ATP on MSCs dose dependently. Kynurenine levels dropped to initial levels (without ATP adding) using 1.25 and 2.5 U apyrase/ml.

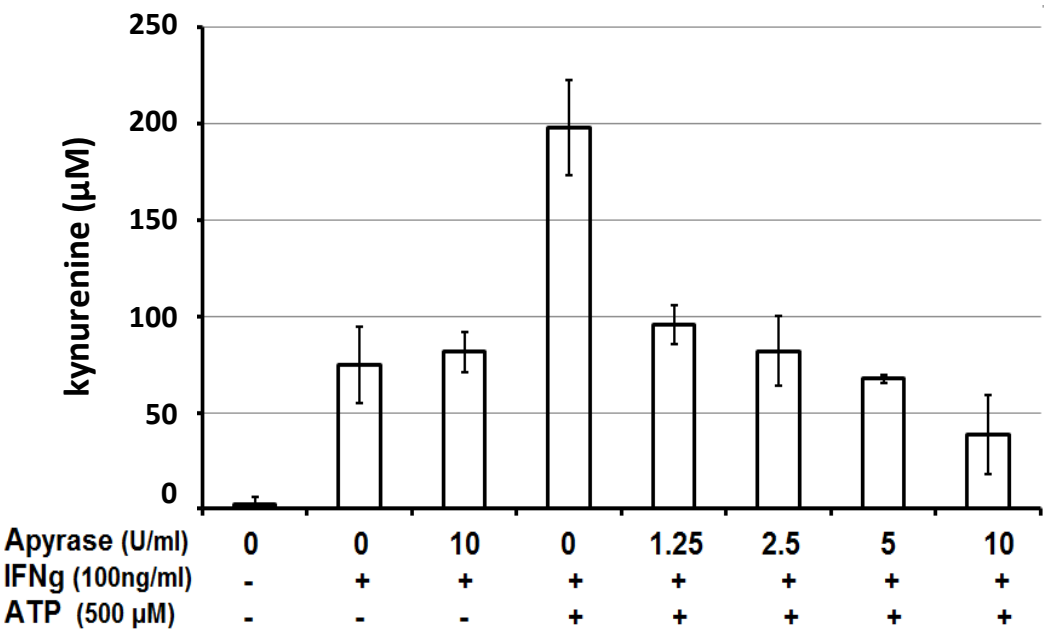


Figure 5. Apyrase dose-dependently inhibits adenosine tri phosphate (ATP) promoted kynurenine production in interferon-γ (IFN-γ) stimulated mesenchymal stromal cells (MSCs).

In the presence of 100 µg tryptophan/ml culturing media, bone marrow derived MSCs ($15x10^3$ cells/cm^2) were stimulated with IFN-γ (100 ng/ml) and ATP (500 µM) in the presence of indicated concentration of ATP-degrading enzyme apyrase. Results were derived by the colorimetric kynurenine assay. Shown are representative results (mean ± standard deviation) from 1 out of 4 individual experiments.

Getting these high amounts of MSC kynurenine production with ATP stimulus any direct impact on the proliferation rate of competent immune cells applying the MSC derived supernatants was to be investigated. Thus supernatants of ATP and IFN-γ stimulated MSCs were added to culturing media of PBLs, which comprised the standard media.

After 4-day of culture about 65% of PBLs proliferated following stimulation with anti-CD3/anti-CD28 beads (Fig. 6A). This proliferation could be reduced to 41% in the presence of conditioned media from IFN-γ stimulated MSCs (Fig. 6B). Compared to stimulation with IFN-γ alone (Fig. 6 B), supernatants from MSCs stimulated with both

IFN-γ and ATP showed a significantly higher antiproliferative effect on PBL, leading to further reduction of PBL proliferation by nearly 50% (from 16% to 65%) compared to completely not stimulated PBMCs (Fig. 6C).

The antiproliferative effect of conditioned media from MSCs could only be demonstrated following stimulation of MSCs with IFN-γ, with or without ATP (Fig. 6B & C). It was not detectable using supernatants from MSCs cultured in the absence of IFN-γ (Fig. 6A).

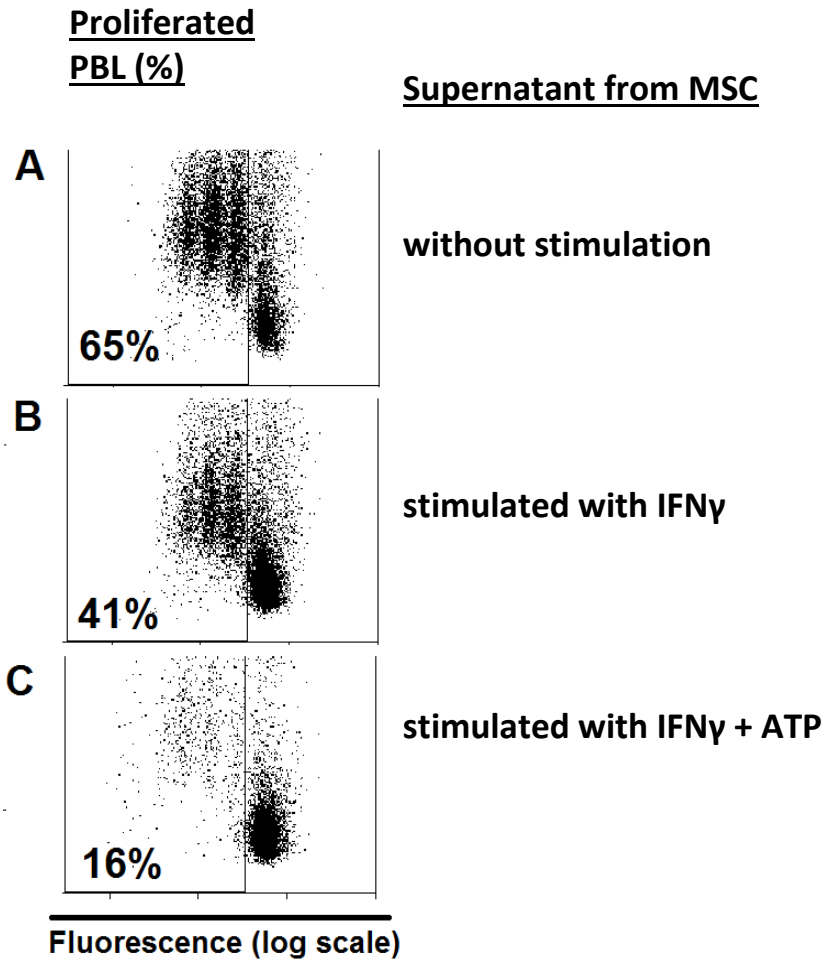


Figure 6. Supernatants derived from Adenosine tri phosphate (ATP) stimulated mesenchymal stromal cells (MSCs) enhance interferon-γ (IFN-γ) promoted inhibition of peripheral blood lymphocyte (PBL) proliferation.

In the presence of anti-CD3/anti-CD28-beads with a ratio of 1 bead per 12.5 PBLs, carboxyflourescein succinimidyl ester (CFSE)-labelled PBLs were cultured for 4 days in conditioned medium obtained either from MSCs without stimulation **(A)**, stimulated with IFN-γ (100 ng/ml) alone **(B)**, or in combination with 250 µM ATP **(C)**. MSCs were cultured in media containing 100 µg/ml tryptophan in order to allow MSCs to produce kynurenine. By performing fluorescence-activated cell sorting (FACS), lymphocyte proliferation was assessed**.** Shown is the ratio of proliferating lymphocytes from one representative out of at least 3 independent experiments.

In order to confirm kynurenine as a crucial factor within the conditioned media responsible for inhibition of lymphocyte proliferation, we supplemented conditioned media from non-stimulated MSCs with graded concentration of kynurenine and could demonstrate a dose-dependent antiproliferative direct effect of kynurenine (Fig. 7B) on PBLs.

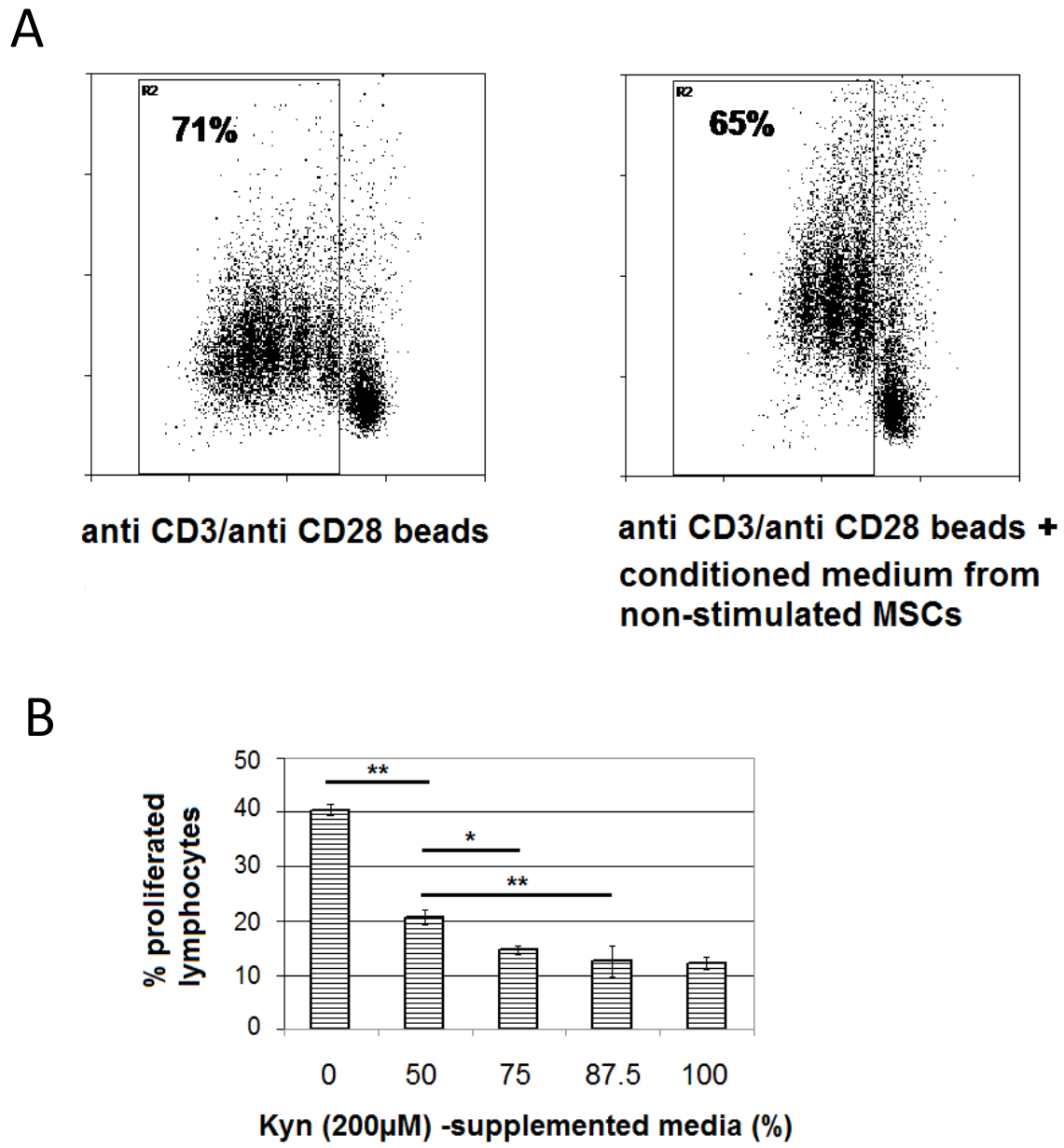


Figure 7. Kynurenine (Kyn) plays an important role in mesenchymal stromal cell (MSC) induced inhibition of lymphocyte proliferation.

(A) Conditioned media from non-stimulated MSCs does not inhibit lymphocyte proliferation. In the presence of anti-CD3/anti-CD28-beads, carboxyflourescein succinimidyl ester (CFSE)-labelled lymphocytes were incubated for 4 days in fresh media (left) or in conditioned media (right) obtained from non-stimulated MSCs (media containing 100 µg/ml tryptophan, but no IFN-γ or DAMP). Lymphocyte proliferation was measured by performing fluorescence-activated cell sorting (FACS). Shown is the ratio of proliferated lymphocytes. Panel displays representative out of at least 3 independent experiments.

(B) Kynurenine dose-dependently inhibits lymphocyte proliferation within 4 days. Culturing media was supplemented with 200 µM kynurenine and added at indicated concentrations to lymphocytes suspended in conditioned media from non-stimulated MSCs, in order to mimic kynurenine production by stimulated MSCs. Shown is 1 representative experiment out of 3 independent experiments. Error bars display standard deviation (±SD). Asterisks express significances * $p \leq .05$; ** $p \leq .01$

3.2.4 Uric acid lowers Kynurenine Production in MSCs

Uric acid is yet another ubiquitous present substance noted for its association with necrotic cell death. It further acts as a potent antioxidant. Given that the biologic activity of certain DAMP members can be abolished by oxidation, it seemed interesting to determine in which manner uric acid influences kynurenine production by MSCs, when added to media with above mentioned DAMPs.

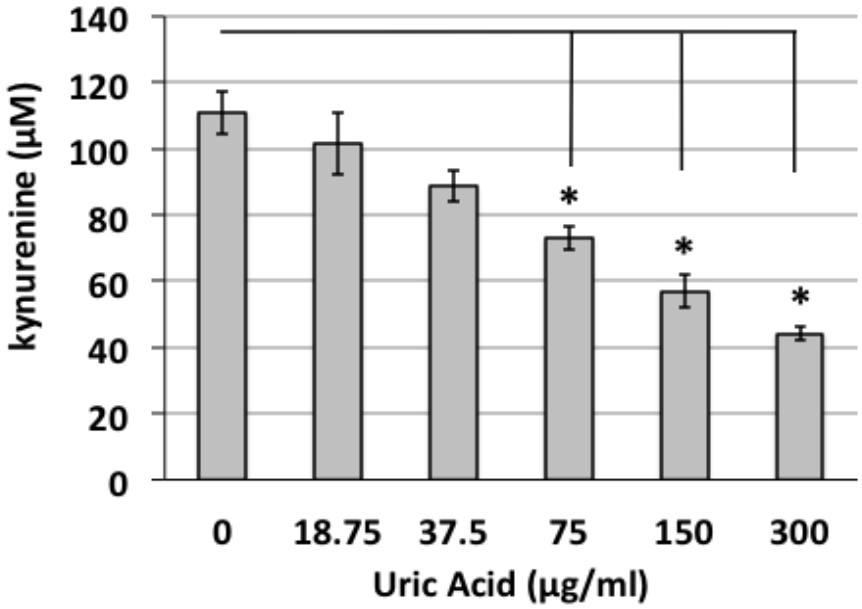


Figure 8. Uric acid decreases kynurenine levels in supernatants derived from interferon-γ (IFN-γ) stimulated mesenchymal stromal cells (MSCs) in a dose dependent fashion.

Uric acid was prepared by resolution in 1M sodium hydroxide (NaOH) and heating for 1 hour at 60°. Afterwards pH was adjusted to 7.4 adding 1M hydrochloric acid (HCl.). The x-axis displays graded concentration generated from the working stock. IFN-γ (100ng/ml) stimulated MSCs ($15x10^3$ cells/cm^2) were cultured in tryptophan-enriched media (100 µg/ml) with the indicated concentrations of uric acid. After for 4 days supernatants were harvested. To detect kynurenine the colorimetric assay was performed. Figure shows 1 out of 5 individual experiments with SD as error bars. Asterisks depict significance * $p \leq .05$

In contrast to previous examined DAMPs, uric acid decreased kynurenine levels within MSC culture supernatants in a dose dependant manner, with significant results using concentrations above 75 µg/ml. A maximal inhibition of kynurenine

production of about 60% compared to conditions without uric acid could be triggered using 300 µg/ml of uric acid (Fig. 8).

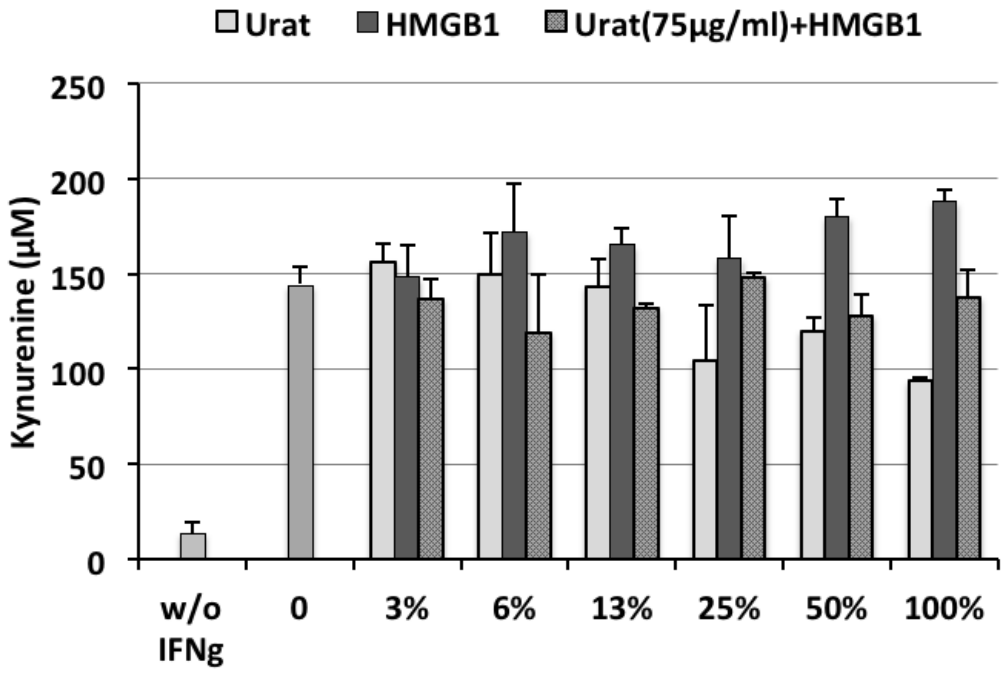


Figure 9. Uric acid abolishes high mobility group box 1 (HMGB1) triggered gain in kynurenine concentration by interferon-γ (IFN-γ) stimulated mesenchymal stromal cells (MSCs).

The x-axis displays graded concentrations of uric acid and HMGB1 ranging from 0% to 100%. The highest concentrations (100%) represent an absolute value of 300 µg uric acid/ml or 1000 ng HMGB1/ml respectively. The very left bar shows MSCs ($15x10^3$ cells/cm^2) without any stimulation (w/o IFN-γ). Patterned columns stand for a steady concentration of uric acid (75 µg/ml) added to rising levels of HMGB1 (100% corresponding to 1000 ng/ml). After 4 days of culture in tryptophan (100 µg/ml) saturated media, supernatants were taken. Colorimetric kynurenine assay determined kynurenine concentration via absorbance. Shown are means and SDs of 1 representative experiment out of 3 individual ones.

Applying the combination of uric acid and HMGB1, the inhibitory effect of uric acid dominated. It was pivotal as yet small amounts of 75 µg/ml uric acid (Fig. 9, patterned bars) impaired the HMGB1 induced kynurenine increase. Even high concentrations of HMGB1 (cf. Fig.9, patterned bars, x-axis 100%) could not lift kynurenine concentrations atop basic IFN- γ stimulation when uric acid was present.

4. Discussion

Solid tumor formations distinctly and visibly rely on their specific microenvironment at all stages of their development comprising initiation, progression and metastatic spreading (Yagi et al., 2010; Hall et al., 2006). The prevailing conditions of chronic inflammation are harnessed by the tumor to shield itself from attacks of the host's immune system and antitumor drugs. MSCs can actively migrate into tumor tissue (Spaeth et al., 2009). Their presence was shown to be associated with higher rates of metastasis and poor prognosis regarding survival (Karnoub et al., 2007; Spaeth et al., 2009). One explanation to this might be found in the interplay between MSCs and lymphocytes, with regard to kynurenine levels and IDO expression, causing alterations in lymphocyte proliferation and mode of action.

HCT-116 lysate, representative for necrotic material within tumor tissue inhibited kynurenine production in MSCs in a dose depended manner (Fig. 1). However, we did not test if impaired MSC proliferation or metabolism is responsible for our observation. Furthermore, lysates derived from different tumor lines should be compared regarding their potential to influence kynurenine levels, as previous studies have shown that there are differences between the stimulatory strength of lysates from different tissue origins with regard to chemoattractive strength (Eisenbacher et al., 2014). In opposition to the tumor lysates' sum effect on MSCs, the three single DAMP members S100A4, HMGB1 and ATP raised kynurenine production by MSCs. As already been done for S100A4 (Eisenbacher et al., 2014) by the use of enzyme-linked immunosorbent assay (ELISA), the concentrations of ATP, HMGB1 and uric acid within HCT-116 tumor lysate need to be determined, in order to be able to draw conclusions on the their roles and to explain the overall effect of HCT lysate on MSCs' kynurenine production.

Three tested DAMP-representatives showed a positive impact on MSCs' kynurenine production, however their effect on MSC proliferation and metabolism was individually different. ATP strongly enhanced kynurenine production and metabolism in MSCs (Fig. 4A, B). This is consistent to findings with ATP exposed dentiritic cells (DCs) (Marteau et al., 2005). ATP is a ubiquitous intracellular molecule. Due to its short shelf life, ATP has a rather small distance of action influencing cells in close proximity to its release. This could indicate that MSCs rely on this pervasive molecule to strongly suppress immune response locally. S100A4 and HMGB1 also both enhanced

kynurenine levels, but without any impact on MSC metabolic activity (Fig. 2A, B & Fig. 3A, B). So the effect on metabolism in MSCs under ATP stimulus seemed to be consistent to the strong effect on kynurenine production in MSCs, possibly due to an overall cell activation with subsequent enzyme induction. Intracellular measurement of IDO levels, e.g. by FACS scan, will be needed to strengthen this suggestion. Neither ATP nor HMGB1 influenced MSC proliferation (Fig. 3C & Fig. 4C). It is noteworthy mentioning that S100A4 enhanced kynurenine production in MSCs (Fig. 2A), even though this protein inhibited their proliferation (Eisenbacher et al., 2014), which may possibly be explained by comparatively strong enzyme induction. Again measurements of intracellular IDO concentration will be required as a following step of proof.

Our results support the idea that the presence of DAMPS within necrotic tissue has a significant impact on microenvironmental cellular immune regulation. The results obtained from MSC stimulation with S100A4 and co-culture with PBMCs emphasized this notion (Fig. 2C). The co-culture of MSCs with PBMCs in the presence of a DAMP member S100A4 provided supernatants with significantly higher kynurenine concentrations compared to the supernatants from MSCs monoculture stimulated with S100A4, while the PBMCs alone did not raise kynurenine concentrations in the presence of S100A4. So not only DAMPS themselves, but the surrounding immune cells dictated the amount of kynurenine production. PBMCs induced an increase in kynurenine concentration by mere proximity to MSCs, followed by their own down regulation. Kynurenine is also capable to induce a shift from Th1 immune response to Th2 (Odemuyiwa et al., 2004; Fallarino et al., 2002). So this molecule acts as an important junction in at least two different modes of immune regulation.

When compared to the other tested DAMP members, uric acid provided quite the opposite effect, making kynurenine production in MSCs decline (Fig. 8). This points towards our findings concerning the HCT-116 lysates' sum effect (Fig. 1). We exposed MSCs to graded concentrations of HMGB1 in the presence of a stable concentration of 75 µg uric acid/ml (Fig. 9), being the smallest concentration to achieve significant inhibition of kynurenine production with. Here even high HMGB1 concentrations could not stop the uric acid effect to be abrogated. So uric seems to neutralize HMGB1 effect regarding kynurenine production by MSCs. This might be because of the proliferation impairing effect of uric acid on MSCs we could observe in prior experiments (Lotfi et al., 2011).

The completing step would be to show if there are any influences on MSCs' metabolism. Uric acid is known to be a reducing agent. Given that certain DAMPs (e.g. S100A4 and HMGB1) loose their bioactivity following oxidation processes, uric acid might serve as a protective factor for these DAMPs. Small amounts, not able to inhibit MSC proliferation (ultimately resulting in lowered kynurenine levels), might hence be sufficient to promote kynurenine production when S100A4 or HMGB1 is present. This has already been shown in the context of S100A4's chemotactic potential in uric acid-enrich surrounding (Lotfi et al., 2011).

We could verify the effect of IFN-γ stimulated MSCs – mirroring inflammatory conditions – interfering with lymphocyte proliferation (Fig. 6B). This was consistent with previous observations from other groups (Di Nicola et al., 2002; Li et al., 2009). Our data revealed that this effect could be boosted in the presence of ATP (Fig. 6C), leading to even higher inhibition of lymphocyte proliferation by MSCs. Given the fact, that combination of ATP with IFN-γ enhanced kynurenine production by MSCs, when comparing to conditions without ATP, IDO might be the key or at least a crucial enzyme in the context of MSC induced inhibition of lymphocyte proliferation in this setting. The anti-proliferative effect of conditioned media from MSCs could not be detected using supernatants from non-stimulated MSCs (Fig. 6A, B), excluding the possibility that inhibition of lymphocyte proliferation may have been due to using a media deprived of nutrients by MSCs. By directly applying kynurenine to PBLs we could not only strengthen this hypothesis, but also directly identify kynurenine to play a major part in this inhibition of lymphocyte proliferation (Fig. 7B). Target receptors on PBLs, which are involved in kynurenine-mediated effects are to be identified, with aryl hydrocarbon receptor (AHR) being a promising candidate in this regard (Di Natale et al., 2010). Experiments with AHR receptor blockers and further possible candidates, e.g. members of Toll-Like receptor (TLR) family, might uncover the receptors required for kynurenine signalling in PBLs.

As the tumor milieu consists of a heterogeneous mix of different soluble factors, further important DAMPs, but also other bioactive factors (e.g. chemokines and cytokines) with an impact on kyurenine production by MSCs need to be identified. Isolated effects of single DAMP representatives on MSCs may not mirror physiological processes in the necrotic microenvironment. The interplay of the representatives of the DAMP group together with inflammatory chemokines seems to be responsible for creating a certain sum effect on kynurenine levels in the microenvironment.

MSCs are also known for their role in cellular crosstalk with other immunogenic cells (Wang et al., 2012), as we could observe in co-culture with PBMCs (Fig. 2C). Thus, different combinations of bioactive factors found within tumor milieu and different immune competent cell types must be considered in future experimental arrangements.

5. Summary

Mesenchymal stromal cells (MSCs) have already been reported to crucially influence immune response in inflammatory settings. One mechanism is influencing tryptophan metabolism. Through triggering a special path of tryptophan breakdown, namely IDO (indolamine 2,3-dioxygenase) activation, kynurenine is produced. The kynurenine itself acts immunosuppressive. Accumulation of MSCs within tumor tissue is associated with tumor progression and poor prognosis. Necrotic cell death with subsequent release of damage associated molecular patterns (DAMPs) is a characteristic feature of advanced solid tumors. Adenosine triphosphate (ATP), high mobility box 1 (HMGB1), S100A4 and uric acid represent known DAMP family members. It was already shown that DAMPs act as chemoattractants on MSCs.

The present work aimed at determining if DAMPs have any impact on immunosuppressive capacities of MSCs, with specific regard to IDO-activity and subsequent production of kynurenine.

Our data derived from the kynurenine assay revealed S100A4, HMGB1, and especially ATP to promote an effect of enhanced kynurenine production by MSCs, whereas uric acid lowers kynurenine levels in MSC culture. Corresponding to the most significant results under MSC stimulation with ATP, an elevated metabolism rate in MSCs under this culturing condition could be observed with WST-1-assay. ATP even enhances interferon-γ (IFN-γ) promoted inhibition of peripheral blood lymphocyte (PBL) proliferation by MSCs, measured by fluorescence-activated cell sorting (FACS).

It further could be shown that kynurenine directly leads to the inhibition of immune cell proliferation (e.g. PBLs) in FACS-analysis.

The local composition of DAMPs - released via necrotic cell disruption - determines kynurenine levels produced by MSCs, alongside with prevailing cytokines and immune cells within inflammatory tissue. Cytotoxic immune response to tumor enhances necrotic tumor cell death with subsequent release of DAMPs, thus interfering with effective further immune response. Local fluctuation in the concentrations of these DAMP representatives may create a kynurenine gradient throughout the necrotic environment having an impact on local immune response, causing changes in lymphocyte proliferation e.g. shifting from Th1 to Th2 response. Bearing this in mind, studies, targeting those DAMPs by the application of antibodies (aHMGB1, aS100A4), enzymes (apyrase) or direct receptor interaction (aRAGE,

aTLR2, aTLR4, aP2X7) on MSCs, may provide helpful strategies to attenuate this vicious circle of immune suppression, paradoxically induced by a primary effective cytotoxic immune response to tumor.

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Acknowledgements

First of all I need to utter my thanks to Prof. Dr. Ramin Lotfi. Under his inspiring lead, I was able to develop the spark of ambition, which provided the stamina needed to perform valid research. Thank you Ramin for your apparently endless patience!

Furthermore I feel grateful for having had such a great team alongside my way.
Thank you Tatjana Geier, Gisela Baur, Dorothe Erz, Alexander Erle and Thomas Becker. You were always available for counsel and provided assistance not only in helping me in gathering all materials needed, but also in mastering methods.

Thank you Lena Steppe for your support. Your sedulous work contributed a lot to the overall project.

Finally I would also show my gratitude to Prof. Dr. Hubert Schrezenmeier, whom patronage without, this project would not have been realizable.

VII. Curriculum Vitae

Personal Data

Removed for privacy reasons.

Academic Education

Abridged for privacy reasons.

WT 2009 – ST 2016	Study of human medicine, University of Ulm (graduation: State Exam)

Professional Experience

Removed for privacy reasons.

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Japanese A2 level

Parts of the dissertation have already been published in the following journal article:

Lotfi R, Steppe L, Hang R, Rojewski M, Massold M, Jahrsdörfer B, Schrezenmeier H: ATP promotes immunosuppressive capacities of mesenchymal stromal cells by enhancing the expression of indoleamine dioxygenase. *Immunitiy, Inflammation and Disease.* 6: 448-455 (2018)

DOI: https://doi.org/10.1002/iid3.236