From the Institute for Transfusion Medicine

of the University of Ulm

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# Mesenchymal Stromal Cells Respond to Necrotic Material within Tumor Microenvironment with Regard to Proliferation and Chemotaxis

Dissertation

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For my parents and my sisters, who have been there for me in every possible way.

You're the best!

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# List of Abbreviations

| | |
|---|---|
| α-MEM | Alpha-Modified Minimal Essential Medium |
| ATP | Adenosin Triphosphate |
| CFSE | Carboxyfluorescein Succinimidyl Ester |
| DAMP | Damage Associated Molecular Pattern |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Desoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetate |
| EGFR | Epidermal Growth Factor Receptor |
| ELISA | Enzyme-linked Immunosorbent Assay |
| FACS | Fluorescence Activated Cell Sorting |
| FBS/FCS | Fetal Bovine/Calf Serum |
| FITC | Fluorescein Isothiocyanate |
| FSC | Forward Scatter |
| F/T | Freeze/Thawing |
| $H_2O_2$ | Hydrogen Peroxide |
| $H_2SO_4$ | Sulfuric Acid |
| HCl | Hydrochloric Acid |
| HMGB1 | High Mobility Group Box 1 |
| HRP | Horse Radish Peroxidase |
| HSP | Heat Shock Protein |
| IDO | Indolamine-2,3- dioxygenase |
| IgG | Immunoglobuline Class G |
| IL | Interleukin |
| MFI | Mean Fluorescence Intensity |
| MSC | Mesenchymal Stromal Cell |
| NaCl | Sodium Chloride |
| NaOH | Sodium Hydroxide |
| Osm | Osmolarity |
| PBS | Phosphate Buffered Saline |

| | |
|---|---|
| PE | Phycoerythrin |
| PerCP | Peridinin Chlorophyll |
| PL | Platelet Lysate |
| RAGE | Receptor for Advanced Glycation End Products |
| ROS | Reactive Oxygen Species |
| SD | Standard Deviation |
| sRAGE | Soluble Receptor for Advanced Glycation End Products |
| TLR | Toll-Like Receptor |
| TMB | Tetramethylbenzidine |
| TNF | Tumor Necrosis Factor |

# 1 Introduction

## 1.1 Tumor Microenvironment

### 1.1.1 Damage Associated Molecular Patterns (DAMPs)

The progress of solid tumors is often associated with chronic inflammation and necrosis [9, 24]. Disregarding the origin and localization of neoplastic cells, necrosis is a characteristic feature of advanced solid tumors especially due to the following conditions:

1) lacking nutrient supply to tumor cells as a consequence of imbalance between tumor growth and angiogenesis,

2) the host's cytotoxic immune response to the tumor, and

3) downregulation of programmed (apoptotic) cell death by the tumor, itself [9, 24].

Released factors following necrotic cell death are also referred to as damage associated molecular patterns (DAMPs) and can act as danger signals to surrounding tissue and immune cells [9, 24].

Besides DAMPs the tumor microenvironment consists of extracellular matrix, cytokines, chemokines, and two categories of tumor-associated cells:

1) tissue resident cells like healthy endothelial/epithelial cells, fibroblasts, (mesenchymal) stromal cells (MSCs) and leukocytes (e.g. macrophages and dendritic cells) as well as

2) migrated cells like leukocytes and MSCs responding to local signals such as chemokines and cytokines, which induce their chemotaxis to the tumor site.

The host's wound healing associated mechanisms - like angiogenesis and immunosuppression - become activated due to the predominance of DAMPs within the tumor microenvironment [9, 24]. Hence, neoplastic cells harness the regenerative capacities of the host for their own survival, proliferation and metastasis [26, 27]. This circumstance protects the tumor from being eradicated by the host's immune cells and promotes its spreading due to neovascularisation, on the one hand, but renders the tumor totally dependent on its microenvironment [48, 51], on the other hand. Two common observations support the notion of tumor's reliance on its microenvironment *in vivo:* 1. It is very difficult - and some times even impossible - to cultivate tumor cells ex vivo, even though presumably optimal conditions as continuous and sufficient nutrient supply, controlled pH,

humidity, and temperature are provided *in vitro* [45, 49], and 2. Tumor cells do not metastasize randomly but rather follow a specific pattern, which does not always match with the infrastructure of lymph or blood vasculature [52]. Detailed knowledge on the interaction and interdependence [47] of tumor with its microenvironment opens new therapeutic options aiming to target and modulate the tumor microenvironment. Therefore, it is important to better characterize the bioactive factors responsible and indispensible for these effects and for tumor survival. DAMPs in particular can critically impact the tumor microenvironment by enhancing angiogenesis or influencing the immune response. DAMPs interact with their target cells by binding to surface receptors, of which some are well described, like some of the toll-like receptors (TLRs) [5, 8], the epidermal growth factor receptor (EGFR) [17] and the receptor for advanced glycation end products (RAGE) [4, 14, 24]. As "danger signals", DAMPs have also chemoattractive and activating effects on leukocytes; regarding granulocytes for instance, DAMPs enhance the oxidative burst of eosinophils leading to production of reactive oxygen species (ROS) [25], which in turn are able to oxidize DAMPs. Oxidized DAMPs lose their biologic activity [24, 25]. Identified DAMPs – shared by almost all cell types - include S100 proteins, uric acid, ATP, hyaluronan, heat shock proteins (HSP), heparan, syndecan, versican, and high mobility group box 1 (HMGB1) [25, 28].

### 1.1.2 HMGB1

HMGB1 is a highly conserved, intracellular protein that is physiologically involved in transcriptional processes. It is found in almost all cell types and located in both nucleus and cytoplasm [14, 18, 36]. As a prototypic DAMP member, it is released upon necrotic, but not upon apoptotic cell death [14, 36, 39]. Cells that have been activated by HMGB1 (which are mainly leukocytes) are known to produce cytokines and express adhesion molecules [28]. Furthermore HMGB1 is associated with autoimmune diseases like systemic lupus erythematosus or rheumatoid arthritis [14, 36]. HMGB1 is known to be sensitive to oxidation, thus requiring reducing conditions in order to maintain its biologic activity.

### 1.1.3 S100A4

S100A4 is a member of the heat labile S100-calcium-binding family, and - like other DAMPs – is released following necrosis [9]. It is involved in regulation of angiogenesis, cell motility, invasion and cell survival [4, 9]. The expression of S100A4 is known to be increased in colorectal cancer cells, and a higher serum level of S100A4 is associated with increased risk of metastasis in colorectal carcinomas [4, 9, 32]. Furthermore, elevated levels of S100-proteins in the serum can be detected during inflammatory conditions and trauma [9, 40]. Like HMGB1, S100A4 is very sensitive to oxidation and is dependent on reducing conditions – as found within tumor microenvironment – in order to maintain its biologic activity [23].

### 1.1.4 Uric Acid

Uric acid is the end product of the purine nucleotide-breakdown and is found in higher concentrations whenever local conditions demand a higher cellular turnover as can be found following cell injury and subsequent proliferation or neoplastic transformation [3]. Uric acid is not only capable of inducing an acute inflammatory reaction, but is also responsible for maintenance of chronic inflammatory conditions as can be found in gout [9, 42]. Local and systemic concentration of uric acid is elevated in cancer patients due to inflammation, necrosis and high cellular turnover [9]. Of note, uric acid is a strong antioxidant. In humans, over half the antioxidant capacity of blood plasma comes from uric acid [29], thus higher concentrations of uric acid within tumor tissue and blood of cancer patients may critically contribute to providing a reducing microenvironment (which possibly protects DAMPs from being oxidized and thus becoming inactivated).

## 1.2 Mesenchymal Stromal Cells

### 1.2.1 Physiology

MSCs are pluripotent progenitor cells that contribute to the maintenance and regeneration of a variety of connective tissues [34]. Although MSCs mainly home

in the bone marrow, they are also distributed throughout many other tissues, where they are believed to serve as local sources of dormant stem cells [24, 50]. The participation of MSCs in tissue formation becomes apparent in cases of tissue remodelling after injury or chronic inflammation [50]. In these conditions MSCs are mobilized from the bone marrow and recruited to the site of cell and tissue damage. MSCs have been shown to contribute to the formation of fibrous scars after injury [10]. In the setting of wound healing, MSCs can act as immunoregulatory cells by releasing IL-10 and prostaglandin E2, or by expressing the enzyme indolamine-2,3-dioxygenase (IDO) [19, 31, 44]. IDO converts the essential amino acid tryptophan to kynurenine. Local decrease of tryptophan and increase of kynurenine is associated with immunosuppression and a shift from a Th1 to a Th2 response [9, 24, 33]. Expression of IDO in MSCs is induced by IFNγ [31], which is generally found within inflammatory tissue. Especially T-cells, but also other leukocytes, can be inhibited in their proliferation and activation due to IDO expression and activity [33].

The receptor for advanced glycation end products (RAGE) [4, 14], epidermal growth factor receptor (EGFR) [17], and toll-like receptor 4 (TLR-4) [5, 8] are extracellular receptors for S100A4, all three receptors are reported to be expressed on MSCs [17, 21, 35, 41]. RAGE has also been described as a receptor for HMGB1, thus DAMPs seem to share receptors [25].

### 1.2.2 MSCs in the Tumor Microenvironment

MSCs are often found within tumors [13] and have been reported to promote cancer progression and metastasis [16, 43]. MSCs can migrate to the sites of inflammation [50] and damage as can be found in tumor tissue and thus participate in the tumor microenvironment [1, 2, 11, 18]. Systemically transferred MSCs have been described to migrate into colon carcinomas [9, 15, 24]. By expressing connexin-43, MSCs reduce local concentrations of ROS [46] and thus provide a niche for tumor cells protecting them and released DAMPs from ROS and thus oxidation. Furthermore MSC-mediated local immunosuppression could possibly be one reason for insufficient anti-tumor immune response [16].

## 1.3 Aims

Based on the fact that accumulation of MSCs is associated with a poor prognosis regarding tumor metastasis and the survival of cancer patients [16, 43] (probably due to MSC-initiated immunosuppression), the aim of this study was to identify factors within the tumor microenvironment, which could be responsible for the MSC migration to the site of tumor and the local proliferation of MSCs. Given that DAMPs are found in basically all advanced tumor tissues and that they are known to promote tumor angiogenesis and metastasis [48, 51], this study sought to determine if there was any association between DAMPs and MSC biology, in terms of MSC chemotaxis and proliferation. According to the published observations that oxidized DAMPs lose their stimulatory capacity on granulocytes [25], it was also important to investigate, if the same was true for the biologic activity of DAMPs on MSCs. Three specific DAMP family members, namely HMGB1, S100A4, and uric acid, seemed to be promising candidates to be further analyzed, given that:

1) Higher concentrations of all three above mentioned DAMP family members are found in cancer patients [20, 27, 32, 36, 38]

2) S100A4 is reported to be associated with higher metastasis rates in cancer patients [4, 32]

3) S100A4 and HMGB1 are both known to be sensitive to oxidation and thus requiring reducing conditions to maintain their biologic activity [23, 24, 25]

4) Uric acid is a potent anti-oxidant possibly acting as a protector for HMGB1 and S100A4 [29].

# 2 Material and Methods

## 2.1 Material

### 2.1.1 Instruments

| | |
|---|---|
| Biofuge A Centrifuge | Heraeus, Hanau, Germany |
| Eppendorf Centrifuge 5810R | Eppendorf, Hamburg, Germany |
| Incubator for Cell Cultures | Heraeus, Hanau, Germany |
| FACScan™ | Becton Dickinson, Heidelberg, Germany |
| POLARstar Omega | BMG Labtech, Ortenberg, Germany |
| Zeiss Microscope | Zeiss, Oberkochen, Germany |

### 2.1.2 Plastic Materials

| | |
|---|---|
| BD Falcon™ HTS Fluoroblok™ 96-Multiwell Insert System | Becton Dickinson, Heidelberg, Germany |
| BD Falcon™, 5ml polystyrene round bottom tubes for FACS measurement | Becton Dickinson, Heidelberg, Germany |
| Eppendorf tubes | Eppendorf, Hamburg, Germany |
| NUNC Immunomodule MultiSorp™ | Nunc, Wiesbaden, Germany |
| NUNClon™, surface, disposables for cell cultures | Nunc, Wiesbaden, Germany |
| NUNC multidish 96 wells | Nunc, Wiesbaden, Germany |

### 2.1.3 Chemicals

| | |
|---|---|
| Alpha-Modified Minimal Essential Medium (alpha-MEM) | Lonza, Cologne, Germany |
| Ampuwa™ Destillated 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 | AbD Serotec, Duesseldorf, Germany |
| Anti-human EGFR-PE | Becton Dickinson, Heidelberg, Germany |
| Anti-human HMGB1 | Abcam, Cambridge, England |
| Anti-human IgG | Jackson ImmunoResearch, Newmarket, Suffolk, England |
| Anti-human IgG | Abcam, Cambridge, England |
| 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 | R&D Systems, Wiesbaden, Germany |
| Anti-human IgG2a-PE | eBioscience, Frankfurt, Germany |
| Anti-human IgG2b-PE | Becton Dickinson, Heidelberg, Germany |
| Anti-human RAGE | Abcam, Cambridge, England |
| Anti-human S100A4 | Aldevron, Freiburg, Germany (formerly Genovac) |

| | |
|---|---|
| Anti-human S100A4 | Lifespan Biosciences, Eching, Germany |
| Anti-human TLR4-PE | eBioscience, Frankfurt, Germany |
| Anti-mouse IgG-HRP | Abcam, Cambridge, England |
| Anti-rabbit IgG-PE | Abcam, Cambridge, England |
| Bovine Albumin Fraction V | Carl Roth, Karlsruhe, Germany |
| CellTrace™ (CFSE) | Life Technologies, Darmstadt, Germany |
| CyQuant™ | Life Technologies, Darmstadt, Germany |
| Dulbecco's Modified Eagle Medium (DMEM) | Life Technologies, Darmstadt, Germany |
| EDTA | Sigma-Aldrich, Seelze, Germany |
| Fetal Bovine Serum (FBS) | Life Technologies, Darmstadt, 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 (HCl) | AppliChem, Darmstadt, Germany |
| Hydrogen Peroxide ($H_2O_2$) | Fischar, Saarbruecken, Germany |
| Penicillin-Streptomycin (PenStrep™) | Life Technologies, Darmstadt, Germany |
| Phosphate Buffered Saline (PBS) | Lonza, Cologne, Germany |
| Platelet Lysate (PL) | 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, Seelze, Germany |
| Sodium Azide | Sigma-Aldrich, Seelze, Germany |
| Sodium Hydroxide (NaOH) | Merck, Darmstadt, Germany |
| Soluble RAGE HEK | ProSpec, Rehovot, Israel |
| Sulfuric Acid ($H_2SO_4$) | Sigma Aldrich, Seelze, Germany |

| | |
|---|---|
| Tetramethylbenzidine (TMB) Substrate Kit | Thermo Fisher Scientific, Rockford, IL, USA |
| Trypan Blue Solution | Sigma-Aldrich, Seelze, Germany |
| Trypsin (2.5%) | Life Technologies, Darmstadt, Germany |
| Tween 20 | Sigma-Aldrich, Seelze, Germany |
| Uric Acid (UA) | Sigma-Aldrich, Seelze, Germany |

### 2.1.4 Cell Culture Medium and Culturing Conditions

As standard cell culture medium (if not otherwise indicated) phenol-red free DMEM supplemented with 10% human serum of blood group AB was used. Considering the fact that S100A4 is present in human serum [4, 14], all experiments focussing on the bioactivity of S100A4 were performed with heat-inactivated serum in order to inactivate S100 within serum and thus decrease the background noise. All cells were incubated in a humidified atmosphere at 37°C with 5% $CO_2$.

### 2.1.5 Other Reagents

EDTA-Trypsin: EDTA 0.02% supplemented with 0.05% trypsin.

## 2.2 Methods

### 2.2.1 Isolation and Characterization of MSCs

Heparinized bone marrow was taken from healthy donors and cultured in alpha-modified minimal essential medium (α-MEM) supplemented with 10% human platelet lysate (PL) and containing 100 U/ml penicillin, and 100 µg/ml streptomycin. At a confluence below 80%, adherent cells were harvested and passaged by trypsinizing. 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 PBS for 15 minutes at room temperature using the following antibodies according

to the manufacturer's recommendations:
CD105-FITC, CD3-PerCP, CD34-PE, CD45-PerCP, CD73-PE, CD90-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. Experiments were performed only on MSCs which were cultured at least 24hrs in standard cell culture media as described above. In order to assess the expression of S100A4 receptors on MSC, the cells were stained with the following antibodies: Rabbit anti human RAGE as primary and PE-labelled goat anti-Rabbit IgG as secondary antibody, PE-labelled mouse anti-human TLR4 and IgG2a as corresponding isotype control, PE-labelled mouse anti-human EGFR and mouse IgG 2b as corresponding isotype control. FACS analysis was performed using FACScan™, data were analyzed using FlowJo™ software (version 9.3.1; Tree Star, Stanford, CA). For every assay, at least 50,000 MSCs were collected.

### 2.2.2 Tumor Cell Lines

The colorectal tumor cell lines HCT-116, CACO-2 and COLO-678 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and cultured according to our standard culturing conditions. For cell passaging purposes, cells were harvested by using EDTA-Trypsin.

### 2.2.3 Preparation of Cell Lysates and Soluble DAMPs

EDTA only (without trypsin) was used to detach adherent cells, in order to protect surface proteins from enzymatic degradation due to exposure to trypsin.
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-thawing (F/T). For platelet lysates (PL) regular platelet concentrates containing about $10^9$ platelets per ml autologous plasma were used. The viability following F/T treatment was assessed using trypan blue exclusion and was always below 0.1%. In order to obtain soluble DAMPs, lysates were spun down hard (16.300 x g) twice and the supernatants

(soluble DAMPs) were used at indicated concentrations.

### 2.2.4 Oxidation of Soluble DAMPs

In order to oxidize DAMPs, soluble DAMPs (see above) were incubated for 2 hours at room temperature with $H_2O_2$, at concentrations that are usually found within inflammatory tissue, i.e. 0.01mM or 0.02mM [24]. Sham treatment of native DAMPs was performed using with PBS instead of $H_2O_2$.

### 2.2.5 Depleting Specific DAMPs within Necrotic Material/Soluble DAMPs

Soluble DAMPs which were obtained as described above were preincubated with either anti-human S100A4, anti-human HMGB1, soluble RAGE or the appropriate isotype controls at indicated concentrations for 30 minutes at room temperature to deplete S100A4 and/or HMGB1 from the necrotic material, respectively.

### 2.2.6 Preparation of Uric Acid

Uric acid was dissolved in 1M NaOH by heating at 60°C for 1 hour to obtain a stock solution of 3mg/ml. Afterwards the pH was adjusted to 7.4 by adding 1M HCl. NaCl was substituted for uric acid in osmolarity and pH controls.

### 2.2.7 Proliferation Assay

MSCs were cultured in a sterile 96-well plate (200 MSCs/well) in phenolred-free DMEM containing indicated concentrations of heat-inactivated pooled human serum in combination with soluble DAMPs obtained from lysed cells, or alternatively with recombinant human S100A4, recombinant HMGB1, or uric acid as stimulus at indicated concentrations. After 5 or 7 days adherent MSCs were washed with PBS and the cell count was assessed by staining for DNA with CyQuant™ according to manufacturer's guidelines and using a microplate reader to measure fluorescence.

Alternatively, MSC proliferation was measured performing FACS analysis of CFSE labelled MSCs using CellTrace™ to stain the cells according to manufacturer's guidelines. About 50000 CFSE-labelled MSCs were added to each well of a 24 well plate with a final volume of 1ml / well. After 5 days of incubation in the presence of indicated concentration of DAMPs MSCs were harvested using EDTA-Trypsin and their fluorescence was assessed by flow cytometric means. Loss of fluorescence was considered as cell division, thus proliferation.

### 2.2.8 Chemotaxis Assay

For chemotaxis assays BD Falcon™ HTS Fluoroblok™ Insert systems with a pore size of 8µm were used. MSCs were stained with CFSE using CellTrace™ according to manufacturer's recommendations. About $75x10^3$ CFSE stained MSCs were added on the top of the membrane of each chemotaxis chamber. Soluble DAMPs, or specific DAMP members - i.e. rhHMGB1, uric acid, rhS100A4, or the combination of uric acid and S100A4 - was solved in 0.05% human serum albumin in PBS at at indicated concentrations and added into the lower chamber of the chemotaxis chamber. Human serum albumin (0.05%) in PBS and fetal bovine serum at a concentration of 50% served as negative and positive controls, respectively. The assay was performed for 45 to 90 minutes at 37°C. The fluorescence within the lower migration chamber, which correlated with the number of migrated MSCs, was measured using a microplate reader (POLARstar Omega, BMG Labtech).

### 2.2.9 ELISA for S100A4

In-house established ELISA for S100A4 was performed using Nunc Immunomodule MultiSorp™ plates, which were coated with rabbit polyclonal anti-S100A4 at a concentration of 2µg/ml as capture antibody. Non-specific protein binding sites on the plate were blocked with 4% bovine serum albumin before adding the samples. Monoclonal mouse anti-S100A4 (Clone NJ-4F3-D1) at a concentration of 2µg/ml was used as detection antibody. Finally, the plates were incubated with horse radish peroxidase (HRP) labelled rabbit polyclonal anti-mouse antibody at a dilution of 1:5000. TMB subtrate kit was used as substrate for

HRP following the manufacturer's instructions. The reaction was stopped with 1 N sulfuric acid and absorption was measured at 450 nm using PolarStar Omega plate reader.

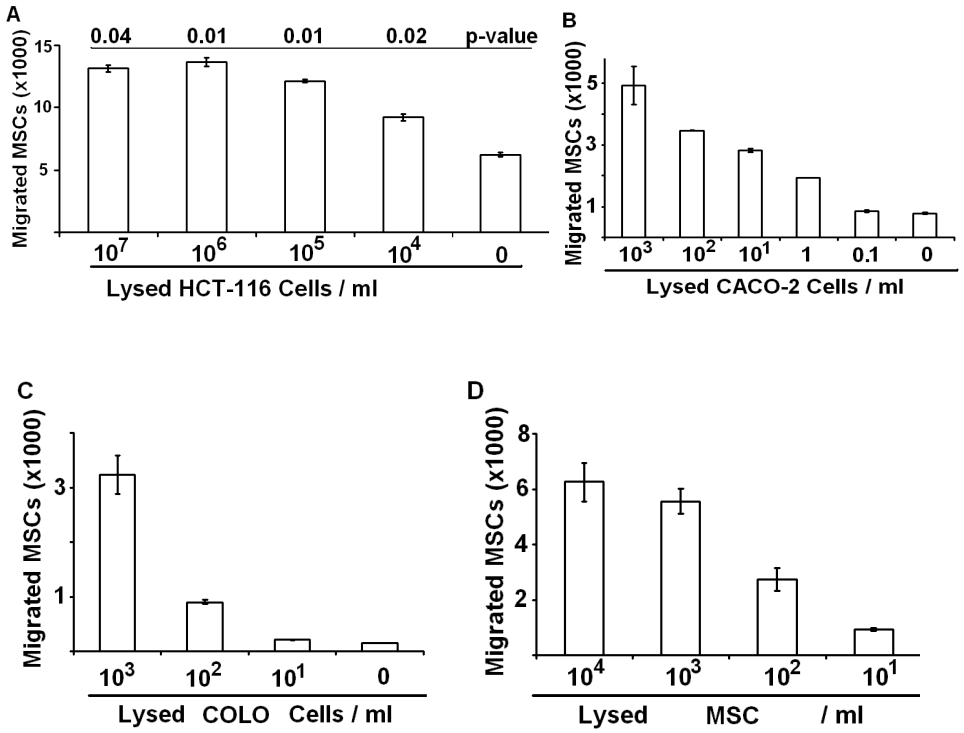
### 2.2.10 Statistics

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

# 3 Results

## 3.1 DAMPs Act as Chemoattractants on MSCs

Chemotaxis assays were performed with DAMPs obtained from lysed cells at graded concentrations between $10^1$ and $10^7$ lysed cells/ml. MSCs showed a dose-dependent chemotaxis towards native DAMPs regardless of their cellular origin with the optimal chemotactic concentration between $10^3$ and $10^6$ lysed cells/ml. Even though the dose-response was reproducible irrespective of the origin of cell lysate, the chemotactic activity was dependent on the origin of cell lysate suggesting the presence of shared chemottractants with different concentrations within cell lysates obtained from neoplastic (lysed tumor cells) (Figure 1A, B, C) or from normal (lysed MSCs) cells (Figure 1D).



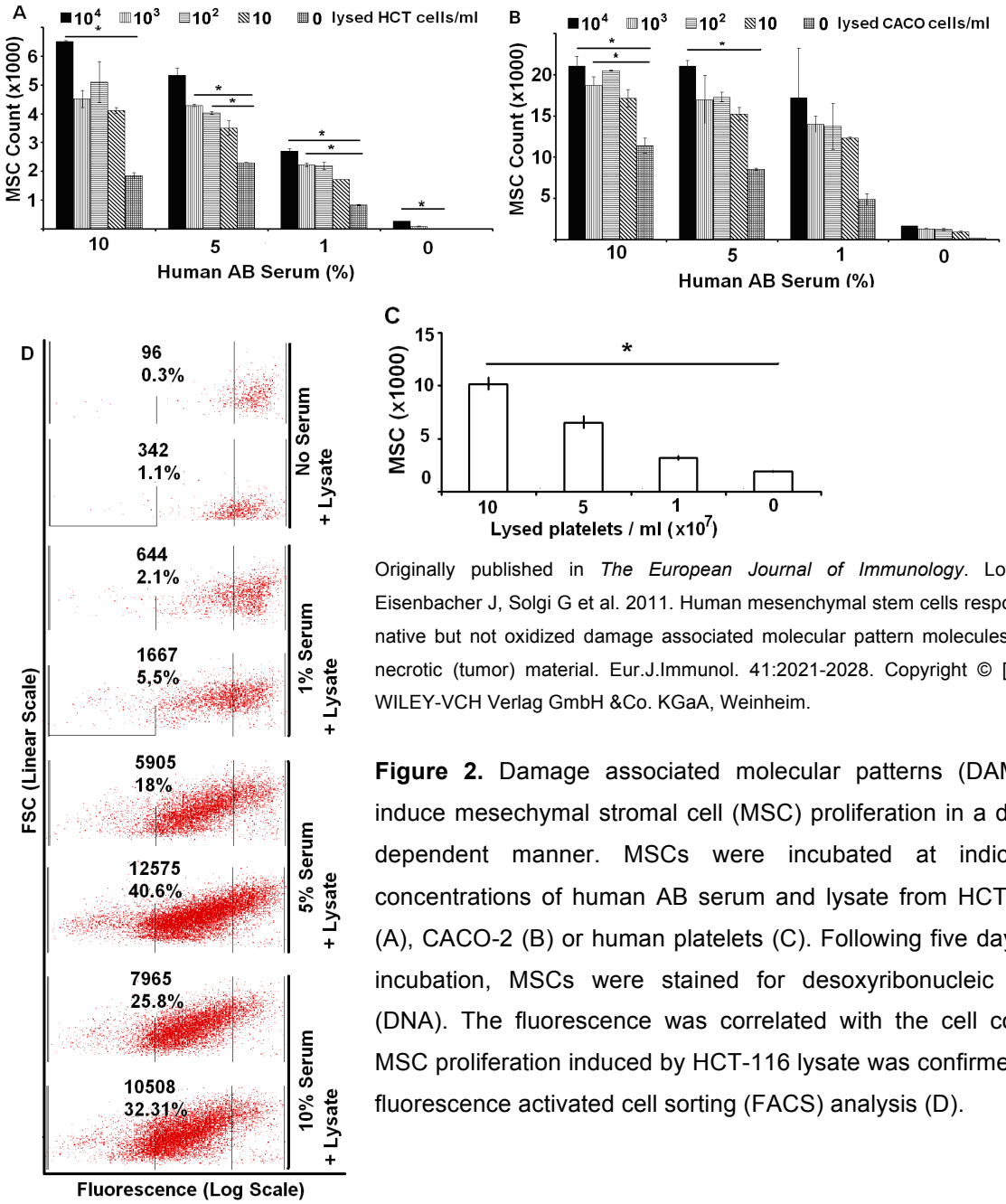

Originally published in *The European Journal of Immunology*. Lotfi R, Eisenbacher J, Solgi G et al. 2011. Human mesenchymal stem cells respond to native but not oxidized damage associated molecular pattern molecules from necrotic (tumor) material. Eur.J.Immunol. 41:2021-2028. Copyright © [2011] WILEY-VCH Verlag GmbH &Co. KGaA, Weinheim.

**Figure 1.** Damage associated molecular patterns (DAMPs) act as chemoattractants towards mesenchymal stromal cells (MSCs). DAMPs were obtained from lysed human cell lines of different origins (A: HCT-116, B: CACO-2, C: COLO-678, D: MSCs) and used as chemoattractants. Appromximately 75,000 fluorescently labelled MSCs were added to the upper chamber of the

chemotaxis system and allowed to migrate through the chemotaxis membrane towards graded concentrations of necrotic material as indicated. After 45 minutes at 37°C, fluorescence in the lower chamber was measured and correlated with the cell count. Representative data from at least three individual experiments are shown. Error bars indicate standard deviation (SD).

## 3.2 DAMPs Induce Proliferation of MSCs

The presence of individual concentrations of soluble DAMPs within culturing media enhanced the proliferation of MSCs dose-dependently within five days of incubation (Figure 2).




Originally published in *The European Journal of Immunology*. Lotfi R, Eisenbacher J, Solgi G et al. 2011. Human mesenchymal stem cells respond to native but not oxidized damage associated molecular pattern molecules from necrotic (tumor) material. Eur.J.Immunol. 41:2021-2028. Copyright © [2011] WILEY-VCH Verlag GmbH &Co. KGaA, Weinheim.

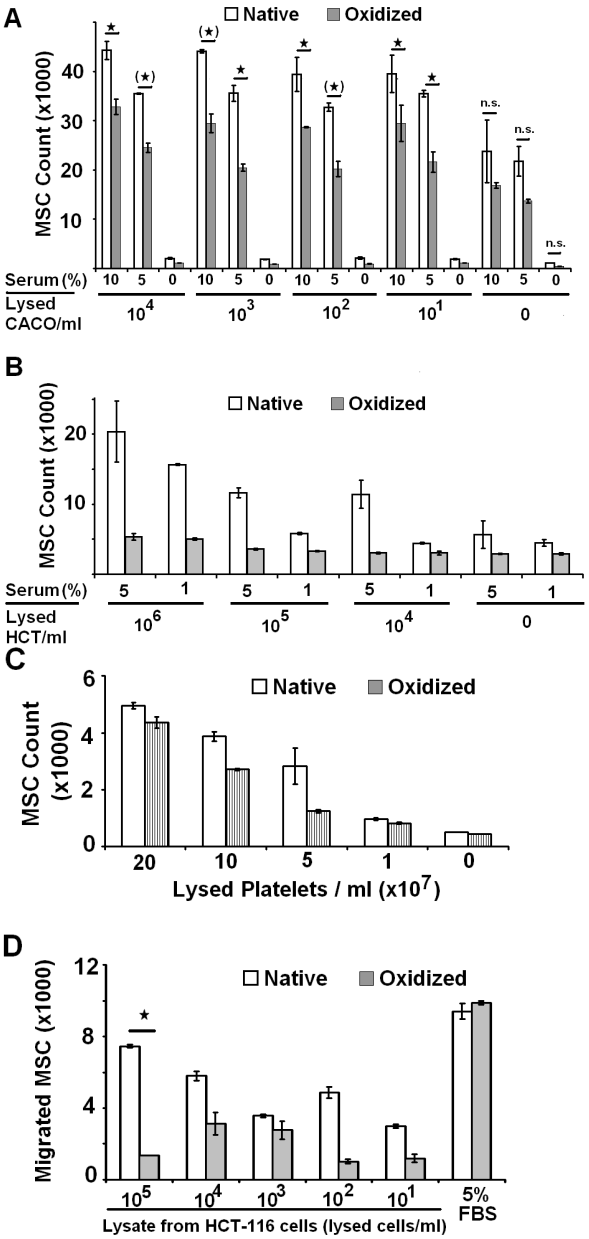
**Figure 2.** Damage associated molecular patterns (DAMPs) induce mesechymal stromal cell (MSC) proliferation in a dose-dependent manner. MSCs were incubated at indicated concentrations of human AB serum and lysate from HCT-116 (A), CACO-2 (B) or human platelets (C). Following five days of incubation, MSCs were stained for desoxyribonucleic acid (DNA). The fluorescence was correlated with the cell count. MSC proliferation induced by HCT-116 lysate was confirmed by fluorescence activated cell sorting (FACS) analysis (D).

Here, the MSCs were stained with carboxyfluorescein succinimidyl ester (CFSE) and cultured for five days. Cells cultured without any serum or DAMPs (top panel) served to set the threshold for non-proliferating cells. Loss of fluorescence was considered cell division and thus as proliferation. Y-axis shows forward scatter (FSC). Indicated is cell number and ratio of proliferated cells. The figure shows representative data from at least six individual experiments. Error bars indicate standard deviation (SD).

Consistent with results from chemotaxis assays the response of MSCs to DAMPs was independent of the cellular origin of lysates, which were not only obtained from neoplastic but also from healthy cells. The optimal DAMP concentration enhancing MSC proliferation was generally between $10^4$ and $10^6$ lysed cells/ml. Results from proliferation assays using CyQuant™ kits could be confirmed by flow cytometric means as an alternative method to measure MSC proliferation (Figure 2 D).

## 3.3 Oxidation of DAMPs Impairs Their Biologic Activity

It was already shown that DAMPs can act as chemoattractants and survival factors for granulocytes [24, 25, 29]. Interestingly, the chemotactic activity of DAMPs was abolished once the DAMPs were oxidized. Therefore, it seemed conceivable that oxidation could also affect the biologic activities of DAMPs on MSCs. Preincubation of DAMPs with $H_2O_2$ significantly reduced their stimulatory capacity on MSCs in terms of induction of proliferation and chemotaxis (Figure 3). This observation gave rise to hypothesize that those DAMPs which are sensitive to oxidation are mainly responsible for the stimulatory sum effect of necrotic material on MSCs. Accordingly, S100 proteins and HMGB1 seemed to be the most promising candidates from the DAMP family members.

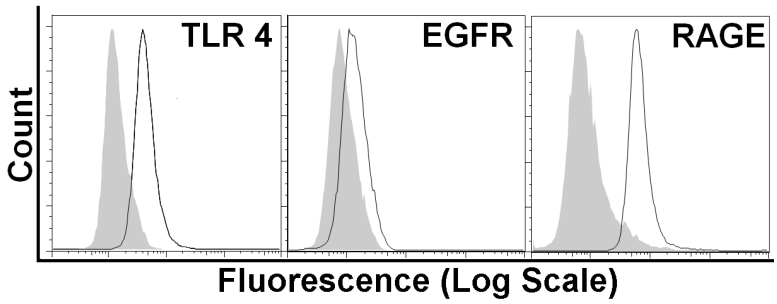



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**Figure 3.** Oxidation impairs biologic activity of damage associated molecular patterns (DAMPs). Open bars indicate mesenchymal stromal cell (MSC) count after stimulation with native DAMPs obtained from lysed CACO-2 cells (A), HCT-116 cells (B), or platelets (C). Reduced stimulatory capacity of oxidized DAMPs is shown with grey bars (A-C). Herefore, DAMPs were incubated with 0.02mM $H_2O_2$ for 30 minutes. Proliferation induction of DAMPs was significantly decreased once oxidized. Furthermore, chemotactic stimulation of HCT-116 lysate was impaired when preincubated with hydrogen peroxide ($H_2O_2$) (D). Fetal bovine serum (FBS) served as positive control for MSC migration. Representative results from three individual experiments. Error bars indicate standard deviation (SD). Asterisks indicate significance.

## 3.4 HMGB1 and S100 Receptors Are Expressed on MSC Surface

FACS analysis was performed on MSCs that were specifically stained for the cell surface expression of TLR4, EGFR and RAGE which are known receptors for HMGB1 and S100. MSCs of three different passages were used, in order to demonstrate the stable expression of RAGE, TLR 4, and EGFR (Figure 4).

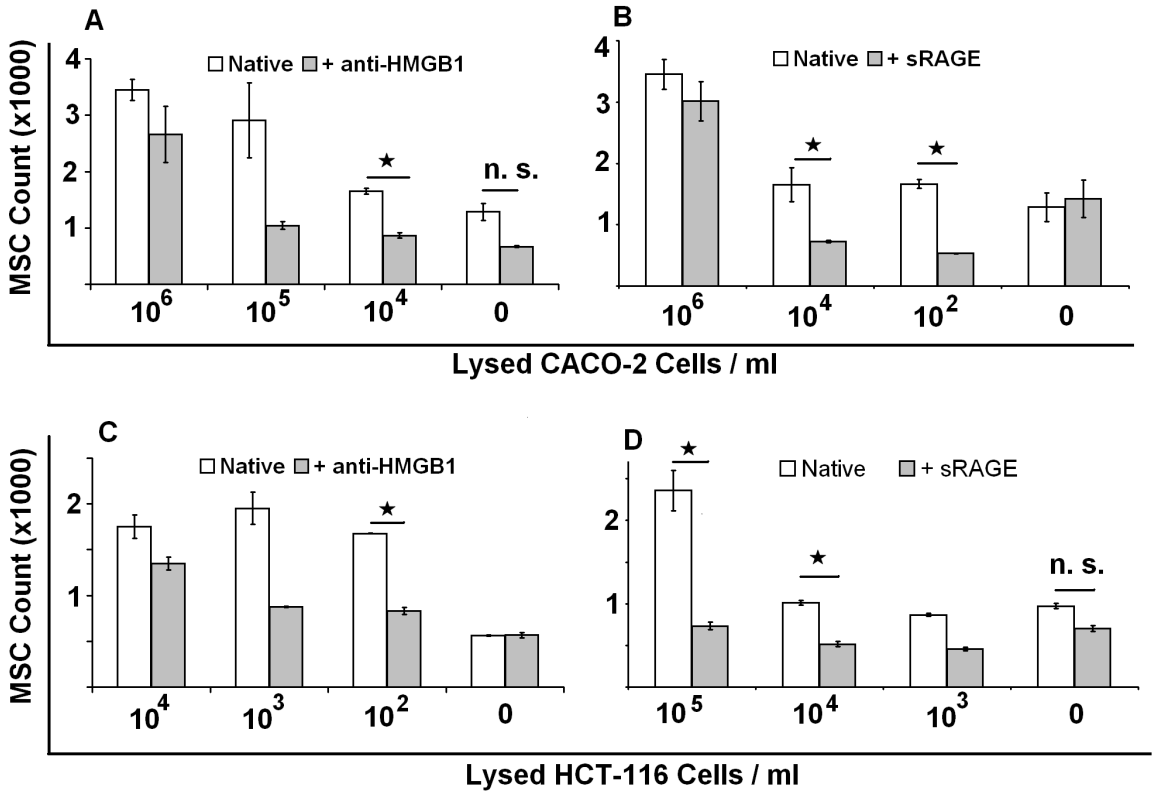



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**Figure 4.** Mesenchymal stromal cells (MSCs) show a stable expression of S100 and high mobility group box 1 (HMGB1) receptors. Flow cytometric analysis of MSCs which were stained with either isotype controls (grey curve) or specific antibodies against toll-like receptor 4 (TLR4), epidermal growth factor receptor (EGFR) and receptor for advanced glycation end products (RAGE) (open curve). Shown is one representative out of three individual experiments with different MSC passages.

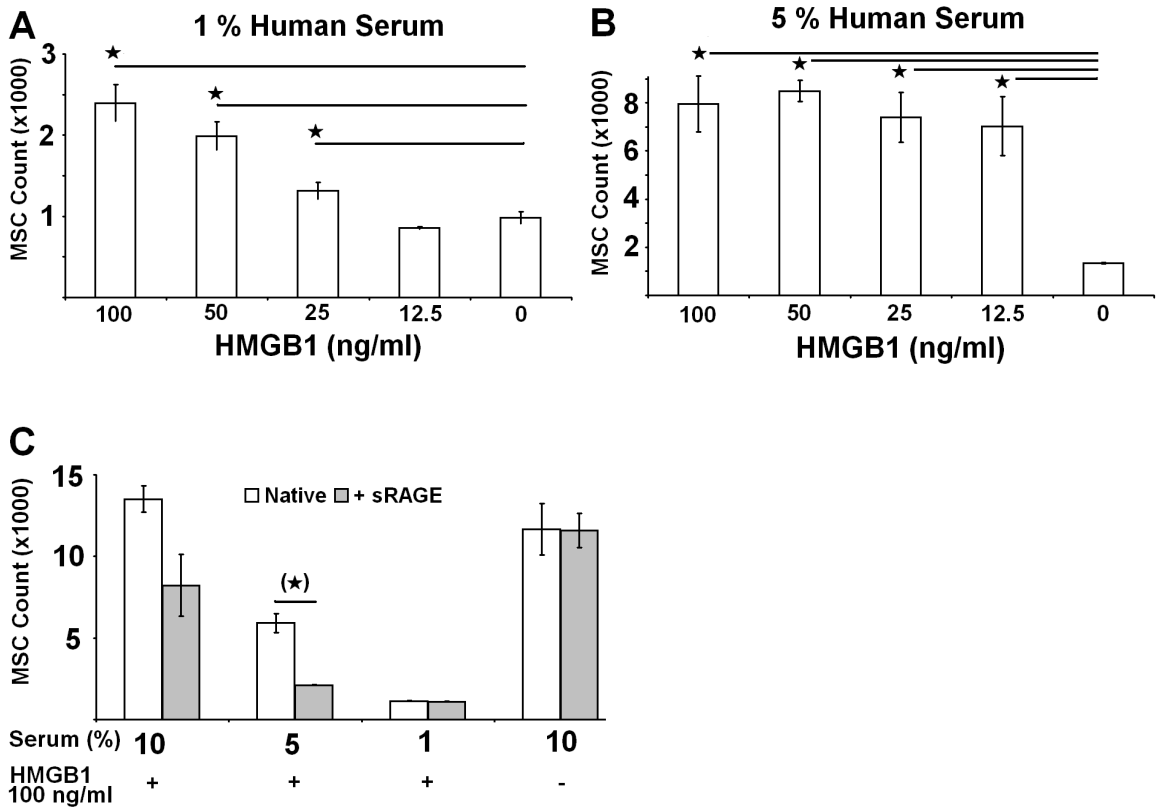
## 3.5 Depletion of HMGB1 within Lysate Reduces DAMP Mediated Effects

Depletion of HMGB1, a well-known member of the DAMPs, within tumor cell lysate by preincubation of lysate with anti-human HMGB1 or soluble RAGE (sRAGE), significantly inhibited the DAMPs mediated effects on MSCs. (Figure 5). This observation suggested that HMGB1 is a member of the DAMP family that supports the main effects of DAMPs on MSCs. Proliferation and chemotaxis assays with recombinant human HMGB1 instead of DAMPs confirmed this suggestion (Figure 6, Figure 7). However, HMGB1 alone did not count for the complete DAMP mediated effects.



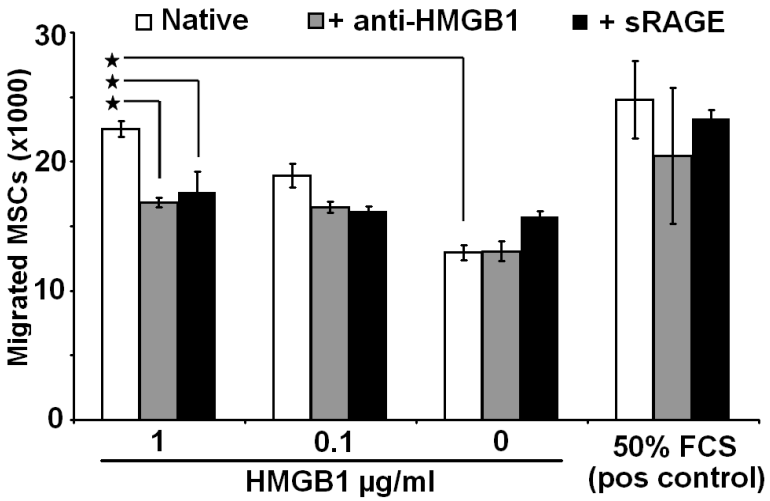

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**Figure 5.** High mobility group box 1 (HMGB1) plays a crucial role in the proliferation-inducing effect of damage associated molecular patterns (DAMPs) on mesenchymal stromal cells (MSCs). MSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5% heat-inactivated pooled human serum and in the presence of individual concentrations of native DAMPs obtained from lysed colorectal cells CACO-2 (A and B) or HCT-116 (C and D). On day five proliferation of MSCs exposed to native DAMPs (open bars) was measured and compared with proliferative capacity of MSCs exposed to DAMPs from the same origin but pre-incubated for 2h (grey bars) with specific mouse polyclonal antibodies to human HMGB1 protein (A and C) or 1mg/ml soluble receptor for advanced glycation end products (sRAGE) (B and D). Representative results from one out of four independent experiments with standard deviation (SD) as error bars are shown.




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**Figure 6.** Recombinant human high mobility group box 1 (HMGB1) enhances proliferation of mesenchymal stromal cells (MSCs). MSCs were incubated for five days in the presence of either 1% (A) or 5% (B) human serum and graded concentrations of HMGB1. After five days MSCs were stained for desoxyribonucleic acid (DNA) and the fluorescence, which correlated with DNA content, was measured. The presence of soluble receptor for advanced glycation end products (sRAGE), which depletes HMGB1 from culturing media abrogates the proliferation inducing effect of HMGB1 on MSCs (C). The figure shows representative data from four (A), two (B) and three (C) experiments. Error bars show standard deviation (SD), asterisks indicate significance.



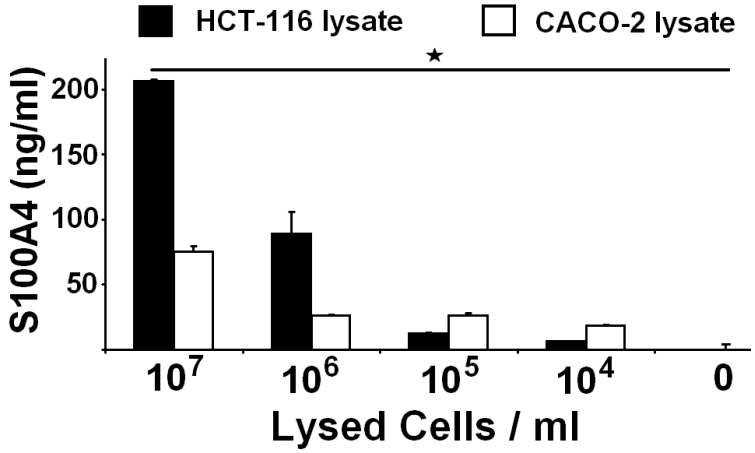

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**Figure 7.** Mesenchymal stromal cells (MSCs) migrate toward high mobility group box 1 (HMGB1) protein in a dose-dependent manner. The chemotactic activity of HMGB1 was blocked in the presence of specific antibodies to HMGB1 or soluble receptor for glycation end products (sRAGE), which is a receptor for HMGB1. 50% fetal calf serum (FCS) served as positive control. Shown are representative data from one out of at least three independent experiments for each cell type with standard deviation (SD) as error bars.

## 3.6 S100A4 Is Present in Tumor Cell Lysates

Given that RAGE also serves as a receptor for S100 proteins, the pre-incubation of DAMPs with sRAGE – as performed in figure 5 - could have depleted S100 proteins besides HMGB1, thus S100 proteins were the next DAMP family members to be investigated for their participation in the sum stimulatory effect of soluble DAMPs on MSCs.

Performing ELISA assays the presence of S100A4 in lysates could be confirmed (Figure 8).

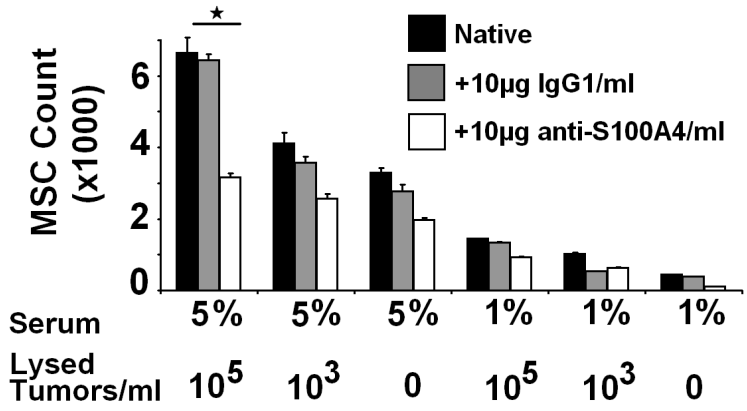



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**Figure 8.** The presence of S100A4 can be detected by enzyme-linked immunosorbent assay (ELISA). In-house established ELISA confirmed different S100A4 concentrations in tumor cell lysates of the cell lines HCT-116 and CACO-2. One representative out of three independent experiments is shown. Error bars show standard deviation (SD).

## 3.7 Depletion of S100A4 within Tumor Lysate Reduces DAMP Mediated MSC Proliferation

Pre-incubation of soluble DAMPs with anti-human S100A4 significantly reduced the proliferative response of MSCs to thus treated lysates suggesting that S100A4 plays a crucial role (Figure 9).



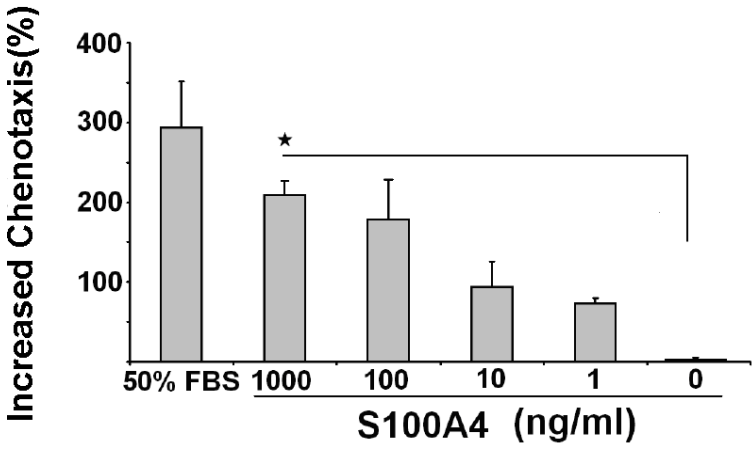

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**Figure 9.** Reduction of S100A4 inhibits proliferation-inducing capacity of damage associated

molecular patterns (DAMPs). Tumor lysate was preincubated with anti-S100A4 or immunoglobuline class G1 (IgG1) as isotype control for 30 minutes. Mesenchymal stromal cells (MSCs) and stimuli were incubated for five days with different concentrations of human serum. MSCs were stained for desoxyribonucleic acid (DNA) and fluorescence was measured. Shown is mean MSC proliferation from three independent experiments with standard deviation (SD) as error bars, indicating inter-assay variability. Error bars show SD, asterisks indicate significance.

## 3.8 S100A4 Crucially Influences MSC Migration and Proliferation

Performing transwell-migration assays using fluorescent-labelled MSCs, the dose-dependent chemotaxis of MSCs towards recombinant human S100A4 could be shown (Figure 10).




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**Figure 10.** S100A4 acts as a chemoattractant towards mesenchymal stromal cells (MSCs). MSCs were stained with carboxyfluorescein succinimidyl ester (CFSE) and then incubated with different concentrations of chemoattractants. 50% of fetal bovine serum (FBS) served as positive control. After 45 minutes at 37°C fluorescence was measured. Data from 3 independent experiments were normalized to random migration (0.1% human serum albumin) and shown as mean increased chemotaxis with standard deviation (SD) as error bars indicating inter-assay variability. Error bars show SD, asterisks show significance.

Next, MSCs were cultured for seven days in the presence of individual concentrations of S100A4 within culturing media demonstrating that concentrations up to 100ng S100A4/ml media have a proliferation-promoting effect

on MSCs, whereas higher concentrations inhibit MSC proliferation down to about 74% compared to the condition without S100A4 (Figure 11).

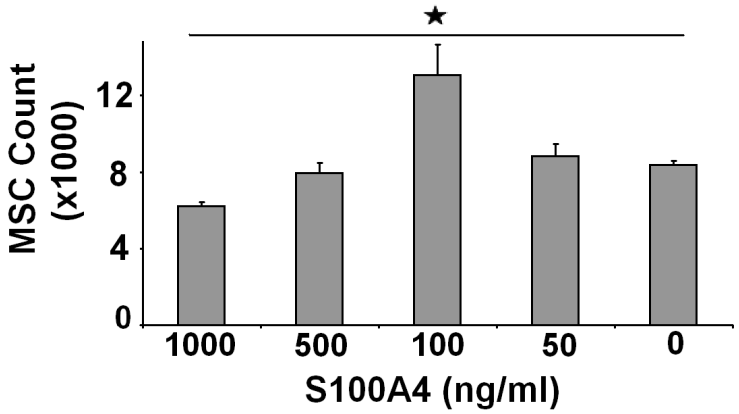



**Figure 11.** S100A4 has both proliferation inducing and inhibiting capacities. In the presence of 5% human serum, mesenchymal stromal cells (MSCs) were stimulated for 7 days with given concentrations of recombinant human S100A4. Shown is the mean MSC count (±standard deviation (SD)) of one representative out of six independent experiments. S100A4 promotes MSC proliferation at concentrations up to 100 ng/ml while it inhibits the same at higher concentrations**.** P-values below 0.05 were considered significant and indicated with asterisks.

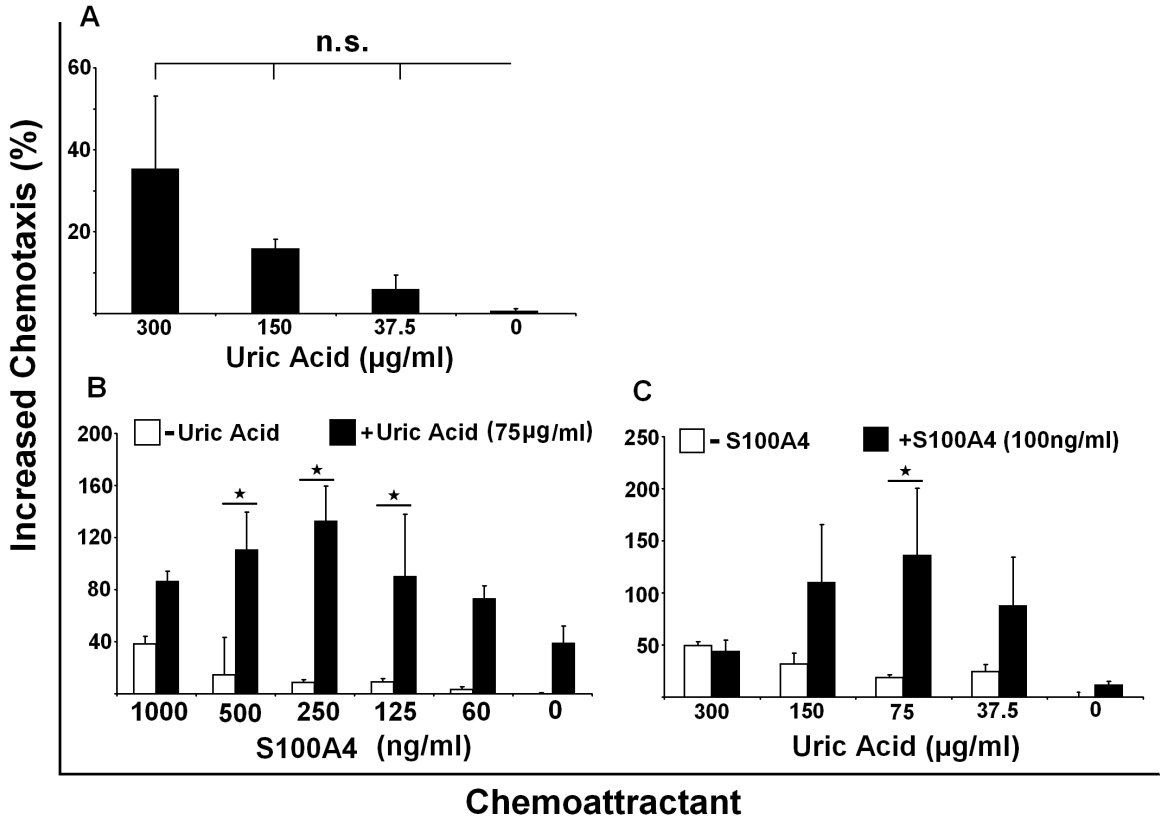
In conclusion S100A4 has divergent effects on MSC proliferation, but acts as a strong chemoattractant on them.

## 3.9 Uric Acid Enhances the Chemotactic Activity of S100A4

The assessment of the effect of uric acid - another member of the DAMP family – on MSCs seemed interesting because of two reasons: 1. There are available drugs influencing the concentration of uric acid in human blood and 2. uric acid is a very strong reducing agent and a potent antioxidant. In humans, over half the antioxidant capacity of blood plasma comes from uric acid [28].

Given that the biologic activity of necrotic material on MSCs is abolished following oxidation it seemed interesting to determine how uric acid participates in the sum effect of necrotic material on MSCs. Thus the biologic activity of uric acid *per se* and in combination with S100A4, was determined (Figure 12), considering the fact that S100 proteins are known to be susceptible to oxidative modifications. While the chemotactic activity of uric acid on MSCs was rather weak and did not reach

significance, the addition of uric acid to suboptimal concentrations of S100A4 (100 ng/ml) enhanced the chemotactic capacity of S100A4. Best results were obtained with 75µg uric acid/ml. These results could be confirmed by adding individual concentrations of S100A4 to 75µg uric acid/ml.

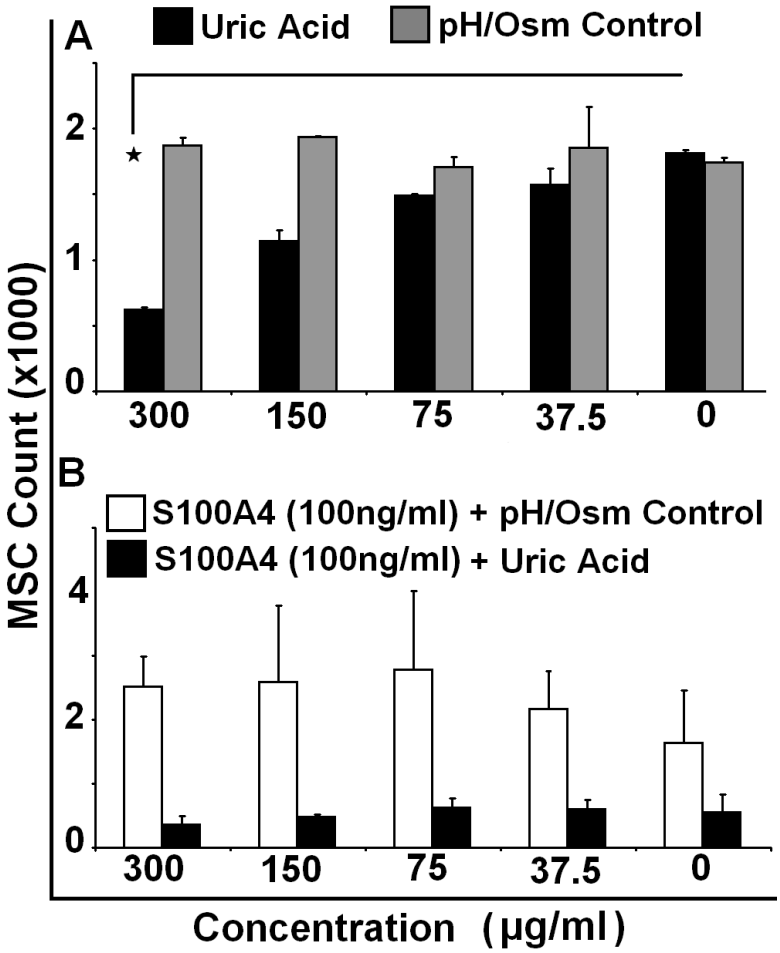



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**Figure 12.** Chemotactic acitivity of S100A4 on mesenchymal stromal cells (MSCs) is enhanced by uric acid.

After 45-90 minutes the chemotaxis of fluorescently labelled MSCs towards indicated concentrations of uric acid (panel A) and the combination of uric acid and S100A4 (panels B and C) was measured. Fetal bovine serum (FBS) (50%) and human serum albumin (0.05%) were used as positive and negative control, respectively. For each panel, data from 3 independent experiments were normalized to random migration (0.1% human serum albumin) and shown as mean increased chemotaxis with standard deviation (SD) as error bars indicating inter-assay variability. P-values below 0.05 were considered significant and indicated with asterisks. P-values above 0.05 were considered as random migration and were indicated with "not significant" (n.s.).

## 3.10 Proliferation-Inhibiting Effect of Uric Acid on MSCs Cannot Be Antagonized by S100A4




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**Figure 13.** Proliferation-inhibiting effect of uric acid on mesenchymal stromal cells (MSCs) cannot be antagonized by S100A4. In the presence of uric acid alone (panel A), or uric acid in combination with S100A4 (panel B) MSCs were cultured in media containing 5% human serum. Cell count was assessed indirectly by measuring desoxyribonucleic acid (DNA) content. The pH/osmolarity (Osm) control contains the same pH and osmolarity as for given concentrations of uric acid. Uric acid inhibits MSC proliferation in a dose dependent manner (A), the inhibitory effect of uric acid cannot be abolished by adding S100A4 to the culturing media (B), while the proliferation promoting effect of S100A4 can be seen in conditions containing pH/osmolarity controls. Shown are mean values (±standard deviation (SD)) from one representative out of three individual experiments for each panel. P-values below 0.05 were considered significant and indicated with asterisks.

Surprisingly, uric acid inhibited the proliferation of MSCs in a dose-dependent manner with best results at 300µg/ml (Figure 13 A).

The combination of uric acid at different concentrations with S100A4 did not support the proliferation-promoting effect of S100A4 on MSCs, but the inhibitory effect of uric acid dominated and was pivotal (Figure 13 B). This observation was inconsistent with the proliferation-enhancing sum effect of necrotic material, as well as with uric acid's synergistic effect on chemotactic capacity of S100A4 on MSCs.

# 4 Discussion

In order to survive and proliferate tumor cells need an optimal microenvironment containing nutrients, angiogenic factors and immunoregulatory cytokines/chemokines released by prevailing and infiltrating tumor-associated leukocytes and MSCs [24]. An inflammatory response by the host may not necessarily be associated with a better outcome [24]. Indeed, leukocyte infiltration into cancer tissues may also imply a poor prognosis for patients when regarding the crucial role of T regulatory cells [24]. The development of a tumor over many years typically leads to reciprocal modifications in the host and the tumor. Often, enhanced tumor growth in the setting of permanent necrosis and inflammation is being observed [24]. This paradoxical observation supports the idea that chronic inflammation and necrosis could rather provide a niche for tumor growth and proliferation [26, 27]. MSCs can be found within tumor tissue and can influence the biological behavior of tumor and the host's immune response to tumor [13]. Their presence within tumor tissue was shown to be associated with higher rates of metastasis and poor prognosis regarding survival [16, 43].

The here presented data demonstrate that DAMPs obtained from different sources of cells (cancer cell lines as well as healthy cells) promote migration and proliferation of MSCs. As a shared factor within these lysates, HMGB1 could be identified as a crucial factor that supports the overall effect of soluble DAMPs.

Interestingly, considerable differences between the stimulatory strength of lysates from different origins could be observed with the greatest differences between lysates from HCT-116 and CACO-2 cells requiring about 1000 times higher concentrations of HCT-116 lysates to induce a comparable chemotactic effect. Consistently, the inhibitory effect of anti-HMGB1, sRAGE, and oxidation differed between individual lysates indicating that further factors (i.e. cytokines or other DAMP members like S100 or heat shock proteins) may participate in the demonstrated biological/stimulatory effect of DAMPs on MSCs. These results demonstrating chemotactic activity of DAMPs — seen specifically with HMGB1— are consistent with already published data [24, 25].

The here presented data support the notion that DAMPs critically influence the tumor microenvironment. The tumor microenvironment is characterized by reducing/hypoxic conditions that protect DAMPs from losing their biological activity

due to oxidation [7]. Consistent with these results showing diminished biological activity of DAMPs following oxidation, HMGB1 and S100 are both known to lose their activity after oxidation [23, 25]. The effect of $H_2O_2$ within inflammatory tissue is still discussed controversial; $H_2O_2$ at low levels is know to act as ‘‘second messenger’’ for lymphocyte activation [37]; it has also been observed to have both proliferative and apoptotic effects on T cells through various signalling pathways [6, 12, 22].

As demonstrated here, DAMPs - especially HMGB1 - can act as chemoattractants to MSCs, nevertheless, HMGB1 alone was not sufficient to reach the same level of stimulation compared to the whole cell lysate, leading to the question if there is a more potent DAMP member strong enough to count for the sum effect of whole cell lysate.

S100A4 seemed promising because of the following reasons:

1. S100A4 has been shown to be overexpressed in colorectal carcinomas and elevated S100A4-levels are associated with a poor prognosis regarding to patient’s survival [4, 32]
2. RAGE, which is the extracellular receptor for S100A4 [4], has already been described on MSCs [21], and it could also be shown that the depletion of RAGE ligands in necrotic material could significantly reduce the sum effect of DAMPs on MSCs [24]
3. S100 proteins are known to be sensitive to oxidative environment [23] as are DAMPs concerning their stimulatory effect on MSCs and granulocytes [24, 25]. Additionally, induction of an oxidative microenvironment is a feasible condition to be generated locally in the setting of targeted tumor therapy.

Uric acid too, seemed to be a promising candidate from the DAMP family because of several circumstances, such as its antioxidant capacities.

Considering the fact that the biologic activity of necrotic material on MSCs is abrogated following oxidation it seemed interesting to determine how uric acid participates in the sum effect of necrotic material on MSCs and if uric acid - acting as reducing agent - could enhance S100A4 activity.

Consistent with the sum effect of necrotic material S100A4 acted dose-dependently as a chemoattractant on MSCs, this effect could be enhanced by adding uric acid, which by itself had a weak (if any) chemoattractive capacity. This observation supports the antioxidative impact of uric acid within necrotic material

and specifically on S100A4 but needs to be addressed and confirmed in future experiments assessing oxidated versus reduced states of S100A4 in the presence or absence of uric acid (or ascorbic acid) and correlating its redox-status with its biologic activity.

S100A4 in concentrations up to 100ng/ml enhanced the proliferation of MSCs, while inhibiting MSC proliferation when higher S100A4 concentrations were used, leading to the question which concentration is found *in vivo* within (necrotic areas of) tumor tissue and if the inhibition of proliferation is associated with the differentiation of MSCs which may also influence their immunoregulatory functions. The same questions could be asked for uric acid which - in contrast to the sum effect of DAMPs on MSCs - inhibited the proliferation of MSCs in a dose-dependent manner. Considering the fact that uric acid is a potent reducing agent, the question becomes whether or not MSCs rather prefer oxidative conditions for growth and proliferation. Generally, reducing conditions predominate within tumor tissue [7].

Even though S100A4 could enhance MSC proliferation, adding S100A4 to media containing uric acid could not abolish the inhibitory effect of uric acid, opening the question if another DAMP member(s) may be responsible for the sum effect of DAMPs on MSCs. Furthermore, it is conceivable that despite its inhibitory effect on MSC proliferation, uric acid could contribute to the stimulatory sum effect of whole tumor lysate by protecting other factors from oxidation since uric acid is known to be a powerful antioxidant.

Additionally it would be interesting to investigate the interaction between HMGB1 and S100A4, which are factors that have both been shown to support the overall effect of DAMPs. On the one hand, it is imaginable that they show synergistic effects, after all, both of them have MSC proliferation and migration inducing capacities on their own. On the other hand, it should be considered that S100A4 and HMGB1 share at least one receptor on the MSC surface, namely RAGE. It is conceivable that there are more receptors shared by DAMPs. Therefore it is also possible that DAMPs like S100A4 and HMGB1 competitively inhibit each other's activity on MSCs. Following these considerations it becomes obvious that DAMPs influence one another and that it will not be sufficient to investigate them as single factors.

Taking into account the prevailing reducing conditions within tumor

microenvironment which may protect HMGB1 and S100A4 from oxidation and thus inactivation, considerations to create oxidative environment within tumor tissue seem to be reasonable as possible therapeutic strategies focussing the environment which tumor cells need to grow, proliferate and metastasize. It is also conceivable to postulate that chemotherapeutics which induce apoptosis should preferentially be used rather than those which induce necrosis with subsequent release of DAMPs like HMGB1 and S100A4.

## 5 Summary

Necrosis with subsequent release of damage associated molecular patterns (DAMPs) is a characteristic feature shared by advanced solid tumors. DAMPs impact tumor microenvironment by initiating wound healing processes like angiogenesis or modulating the immune response. The presence of tumor-infiltrating mesenchymal stromal cells (MSCs) is associated with higher risk of tumor progression and metastasis. Aim of the presented study was to characterize the impact of DAMPs on MSCs and furthermore to indentify specific DAMPs which influence MSC biology in the setting of tumor necrosis. Additionally, the question concerning the importance of redox conditions for the biologic activity of DAMPs on MSCs was addressed here.

Necrotic material (DAMPs), regardless of its tissue of origin, promoted migration and proliferation of MSCs. This effect could be diminished once the DAMPs where oxidized.

High mobility group box 1 (HMGB1) could be identified as a crucial member of the DAMPs that supports the sum effect of whole cell lysates. However, the effect of HMGB1 alone could not explain the extent of migration and proliferation enhancing effect of whole necrotic material on MSCs.

S100A4, another member of the DAMP family, showed a dose-dependent chemotactic activity (from 1 to 1000 ng/ml) on MSCs, while it had divergent effects on MSC proliferation: S100A4 at concentrations up to 100 ng/ml enhanced the proliferation of MSCs, while higher concentrations had an adverse effect.

Uric acid, the last DAMP member that was investigated, showed minimal chemotactic capacities by itself, but significantly enhanced the chemotactic activity of S100A4. In contrast to S100A4, Uric acid dose-dependently inhibited the proliferation of MSCs, this effect could not be abolished by adding S100A4.

MSCs found within tumor tissue can act as immunoregulatory cells and are able to promote tumor metastasis, thus playing a crucial role within the tumor microenvironment. Here, DAMPs - especially HMGB1, S100A4 and uric acid - were revealed to be crucial factors in the setting of MSC biology within the tumor microenvironment. The tumor microenvironment is characterized by reducing and hypoxic conditions that protect DAMPs from oxidation. Based on these results, oxidizing conditions should be considered for therapeutic approaches that target

the tumor microenvironment.

The results presented here have implications for therapeutic strategies targeting the tumor microenvironment.

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| 1994-1996 | Elementary School "Berkenschule", Holzgerlingen, Germany |
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| 10/2010-07/2011 | Scholarship in the Doctorate Programm „Experimental Medicine“ of the "International Graduate School" of the University of Ulm, Germany |
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Eisenbacher J, Yildiz T, Schrezenmeier H, Lotfi R. 2011. „Damage Associated Molecular Patterns (DAMPs) Including S100 Protein Enhance Chemotaxis and Proliferation of Mesenchymal Stem Cells (MSCs).” Posterpresentation, Cancer Immunotherapy and Immunomonitoring 2011, Budapest, Hungary.

Eisenbacher J, Yildiz T, Schrezenmeier H, Lotfi R. 2011. „The effect of bioactive factors from necrotic tumour material towards proliferation and migration of mesenchymal stem cells". Oral presentation, Research meeting of the German Red Cross Blood Service Baden-Württemberg-Hessen 2011, Lohr am Main, Germany.

Eisenbacher J, Yildiz T, Schrezenmeier H, Lotfi R. 2011. „Shared and Divergent Stimulatory Effects of S100A4 and Uric Acid on Proliferation and Chemotaxis of Mesenchymal Stem Cells (MSCs). Interplay between Necrotic Tumor Material and Immunosuppressive MSCs within Tumor Microenvironment.” Posterpresentation and Receivement of the "ASH Abstract Achievement Award", Annual Meeting of the American Society of Hematology 2011, San Diego, USA.

## Publications

Lotfi R, Eisenbacher J, Solgi G, Fuchs K, Yildiz T, Nienhaus C, Rojewski MT, Schrezenmeier H 2011. Human mesenchymal stem cells respond to native but not oxidized damage associated molecular pattern molecules from necrotic tumor material. European Journal of Immunology 41 (7): 2021-2028.

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