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Expression pattern analysis and activity determination of matrix metalloproteinase derived from human macrophage subsets

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Abstract

The polarization behavior of macrophages determines the clinical outcome after implantation of biomaterials. Formation of classically activated macrophages (CAM) may result in cell fusion to form foreign body giant cells, which induce and support uncontrolled inflammatory responses and can cause undesired material degradation. In contrast, polarization into alternatively activated macrophages (AAM) is assumed to support healing processes and implant integration. The expression of matrix metalloproteinases (MMP) by the different macrophage subsets might play a crucial role for inflammatory and wound healing processes and may subsequently influence the implant integration.

Therefore, it is of importance to characterize the MMP expression pattern by the different macrophage subsets. This knowledge could support the design of biomaterials in which specific MMP cleavage sites are incorporated allowing a controlled cell-mediated degradation of the material. However, it needs to be considered that the pure expression levels may not correlate with the enzymatic activity of the MMP, which depends on a variety of different parameters such as additional co-factors.

For this reason, the differential MMP expression levels and the overall enzymatic activity of *in vitro* generated human non-polarized macrophages (M0), CAM, and AAM are analyzed in this study. While MMP-1, MMP-3, and MMP-10 showed the highest expression levels in CAM, MMP-12 was most strongly expressed by AAM. Interestingly, although various MMP were expressed at high levels in CAM, the enzymatic MMP activity was increased in supernatants of AAM cultures. The data presented here illustrate the importance to combine the measurement of MMP expression levels with the analysis of the enzymatic activity. The observed MMP-12 expression in combination with the higher enzymatic activity detected in AAM supernatants might motivate the design of biomaterials, whose structure could be modified by MMP-12 catalyzed reactions leading to interactive polymers.

Keywords: Biomaterials, Macrophage polarization, Matrix metalloproteinase

1. Introduction

Macrophages as members of the mononuclear phagocyte system exhibit multiple functions during inflammatory reactions, in wound healing processes, tissue homeostasis, and disease pathogenesis including the foreign body reactions (FBR)-induced fibrotic implant encapsulation [4, 23]. Macrophages have a remarkable plasticity and can change their phenotype and associated functions in response to local microenvironmental signals [21]. Several macrophage subsets have been identified and can be roughly categorized as classically activated macrophages (CAM) and alternatively activated macrophages (AAM). CAM, also often referred to as M1 macrophages, are generated by stimulation with interferon (IFN)-γ alone or in combination with microbial stimuli such as lipopolysaccharide (LPS) or pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α. These factors induce an inflammatory phenotype with high microbicidal activity and enhanced production of pro-inflammatory cytokines and reactive oxygen species (ROS) as well as up-regulation of acetylcholinesterase [6, 13]. AAM, also known as M2 macrophages, can be induced by interleukin (IL-4) alone or in concert with IL-13 as well as immunoglobulins via Fc receptor activation, glucocorticoids or IL-10 [19]. AAM are associated with tissue repair, angiogenesis, pro-allergic and anti-parasitic T_H2 immunity, and can exert immunosuppressive functions by the production of anti-inflammatory cytokines [19]. The balance of both macrophage subsets is crucial for the clinical outcome after biomaterial implantation. It is assumed that the formation of AAM supports implant integration and wound healing in the surrounding tissue. In contrast, CAM induction may facilitate inflammatory responses, which can lead to a chronic inflammation of the implant site and subsequently a FBR leading to fibrotic encapsulation of the implant. Additionally, by fusion of CAM foreign body giant cells (FBGC) can be formed, which secrete high levels of inflammatory cytokines, enzymes such as matrix metalloproteinases (MMP) and ROS, thereby influencing the degradation behavior of biomaterials [2]. The chemical composition, the mechanical and physical properties as well as the topography of the biomaterial can influence the fate of macrophages. For example, it could be shown that materials with pore sizes between 30 – 40 μm induced a pronounced formation of AAM and a suppression of FBGC formation with a subsequent amelioration of the FBR [18]. Furthermore, cell free scaffolds generated from extracellular matrix (ECM) compounds seemed to promote the switch from CAM predominantly present immediately after implantation to AAM, which were found to be enriched 7 – 14 days post implantation [5].

The secretion of MMP by the different macrophage subsets could play a crucial role for the implant integration through the modulation of inflammatory and regenerative processes, since

the functions of MMP go far beyond the remodeling of the extracellular matrix by enzymatic cleavage. In contrast to previous observations, recent findings revealed that MMP cleave a much wider range of extracellular compounds and bioactive substrates regulating thereby the functional activity of proteins including the release of growth factors from cell membranes and shedding of cell adhesion molecules [8]. For instance, MMP can regulate the activity of cytokines such as TNF-α and IL-1β by cleaving the surrounding ECM compounds, allowing their physical release. They also generate active TNF-α by mediating cleavage of pro-TNFα [31]. Through the activation of several inflammatory mediators MMP are indirectly able to potentiate or modulate immune reactions. Material-mediated regenerative processes, which depend on the *de novo* synthesis of extracellular matrix by tissue forming cells can be inhibited or delayed, when uncontrolled MMP secretion occurs at the implantation site. In fact, elevated levels of MMP-9 are indicators of inflammation and might be associated with poor wound healing [12]. Additionally, the degradation of collagen- or gelatin-based materials or coatings could be accelerated by MMP secretion. For example, it was shown that macrophages adherent on explanted collagen disks produced MMP-2 and MMP-9 [16].

The MMP expression can also directly be altered by polymeric substrates [27], which could for instance influence healing processes or the structural integrity of the implant by inducing material degradation. The MMP expression of *in vitro* generated macrophage subsets is currently not sufficiently characterized. Therefore, in this study the MMP expression levels were investigated in non-polarized human macrophages (M0) generated from human peripheral blood monocytes by treatment with the macrophage colony-stimulating factor (M-CSF), in CAM polarized from M0 by the addition of LPS and IFN-γ, and AAM, which were polarized from M0 by treatment with IL-4 and IL-13. It was hypothesized that the pure MMP expression values may not reflect the actual enzymatic activity present in the supernatant. To verify this hypothesis the overall enzymatic activity of MMP in the cell culture supernatants was additionally determined, allowing a comparison between the pure MMP expression levels and their biological activity.

2. Material and Methods

2.1 Generation of macrophage subsets

The study was performed in accordance with the Nordkem-workshop and the ethical guidelines of the journal *Clinical Hemorheology and Microcirculation*. The study protocol received an approval by the institutional committee of the Charité Universitätsmedizin Berlin, Germany.

For the generation of human monocyte-derived macrophages, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (DRK-Blutspendedienst Ost, Institut Berlin, Germany) obtained from ten individual apparently healthy donors by density gradient centrifugation as previously described [28]. Monocytes were purified from PBMC by depleting CD14 negative cells using the monocytes isolation KitII (Miltenyi Biotec, Bergisch-Gladbach, Germany), routinely resulting in a monocyte purity of above 95% determined by flow cytometry as previously described [28]. The purified monocytes (2×10^6 cells·mL^{-1}) were cultivated in commercially available polystyrene tissue culture dishes 100 (6 mL cell suspension per tissue culture dish; TPP Techno Plastic Products AG, Trasadingen, Switzerland) for five days in the presence of M-CSF (50 ng·mL^{-1}, Gibco$^®$ by life technologies™, Darmstadt, Germany) to generate macrophages, followed by 24 hours incubation with LPS (*E. coli* O111:B4, 2 μg·mL^{-1}, Enzo Life Sciences GmbH, Lörrach, Germany) and IFN-γ (10 ng·mL^{-1} BioLegend Inc., San Diego, CA, USA) to polarize the macrophages into CAM or IL-4 and IL-13 (both 10 ng·mL^{-1}, Miltenyi Biotec GmbH) to induce polarization into AAM. Non-polarized macrophages (M0) were left untreated for 24 hours. During all cultivation steps, VLE (very low endotoxin)-RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10 % fetal calf serum (Biochrom) was used as culture medium.

2.2 Macrophage phenotype characterization by flow cytometry

The different macrophage subsets were harvested by Accutase™ (EMD Millipore Corporation, Darmstadt, Germany) treatment for ten minutes and subsequent cell scraper usage. The staining procedure was performed as previously described [28]. In brief, after washing cells were labeled for 10 min at 4°C in the presence of FcR-Blocking Reagent (1:100, Miltenyi Biotec) with the following antibodies: anti-CD11b-APC clone M1/70.15.11.5, anti-CD11c-FITC clone MJ4-27G12.4.6, anti-CD86-FITC clone FM95, anti-CD36-PE clone AC106, anti-CD206-APC clone DCN228, anti-CD61-APC clone Y2/51 (all Miltenyi Biotec), anti-CD163 clone GHI/61 (BD Pharmingen™, San Jose, USA), and anti-CD14-PC7 clone RMO52 (Beckman Coulter, Krefeld, Germany). After a final washing the cells were analyzed using a MACSQuant™ flow

cytometer (Miltenyi Biotec). To discriminate live and dead cells, 1 µg·ml⁻¹ 4′,6-diamidino-2-phenylindole (DAPI) was added immediately prior to analysis. Data analysis was performed with the FlowJo software v10(Tree Star, Ashland, USA).

2.3 Macrophage phenotype characterization by Real-time PCR

Total RNA was isolated from 2×10^6 cells using the RNeasy® Minikit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The RNA concentrations and purities were determined at 260 nm and 280 nm using the Infinite®F500 spectrometer (Tecan Group Ltd., Männedorf, Switzerland) in combination with the NanoQuant Plate™. For the reverse transcription of the mRNA into cDNA 800 ng total RNA were used. The reverse transcription was performed with the SuperScript® III RT Kit (Invitrogen™, Darmstadt, Germany) using oligo-dT primers according to manufacturer's instructions. The quantitative real-time PCR was performed with the SYBR® Green Master Mix using the StepOnePlus™ PCR-Cycler (both Applied Biosystems by Life Technologies™, Darmstadt, Germany). The transcripts were quantified with the following forward (FP) and reverse (RP) primers (IBA GmbH, Göttingen, Germany): GAPDH FP: 5'-TGGTATCGTGGAAGGACTCATGAC-3', GAPDH RP: 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'; iNOS FP: 5'- GTGGAAGCGGTAACAAAGGA-3', iNOS RP: 5'- TTGCCATTGTTGGTGGAGTA -3'; IDO FP: 5'- GCGCTGTTGGAAATAGCTTC -3', IDO RP: 5'- CAGGACGTCAAAGCACTGAA -3'; HMOX1 FP: 5'- ACTTTCAGAAGGGCCAGGT -3', HMOX1 RP: 5'- TTGTTGCGCTCAATCTCCT -3'. The expression of GAPDH as housekeeping gene was used to normalize the expression of the target genes. The differential expression of the target genes was determined by the ΔΔCt-method calculated using the StepOnePlus™Software v2.3.

2.4 Detection of MMP expression levels

The expression levels of MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, and Emmprin, were quantified in 1:5 diluted cell culture supernatants of the different macrophage subsets using the Luminex® Performance Human MMP Panel (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The Bio-Plex200® device (BioRad Laboratories, Munich, Germany) was used for the detection of the Luminex® beads.

2.5 Determination of enzymatic MMP activity

The enzymatic MMP activity in cell culture supernatants was determined with the pan-MMP fluorogenic peptide substrate (7-methoxycoumarin-4-yl)Acetyl-Pro-Leu-Gly-Leu-(3-[2,4-

dinitrophenyl]-l-2,3-diaminopropionyl)-Ala-Arg-NH$_2$ (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$) (R&D Systems) as previously described [20]. Recombinant human MMP-9 (R&D Systems) was used as the positive control. The different macrophage supernatants were incubated with the fluorogenic peptide substrate and the fluorescence intensity, which increases after enzymatic cleavages, was followed over 100 min with a 5 min detection interval using the Infinite® F500 spectrometer (Tecan). To calculate the enzymatic activity, the MCA-Pro-Leu-OH was used as calibration standard (Bachem, Bubendorf, Switzerland) at three different concentrations (0.2 µM, 0.5 µM, 1 µM) and the linear slope gave the conversion factor (CF). The initial reaction velocity V was obtained as the slope of a line fit to the fluorescence increase over time obtained after peptide cleavage by the MMP present in cell culture supernatant. The total reaction volume (vol) was 100 µl. To determine the enzymatic activity (X) of the samples expressed as picomoles substrate hydrolyzed per minute the following formula was used:

$$X \frac{pmoles\ substrate}{min} = \frac{1}{CF} * V * vol.$$

2.6 Statistical analysis

Unless otherwise indicated data shown are the mean of ten individual donors analyzed in three individual experiments. Data were statistically analyzed with the One-Way ANOVA method and the Tukey test for multiple comparisons using Graph Prism 6 (La Jolla, CA 92037, USA). Significance was assumed for p values below 0.05.

3. Results

3.1 Surface molecule expression of the macrophage subsets

The efficiency of the macrophage polarization protocol was verified by flow cytometry analyzing the expression profile of the scavenger receptors CD163 and CD36, the glycoprotein receptor CD200R, mannose receptor CD206, the typical monocyte marker and co-receptor for lipopolysaccharides CD14, the major histocompatibility complex class II (human leukocyte antigen (HLA)-DR), the co-stimulatory molecule CD80, and the β$_2$-integrin CD11b (Figure 1). The surface molecule expression was analyzed on single live macrophages, which were identified by the previously described flow cytometric gating strategy [29]. Briefly, cells were discriminated from debris using the forward (FSC) sideward scatter (SSC) parameters followed by doublet exclusion using a FSC-area versus FSC-height plot (data not shown). Dead cells were defined as DAPI positive and excluded from the analysis (data not shown) [28].
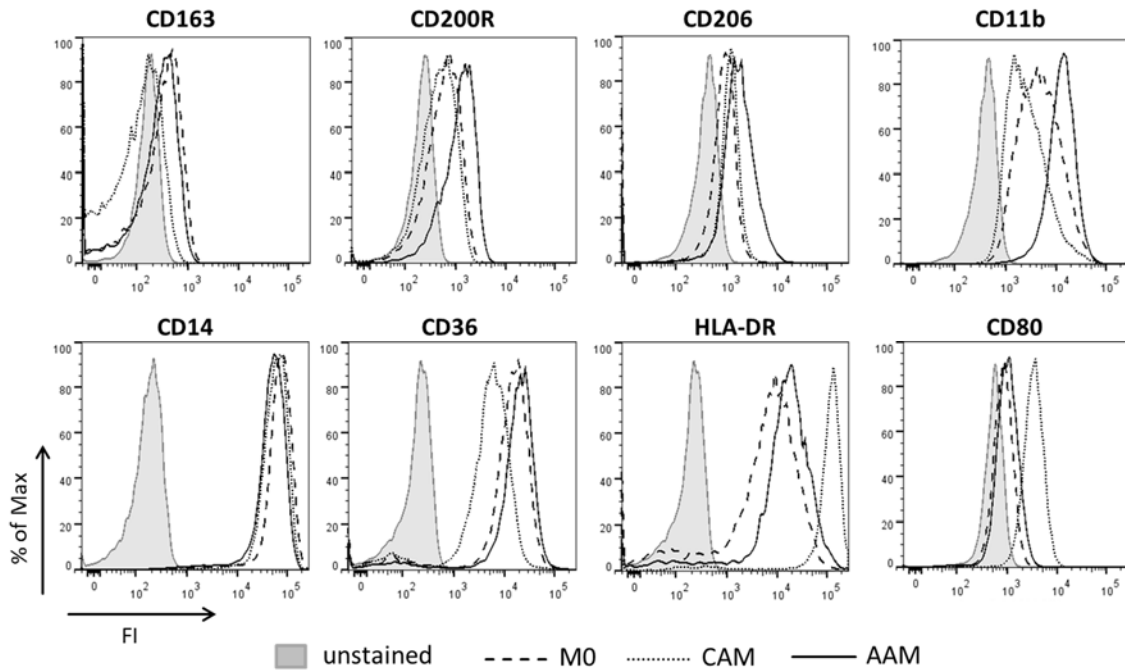
Figure 1: Expression levels of cell surface molecules on non-polarized (M0) and polarized (CAM and AAM) macrophages determined by flow cytometry. The expression levels of CD163, CD200R, CD206, CD11b (top, from left to right), and CD14, CD36, HLA-DR, and CD80 (bottom, from left to right) were analyzed by flow cytometry. Representative histograms of one donor are shown.
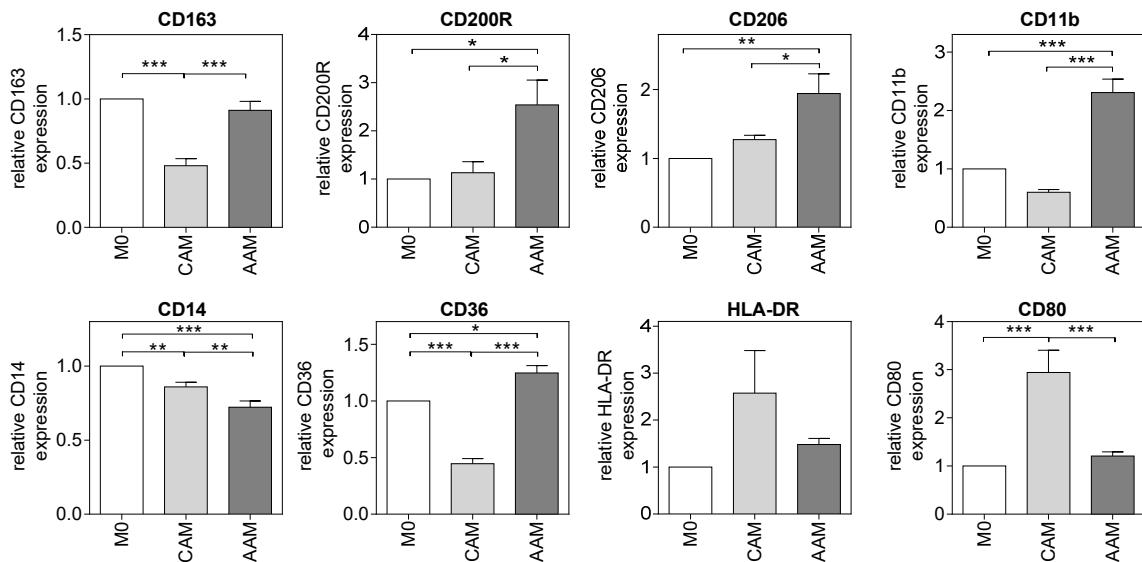
Figure 2. Quantification of surface molecule expression of M0, CAM, and AAM. The expression levels of CD163, CD200R, CD206, CD11b (top, from left to right), and CD14, CD36, HLA-DR, and CD80 (bottom, from left to right) were analyzed by flow cytometry from individual donors. The median FI values from M0 of donor each were used as baseline. The relative expression was calculated as ratio of value for CAM and AAM divided by the M0 baseline. The average relative expression values of macrophage subsets from individual healthy donors (n = 10) isolated and polarized in three independent experiments are shown (mean ± standard error of the mean (SEM)). Results were statistically compared using the one-way Anova analysis with a Tukey posttest (* for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$).

For the donor shown in Figure 1 all surface molecules were clearly expressed by at least one of the different cell types, since the fluorescence intensity (FI) of the stained samples substantially differed from the unstained controls (Figure 1).

The CD163 expression quantification of the macrophage subsets generated from the individual donors showed that it was similarly expressed by M0 and AAM, but seems to be significantly down-regulated in CAM macrophages compared to M0 and AAM (Figure 1, 2).

The expression pattern of CD200R, CD206, and CD11b was very similar, since these molecules were significantly higher expressed by AAM when compared to M0 and CAM (Figures 1, 2). Although significant, the expression of CD14 was only slightly down-regulated following the polarization of M0 into CAM or AAM (Figures 1, 2). The expression of CD36 was significantly down-regulated in CAM, while it was up-regulated in AAM comparing both subsets to M0 macrophages (Figures 1, 2).

HLA-DR seems to be up-regulated in CAM, but showed a high variance among the different donors. It was observed that not all donors responded towards the LPS plus INF-γ induced polarization by up-regulating the HLA-DR molecule (data not shown). Therefore the HLA-DR expression was not significantly different when the three macrophage subsets were compared (Figure 2). However, the co-stimulatory molecule CD80 was strongly induced in CAM, while its expression remained unchanged when AAM were compared to M0 (Figure 2). In summary, the flow cytometric characterization of the different macrophage subsets showed significant differences for most of the analyzed surface molecules. The obtained data are in accordance with the literature and indicate that the applied polarization protocol efficiently induced the polarization of M0 into CAM and AAM [9, 13, 22].
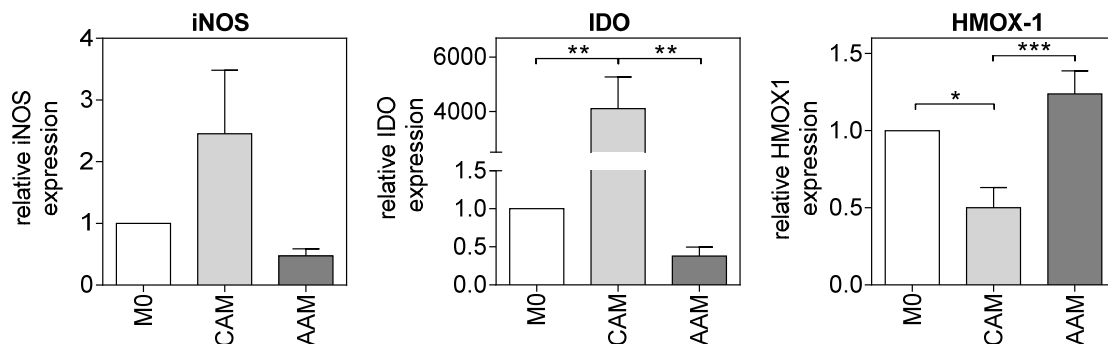
Figure 3. The mRNA expression levels of macrophage polarization markers. Total RNA was isolated from non-polarized and polarized macrophages and transcribed into cDNA. The expression of iNOS (left), IDO (middle), and HMOX1 (right) was analyzed by quantitative real-time PCR. The average relative expression levels of macrophage subsets from ten

individual healthy donors (n = 10) isolated and polarized in three independent experiments are shown (mean ± SEM). Results were statistically compared using the one-way Anova analysis with a Tukey posttest (* for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$).

3.2 Analysis of macrophage polarization on transcriptional level

Macrophage polarization can not only be followed by the analysis of surface molecule expression, but also by gene expression changes on the transcriptional level. Due to their *in vivo* function the metabolic activity of CAM and AAM differs substantially and is accordingly associated with a differential expression of metabolic enzymes, which can be used to monitor the *in vitro* polarization of macrophages [7]. Here, the mRNA levels of inducible nitric oxide synthase (iNOS), which plays a key role for the generation of ROS released by CAM to kill pathogens, the indole amine 2,3-dioxygenase (IDO), an enzyme that catalyzes the conversion of tryptophan into kynurenine and the heme-oxygenase-1 (HMOX-1) [7] were analyzed in the different macrophage subsets.

As expected, the highest iNOS expression was observed in CAM, which was, however, not significant due to the donor to donor variance (Figure 3, left). It was found that IDO is in comparison to M0 strongly up-regulated in CAM and unchanged in AAM (Figure 3, middle). A different expression profile was observed for the HMOX-1. The mRNA levels of HMOX-1 were reduced in CAM compared to M0 and significantly higher expressed in AAM compared to CAM (Figure 3, right). These data confirmed a successful macrophage polarization, since the expression of all three genes was found to be in agreement with the literature [9].

3.3 MMP expression by the different macrophage subsets

One major aim of this study was to analyze the MMP expression of the different macrophage subsets and to identify possible differences among the subsets. To realize this, the cell culture supernatants harvested 24 hours after induction of polarization were analyzed for the expression of various MMP. MMP-1, MMP-3, and MMP-10 were significantly higher expressed in CAM compared to M0 and AAM, whereas no differences were observed when M0 macrophages were compared to AAM (Figure 4). The expression levels of MMP-7, MMP-8, and MMP-9 did not show any significant differences within the three macrophage subsets (Figure 4). MMP-9 showed remarkably high expression levels, which were above the detection limit for all macrophage subsets (Figure 4).
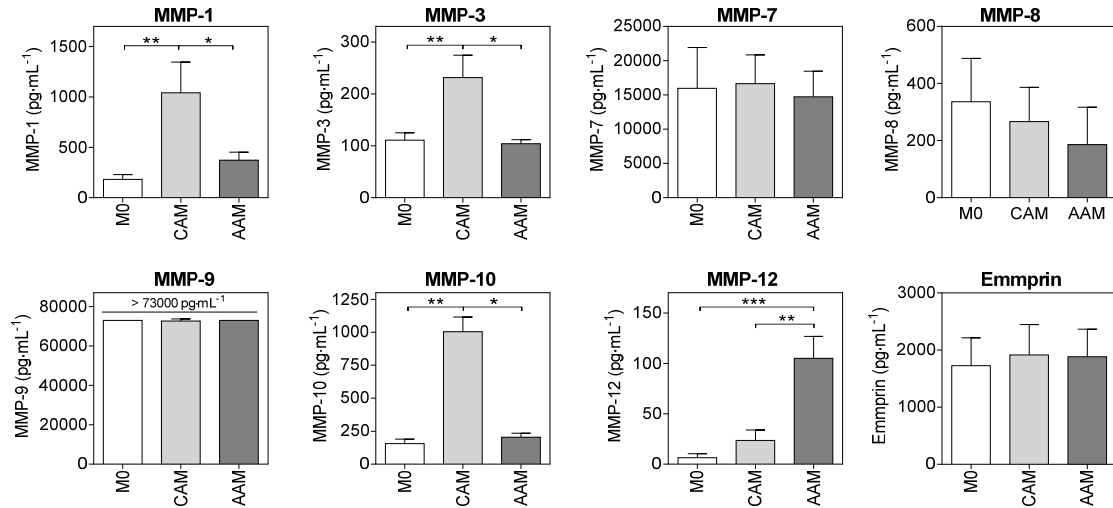
Figure 4. MMP Expression by non-polarized and polarized macrophages. MMP secretion was detected using the BioPlex® multiplex system. The average MMP-1, MMP-3, MMP-7 and MMP-8 (top, from left to right) as well as MMP-9, MMP-10, MMP-12, and Emmprin (bottom, left to right) secretion of macrophage subsets from ten individual healthy donors (n = 10) isolated and polarized in three independent experiments are shown (mean ± SEM). Results were statistically compared using the one-way Anova analysis with a Tukey posttest (* for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$).

The only MMP, which was significantly higher expressed in AAM compared to M0 and CAM was MMP-12. MMP-13 was below the detection limit in the supernatants of the three macrophage subsets (data not shown). In addition to the MMP the expression of Emmprin, which is an inducer of matrix metalloproteinase synthesis, was also determined. The Emmprin levels did not differ between the three macrophage subsets (Figure 4).

In summary, it was shown that the macrophage subsets can secrete various MMP into the cell culture supernatants and that the level of secreted MMP depends on the phenotype of the cells.

3.4 MMP activity determination in macrophage supernatants

In order to investigate whether the MMP secretion correlates with the overall enzymatic MMP activity, supernatants harvested from the different macrophage cultures were incubated with a Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ fluorogenic MMP substrate. The cleavage site in this peptide is the bond between Glycine and Leucine, which is sensitive for MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-12, and MMP-13 [11]. The MMP-mediated cleavage of the amide bond causes an increase in fluorescence intensity due to the release of the quenching group (Dpa) from the fluorescent group (Mca) [11]. It was observed that the MMP activity in CAM was significantly lower than in M0 and AAM and a difference in MMP activity between M0 and AAM could not be observed (Figure 5).
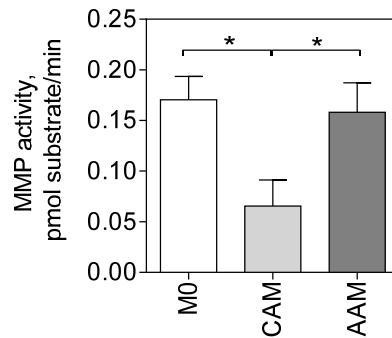
Figure 5. Overall MMP activity in cell culture supernatants of non-polarized and polarized macrophages. The MMP activity was determined using a fluorogenic MMP specific peptide substrate. The peptide cleavage leads to an increase in FI as result of an abrogated quenching of the fluorogenic group. Cell supernatants from ten individual donors were analyzed two times for their MMP activity (mean ± SEM). Results were statistically compared using the one-way Anova analysis with a Tukey posttest (* for $p < 0.05$).

4. Discussion

In this study the differential expression of various MMP by *in vitro* generated human macrophage subsets was investigated. The MMP expression by macrophages could be meaningful for many pathologies, which are associated with tissue remodelling or inflammatory responses [25]. For the *in vitro* polarization M0 were stimulated with LPS and IFN-γ to obtain CAM or with IL-4 and IL-13 to get AAM. The characterization of the different macrophage subsets confirmed a succesful polarization into CAM and AAM, since the expression of CD163, CD200R, CD206, CD11b, and CD86 as well as iNOS, IDO, and HMOX-1 showed characteristic patterns for both subsets in accordance with the current literature and studies using the same polarization protocol [4, 9, 22]. A clear characterization of the *in vitro* generated cells is important to allow comparisons with other studies, since the polarization protocol often differs in terms of cell sources, cell isolation methods, type and duration of stimulation, culture medium and the polymeric cell culture substrates, which can have different chemical compositions or surface and mechanical properties [29]. All of these parameters can have a substantial impact on the macrophage phenotype [9].

Our analysis of the MMP levels in supernatants of M0, CAM and AAM revealed huge differences for MMP-1, -3, -10 and -12. While MMP-1, -3 and -10 were highest expressed in CAM, MMP-12 showed elevated levels in AAM. MMP-7, -8, and -9 were released at similar levels. The difference in MMP expression could not be attributed to Emmprin, since the detected Emmprin levels were very similar for all subsets.

Substrates for MMP are several types of ECM compounds such as collagen type I, II, and III as well as laminin, elastin and proteoglycans [10]. This opens the possibility of introducing proteolytic recognition sites of natural ECM compounds in synthetic polymers [14]. For example, hydrogels formed from multiarmed polyethylene glycol (PEG) and bis-cysteine MMP substrate peptides supported bone regeneration, which was dependent on the proteolytic sensitivity of the matrix [15].

However, the degradation of such materials could strongly depend on the associated inflammatory reaction, possibly induced by the biomaterial after implantation. Therefore, it would be important to test how inflammatory cells such as macrophages respond to MMP sensitive material and whether they facilitate an uncontrolled degradation. Furthermore, the identification of a cell-specific MMP pattern would allow to design biomaterials in which specific MMP cleavage sites can be integrated. For example, the incorporation of a MMP-12 cleavage site could allow the material degradation when AAM are present in close proximity to the material and induced wound healing mechanisms.

Fragments of ECM compounds formed by MMP degradation can be immunologically active and may act as endogenous Toll-like receptor agonists [1, 30]. Therefore, ECM compound-based biomaterials could induce or support the formation of FBGC in an indirect fashion [33]. The pharmacological inhibition of a particular MMP was shown to diminish macrophage fusion and subsequently FBGC formation [10]. For example, blocking of MMP-9 with an anti-MMP-9 antibody and experiments with MMP-9 deficient mice clearly indicated a role for MMP-9 in the formation of FBGC and the subsequent foreign body reaction [17]. However, the macrophages, which seemed to be the major source of MMP-9 in this study, were not phenotypically characterized in more detail [17].

Here, MMP-9 was found to be expressed at very high levels by all three macrophage subsets. However, it remains unclear to which extent MMP-9 contributed to the overall enzymatic activity detected in the cell culture supernatants.

The fact that a higher enzymatic activity was observed in M0 and AAM cell culture supernatants was counterintuitive, since various MMP were expressed at high levels in CAM.

A reason for this discrepancy could be the lack of additional activating factors such as the appropriate zinc ion concentration or proteases, which remove the pro-domain to activate the MMP [25]. The presence of suppressing factors in the cell culture supernatants such as endogenous inhibitors like the so-called tissue inhibitors of metalloproteinases (TIMP) could also influence the MMP activity [25]. The analysis of TIMP-1 and -2 expression in the different macrophage subsets and their subsequent contribution to the enzymatic activity of the cell

culture supernatant will be investigated in the future. By using MMP and TIMP inhibitors it should be possible to identify the MMP responsible for the observed enzymatic activity. Different *in vitro* half-life times or unspecific adsorption onto the polymeric substrate might also explain the differences between the MMP expression levels and biological activity [26]. Besides macrophages, also endothelial cells can express MMP. Both cell types as well as their secreted MMP play a crucial role in angiogenesis but also in cardiovascular pathologies and their interaction with materials intended for cardiovascular implants is therefore of high interest [24, 32]. It could recently been shown that angiogenically stimulated monocytes showed a lower MMP activity than endothelial cells, which was not altered after cultivation of both cell types on soft hydrophobic polymer networks [20].

To predict the *in vivo* behavior of biomaterials, our data indicate that the determination of the expression levels in combination with enzymatic activity of MMP may give additional insights and allows adapting the design of the biomaterial accordingly.

5. Conclusion

It was shown that the determination of MMP expression levels is insufficient to draw conclusions about their actual biological activity. Therefore, we suggest to routinely determine the enzymatic activity in addition to the absolute expression levels of MMP. Insights about the MMP expression and activity of distinct cell types involved in biomaterial-mediated regenerative processes may facilitate the understanding of implant degradation processes and how the tissue remodeling could be regulated.

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