Generation of anti-inflammatory macrophages for implants and regenerative medicine using self-standing release systems with a phenotype-fixing cytokine cocktail formulation

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Abstract

The immediate tissue microenvironment of implanted biomedical devices and engineered tissues is highly influential on their long term fate and efficacy. The creation of a long-term anti-inflammatory microenvironment around implants and artificial tissues can facilitate their integration. Macrophages are highly plastic cells that define the tissue reactions on the implanted material. Local control of macrophage phenotype by long-term fixation of their healing activities and suppression of inflammatory reactions are required to improve implant acceptance. Herein, we describe the development of a cytokine cocktail (M2Ct) that induces stable M2-like macrophage phenotype with significantly decreased pro-inflammatory cytokine and increased anti-inflammatory cytokine secretion profile. The positive effect of the M2Ct was shown in an *in vitro* wound healing model; where M2Ct facilitated wound closure by human fibroblasts in co-culture conditions. Using a model for induction of inflammation by LPS we have shown that the M2Ct phenotype is stable for 12 days. However, in the absence of M2Ct in the medium macrophages underwent rapid pro-inflammatory re-programming upon IFNg stimulation. Therefore, loading and release of the cytokine cocktail from a self-standing, transferable Gelatin/Tyraminated Hyaluronic acid based release system was developed to stabilize macrophage phenotype for *in vivo* application in implantation and tissue engineering. The M2Ct cytokine cocktail retained its anti-inflammatory activity in controlled release conditions. Our data indicate that the direct application of a potent M2 inducing cytokine cocktail in a transferable release system can significantly improve the long term functionality of biomedical devices by decreasing pro-inflammatory cytokine secretion and increasing the rate of wound healing.

Keywords: Gelatin, Cytokine, Macrophage Phenotype Control, Controlled Release, Wound Healing

1. Introduction

Responsive biomedical devices and tissue engineered structures have been becoming increasingly common for the treatment of organ/tissue loss or damage. However, lack of control over the host response to implanted systems significantly affects the clinical outcomes [1]. For example, McConnell et al reported that in an animal model the presence of intracortical electrodes led to chronic inflammation with subsequent dendritic cell and neuron loss [2]. The innate immune response is the first reaction of the host tissue against implanted materials where macrophages play an important role. Macrophages are phenotypically plastic immune cells which have a wide array of responses as a consequence of biophysical and biochemical stimuli in immediate vicinity; they are particularly responsive to the pro- or anti-inflammatory cytokines which determine their phenotype as pro-inflammatory or pro-healing [3].

The presence and accumulation of macrophages during wound healing at the wound site is an important step in the process that leads to wound closure. This has been confirmed by selective ablation of macrophages in transgenic mice by application of diphtheria toxin, which resulted in impaired extracellular matrix (ECM) secretion, lower levels of epithelialization and angiogenesis [4]. Moreover, it has been recently reported that the presence of macrophages is beneficial for facilitation of engineered tissue vascularization [5]. Thus, engineering a microenvironment around implanted devices or artificial tissues that would convert macrophages to a more pro-healing phenotype (generally designated as M2) is a promising approach to improve the integration of the device and attenuate the adverse immune reactions [6, 7]. Furthermore, phenotype-controlled macrophages polarized by anti-inflammatory cytokines have previously been used to treat chronic inflammatory conditions and favored implant tolerance in mouse models [1, 8, 9].

In vitro induction of macrophage phenotype has been regularly achieved by the use of specific cytokines/stimulants such as interferon-gamma (IFNg), interleukin-4 (IL4), bacterial lipopolysaccharides (LPS) etc. [10]. The resulting macrophage phenotypes can be categorized into the conventional M1-M2 classification, or as subgroups of M1 and M2 (such as M2a). However, there is a trend in the immunology field to describe macrophage phenotypes with respect to the actual stimulation

[11]. Furthermore, the physiological signaling conditions are not as clear as one cytokine/one phenotype equivalency and several cytokines can act simultaneously. This has led us to hypothesize that a designed cytokine cocktail might be more potent in establishing and more importantly fixing a desired cytokine phenotype than a single cytokine induction. Recently, human monocyte-derived macrophages with dominant anti-inflammatory phenotype were generated using cytokine cocktail consisting of M-CSF (50 ng/ml), IL4 (20 ng/ml), IL10 (20 ng/ml) and TGFβ1 (20 ng/ml) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) [12].

In order to exert a long term control over the implanted material microenvironment, the establishment of controlled release of desired cytokines is necessary. This can be achieved by methods such as incorporation of nanoparticles or nanocapsules within the implanted device; however, diffusion of the particles itself might hinder the local control [13]. Adhesive, self-standing thin films are attractive delivery systems in this aspect, as they can be applied to the surfaces of implants and engineered tissues and also have the capacity to be loaded and then release cytokines and growth factors in a time dependent manner. We have recently shown the efficacy of immunomodulatory thin films on controlling the behavior of primary human macrophages [14, 15]. However, these films needed to be formed in-situ and could not be transferred. For ensuring wide scale applicability transferable release platforms are desirable.

In this study, we have optimized a cytokine cocktail that ensures high level of M2-like conversion of naive primary human monocytes with long term phenotypic stability in the presence of cocktail components. In order to show the indirect positive effects of such conversion in healing of connective tissue; a wound healing assay was done in the presence of phenotype controlled macrophages with human fibroblasts. Finally, for controlled release of the cytokine cocktail around implantable devices, a transferable release system based on gelatin and tyramine derivative of hyaluronic acid was designed and tested in the presence of primary macrophages.

2. Materials and Methods

2.1 Isolation of human monocyte-derived macrophages, cell stimulations and culture conditions

Monocytes were isolated from buffy coats of healthy donors as described previously [16-18]. Briefly, cells were purified by density gradients followed by positive magnetic selection using CD14+ MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes were cultured at 1×10^6 cell/ml in serum free macrophage-SFM medium (ThermoFisher Scientific) supplemented with 10 ng/ml M-CSF (Peprotech) and 10^{-8}M dexamethasone (dex) (Sigma-Aldrich, Munich, Germany) for the time periods indicated in the relevant sections. Cells were additionally stimulated with human IFNg (100 ng/ml), IL4 (10 ng/ml) or combination of IL4+IL10+TGFβ1 with concentrations of individual cytokines indicated in the relevant sections. All cytokines were from Peprotech. LPS (Invivogen) was added in concentration 1 µg/ml for 24h at the time-points indicated in the relevant sections. For long term analysis of phenotype monocytes were cultured for 12 days without changing the medium and addition of new cytokines. To assess macrophage phenotype changes after cytokine deprivation monocytes were cultured in complete medium with cytokines. On day 6 of culture medium was replaced either by complete medium with freshly added cytokines or basal medium containing 1 ng/ml M-CSF and 10^{-8}M dex and macrophages were cultured for 6 more days.

2.2 Wound healing assay

For wound healing assay, human lung fibroblasts (MRC-5) were routinely cultured as described [19] and 5×10^4 cells/well were seeded in a 12-well plate (Corning) for 5 days to reach 80% confluence. Concurrently monocytes were differentiated into macrophages for 6 days in the presence of 50 ng/ml M-CSF (Miltenyi Biotech) in ultra-low attachment plates (Corning). Naive macrophages were polarized in the presence of the following cytokine mixtures: 20 ng/ml IFNg + 50 ng/ml GM-CSF (M1-like); 3 ng/ml IL4 + 10 ng/ml M-CSF (M2-like) and 10 ng/ml M-CSF + 3 ng/ml IL4 + 10 ng/ml IL10 + 10 ng/ml TGFβ1 (M2Ct) in SFM medium for 48h. Different cytokine-polarized macrophages (1.5×10^5 /well) were co-cultured with fibroblasts in 12 well plates immediately after inflicting a wound using micropipette tip. In order to follow wound closure, MRC5 were pre-labelled with 25 µM of CFDA-SE (Invitrogen) according to manufacturer's instructions. Fluorescence images were taken using a DMIRE2 Fluorescence Microscope (Leica) with a 2.5x objective. Fluorescence intensity of wound area was

quantified using Image J software and data was expressed in percentage of wound closure at 24h and 48h relative to the area of the wound at time point zero.

2.3 Production and characterization of self-standing thin film based controlled release system

Gelatin type B (M_w =2-2.5×10^4 Da, pI = 4.7-5.2) from bovine skin and fluorescein isothiocyanate labeled Bovine Serum Albumin (BSA^{FITC}, M_w = 6.6 × 10^4 Da, pI = 4.7–4.9) were purchased from Sigma Aldrich (St. Quentin Fallavier, France). Microbial Transglutaminase (M-TG) (M_w =3.8×10^4 Da, pI = 9) was obtained from Ajinomoto (Japan). HA-Tyramine (HA-Tyr, M_w = 3,1 x10^5 Da) were produced and characterized by Contipro (Czech Republic).

Gelatin/HA-tyramine films were prepared by spin coating with a protocol described before [20]. Briefly, the films were prepared with a solution of gelatin (14% w/v) mixed with HA-tyramine (1% w/v) in 0.15 M NaCl/10 mM Tris solutions (pH = 7.4). Then 200µL of this solution at 50°C were put on a glass slide (previously installed on the spin coater). The spin coating was done with a rotation speed of 2500 rpm and an acceleration of 1250 rpm for 2 minutes. These films can be double crosslinked through two different processes in order to get an interpenetrated network (IPN) : *i)* gelatin was crosslinked with transglutaminase (TGA) to get amide bond between amine groups on lysine residues and carboxamide groups ($RCONH_2$) on glutamine residues, 10% of TGA in PBS (w/v) were incubated on gelatin/HA-tyramine film; and *ii)* HA-tyramine crosslinking based on dimerization of tyramine to dityramine by horseradish peroxidase mediated reaction, a solution of H_2O_2:HRP (10:1) was prepared with 0.24mg/mL of HRP in PBS and 0.1M H_2O_2 and incubated on top of each film. These films were crosslinked with 100µL of TGA and then with 100µL of HRP solution for 30 minutes for each step. The films were washed 3 times with PBS after each step.

The films were characterized by scanning electron microscopy (SEM) and confocal microscopy. SEM micrograph images were taken by an XL SIRION FEG (FEI Company Eindhoven) with an acceleration voltage of 30 kV. Before imaging, the samples were freeze dried after being frozen in liquid nitrogen. Confocal laser scanning microscopy images were obtained with a Zeiss LSM 710 microscope using a ×20 objective in order to get thickness estimation and 3D reconstructions. The films were stained with a solution of 1 mg/mL of fluorescently labeled BSA^{FITC}. FITC fluorescence was detected after

excitation at λ = 488 nm and an emission band-pass filter of 489-556nm. All the experiments were performed in PBS solution. The BSA loading was studied *in situ* and in real-time using the XNano II nanoplasmonic sensing instrument (Insplorer®, Insplorion, Sweden).

2.4 Effect of controlled release on macrophage phenotype

To generate controlled cytokine release system adhesive coatings were incubated with cytokines in SFM medium for 24h at +4°C. All cytokines were applied in concentrated amounts (10X) compared to standard concentrations mentioned above. Shortly before plating monocytes cytokine containing medium was removed and coatings were washed once with 1 ml SFM medium. Monocytes were cultured on coatings for 6 days in SFM medium supplemented with 1% penicillin/streptomycin followed by stimulation with 1 µg/ml LPS for 24h. In parallel, monocytes were cultured on the same type of coatings in the absence of controlled release system. In this case, cytokines were added in the culture medium after monocyte seeding. As a control, monocytes were cultured for 6 days without coatings.

2.5 Metabolic activity/viability assay

Cell viability was determined using AlamarBlue Cell Viability Reagent (ThermoFisher Scientific) according to manufacturer instructions on day 7 of culture. Briefly, AlamarBlue reagent was diluted 1:10 directly in macrophage culture medium and cells were incubated for 3h, 37°C. Fluorescence was measured in triplicates at 590 nm. Fluorescence of pure AlamarBlue was used as a negative control.

2.6 Light microscopy

Light microscopy photos of macrophages cultured on adhesive coatings for 6 days were taken using Axiovert 100 microscope (Zeiss) equipped with 10X objective and AxioVision 4.8.1 software (Zeiss).

2.7 ELISA

The concentrations of TNF-α, IL1β, IL6, IL1Ra, and CCL18 were measured in macrophage cell culture supernatants using human DuoSet ELISA kits (R&D Systems) according to manufacturer

instructions. Concentration of MMP-7 in supernatants was measured by human Total MMP-7 Quantikine ELISA Kit (R&D systems).

2.8 Statistical analysis

The difference between stimulations was analyzed in GraphPad Prism 6 software using repeated measures one-way ANOVA with Tukey's multiple comparisons or Friedman test with Dunn's multiple comparisons as indicated. Wound healing data were analyzed using Student's t test. The difference was considered significant at $p<0.05$ level.

3. Results

3.1 M2Ct exerts pronounced anti-inflammatory polarization of macrophages

Macrophages are innate immune cells with remarkable phenotypical plasticity. Depending on existing cytokine milieu these cells may induce acute inflammation and tissue injury or conversely resolve inflammation and mediate wound healing. To generate a cytokine cocktail suitable for application on adhesive coatings, we polarized macrophages towards anti-inflammatory state based on the combination of M-CSF, IL4, IL10 and TGFβ1 with certain modifications. First, isolated monocytes were cultured in serum free medium (SFM) to achieve pure effect of polarizing cytokines and avoid additional impact of growth factors that can be present in FBS. In addition, we have supplemented culture medium with corticosteroid dexamethasone in concentration 10^{-8}M to mimic naturally present glucocorticoids in human blood. In an optimization experiment using macrophages of 4 healthy donors the combination of IL4+IL10+TGFβ1 showed pronounced synergistic effect on the suppression of TNF-α production by LPS-stimulated macrophages compared to other cytokine combinations. For instance, average TNF-α levels (pg/ml±SD) were 3112,7±2718,4 for IL4 alone, 3679,7±1993,8 for IL4+TGFβ1, 362,8±213 for IL4+IL10, and 218,4±51 for IL4+IL10+TGFβ1. The combination of IL10+TGFβ1 resulted in low macrophage viability and was excluded from cytokine production analysis (data not shown).

As shown on Fig.1A, B cytokine cocktail consisting of IL4, IL10 and TGFβ1 (M2Ct) strongly inhibited production of pro-inflammatory cytokines TNF-α and IL6 in LPS-treated macrophages.

Importantly, secretion of these cytokines in M2Ct-treated macrophages was significantly lower compared to prototypic M2 stimulation with IL4 (Fig.1A, B). We have also decreased concentration of IL4 from 20 ng/ml to 3 ng/ml that further reduced production of TNF-α in response to pro-inflammatory stimulation with LPS (Supplementary Figure 1). At the same time M2Ct enhanced production of M2-associated chemokine CCL18 and anti-inflammatory cytokine IL1Ra in a similar way as IL4 (Fig.1C, D).

To study whether M2Ct induces prolonged anti-inflammatory macrophage phenotype we cultured macrophages for 12 days in the presence of IFNg, IL4, M2Ct, or without stimulation followed by LPS challenge and TNF-α detection. During this period, culture medium was not changed and fresh cytokines were not added. As shown on Fig.2A stimulation with M2Ct resulted in the lowest levels of TNF-α production compared to other stimulations that was evident for up to 12 days of culture. There was highly significant difference between M2Ct and IL4 stimulations on days 6 and 9 of culture. This trend was also retained on day 12 (Fig. 2A). Since macrophages are phenotypically plastic cells and promptly revert their phenotype upon changes in cytokine milieu, we have examined whether withdrawal of M2Ct components from the medium will restore macrophage responsiveness to LPS back to the level of non-stimulated cells. For this, macrophages were cultured for 6 days followed by replacement of cytokine-containing complete medium for basal medium deprived of cytokines. After 3 and 6 days of culture in basal medium cells were further challenged with LPS and assessed for TNF-α production. Withdrawal of M2Ct components from culture medium for 3 and 6 days resulted in slight elevation of TNF-α secretion upon LPS challenge without reaching statistical significance (Fig. 2B, comparison M2Ct complete vs M2Ct basal). Moreover, even after 6 days of culture in basal medium M2Ct pre-polarized macrophages retained significantly lower levels of TNF-α compared to non-stimulated cells which indicated temporary fixation of anti-inflammatory phenotype (Fig. 2B, comparison ns basal vs M2Ct basal). In contrast, stimulation with IL4 alone was not sufficient to maintain low levels of TNF-α secretion in basal medium (Fig. 2B, comparison ns basal vs IL4 basal). Next, we have assessed whether M2Ct-induced phenotype will be challenged by combination of pro-inflammatory factors IFNg and LPS that can be simultaneously present in case of bacterial infection. When M2Ct pre-differentiated macrophages were deprived of M2Ct-containing medium and re-

stimulated with IFNg followed by additional LPS challenge, they rapidly (in 1 day) restored TNF-α production that was comparable to ns and IL4-stimulated macrophages (Fig. 2C). Remarkably, average increase in TNF-α production after IFNg re-stimulation and LPS challenge was 48.8 times in case of M2Ct pre-differentiated macrophages ($p<0.05$) and 3.4 times for IL4 macrophages ($p<0.05$), whereas changes in TNF-α production by non-stimulated macrophages did not reach significance ($p=0.197$) (Supplementary Figure 2). These results suggested that controlled release system will be required in order to provide prolonged local availability of M2ct components and maintain anti-inflammatory milieu at the implant surface.

3.2 The fibroblast wound healing induced by M2Ct-polarized macrophages correlates with an increase in matrix metalloproteinase-7 (MMP-7)

Adequate wound healing response is an important event upon biomaterials implantation. In order to study the effect of phenotype controlled macrophages on wound healing properties, fibroblast wound closure was studied by co-culturing with diverse cytokine-polarized monocyte-derived macrophages after inflicting a wound. Our results clearly show that co-culture with differentially polarized macrophages resulted in faster wound closure rate compared to single fibroblast culture (Fig. 3A and Fig. 3B). Among all, the fastest wound healing was seen during co-culture with M2Ct- polarized macrophages, which was more obvious in 24h compared to 48h time-point. Fluorescence quantification revealed that, M2Ct mediated approximately 25% faster wound closure rates compared to other types of macrophages (Fig. 3B). In addition to wound closure rate, co-culture with M2Ct polarized macrophages exhibited complete healing of wound edges compared to co-culture with M2 polarized macrophage indicating the ability of M2Ct polarized macrophages in regaining normal tissue architecture. In order to associate improved wound healing mediated by M2Ct with the expression of pro-healing factors such as MMP7, we measured MMP-7 levels in macrophage/fibroblasts co-cultures. Interestingly, MMP-7 was only detected in co-culture with macrophages and the highest MMP-7 level was observed in co-culture with M2Ct polarized cells, which was 3 times higher compared to other co-culture conditions (Fig. 3C). It is therefore reasonable to suggest that different levels of MMP-7

produced from the differentially polarized macrophages were at least partly responsible for the observed differences in the rate of wound healing in different conditions.

3.3 Development and characterization of a self-standing release systems for delivery of M2Ct

Previously, we have reported the controlled release of growth factors from enzymatically crosslinked gelatin based thin films. As hyaluronan is known to interact with many interleukins we have incorporated a hyaluronic acid derivative to the film formulation in order to retain more interleukin components of the cocktail and also to improve the stability of the film by creating an interpenetrating network of separately crosslinked gelatin and hyaluronic acid (Fig. 4A). SEM analysis and 3D confocal images of this material have shown a homogeneous and smooth surface (Fig. 4B, D). The average thickness of this film was evaluated to be approximately 20 μm as shown by confocal images both in 2D (Fig. 4E) and 3D (Fig. 4D). The structure is stable in hydrated form and can be loaded with bioactive molecules. In order to show the ability of the film to absorb proteins, loading of bovine Serum albumin (BSA) was monitored with nanoplasmonic sensing (Insplorion, Sweden) (Fig. 4C). By 4h a significant amount of BSA was absorbed into the film layer, consistent with the confocal microscopy observations. In order to obtain a transferable delivery system, we introduced a sacrificial layer to the production protocol which resulted in self-standing membranes. The films could be easily detached from the substrate by depositing a layer of cellulose acetate on top of the glass slide before the spin coating process. The spin coating was then performed, but once the film is crosslinked, the interaction between the film and the glass slide is so weak that it detached easily once immersed in water as shown in Fig. 4F. This process enabled the transfer of the cytokine cocktail loaded films to target environments.

3.4 Controlled release of M2Ct by adhesive coatings favors anti-inflammatory macrophage state

Due to pronounced anti-inflammatory activity and prolonged effect of M2Ct, this cytokine formulation was selected as an optimal choice for combination with adhesive coatings designed to support controlled cytokine release. First, controlled release system was generated by pre-incubating adhesive coatings with components of M2Ct or M-CSF+Dex only (non-stimulated control) for 24h followed by removal of unbound cytokines and addition of monocytes. In parallel, monocytes were cultured on coatings in the presence of cytokines without controlled release system (cytokines were

added in culture medium). As a control, monocytes were cultured without coatings. After 6 days of culture macrophages were assessed for viability/metabolic activity. The presence of coatings with or without controlled cytokine release was not toxic and did not affect macrophage viability compared to control (Supplementary Figure 3). Furthermore, macrophages cultured on coatings had typical macrophage morphology (Supplementary Figure 4). The assessment of pro-inflammatory cytokine production upon LPS challenge revealed that coatings alone dramatically inhibited production of TNF-α by macrophages and this effect was observed in all groups with and without controlled release (Fig. 5A). As depicted on Fig. 5B and 5C coatings did not significantly influence the production of IL6 and IL1β by macrophages. Controlled release of M-CSF and dexamethasone (group "ns, controlled release") resulted in elevated production of both IL6 and IL1β, which was counteracted by the presence of M2Ct although this effect did not reach statistical significance due to variability in cytokine production between individual donors (Fig. 5B and 5C). As shown on Fig. 5D the highest production of anti-inflammatory cytokine IL1Ra was detected in macrophages differentiated in the presence of controlled release of M2Ct. The concentration of IL1Ra (mean±SD in ng/ml) in M2Ct controlled release group was 257.8±68.4 vs 146.8±42.1 in non-stimulated controlled release group, and increased IL1Ra production was observed in all donors (Fig. 5D). This effect did not reach significance due to individual variations in cytokine production between donors. However, ratio IL1Ra/IL1β was dramatically increased in the presence of controlled release of M2Ct compared to non-stimulated cells (Fig. 5E). This result indicated that M2Ct favored anti-inflammatory phenotype of macrophages cultured on adhesive coatings. In addition, production of M2-associated chemokine CCL18 was negligible in non-stimulated macrophages cultured without coatings (Fig. 5F). However, substantial levels of CCL18 were detected in non-stimulated controlled release group, and were further elevated in M2Ct controlled release group although this effect did not reach significance (Fig. 5F). Overall, controlled release of M2Ct resulted in decreased production of pro-inflammatory cytokines TNF-α, IL6, and IL1β and increase of IL1Ra/IL1β ratio compared to non-stimulated cells cultured in the presence of controlled release system.

4. Discussion

In this study we have shown the availability of potent cytokine combination consisting of IL4, IL10 and TGFβ1 for long-term anti-inflammatory modulation of macrophage phenotype around implants. This cytokine cocktail also favored wound healing *in vitro* and could be combined with self-standing release systems for prolonged local control of macrophage phenotype.

Controlled release systems are perspective tools for long-term local immunomodulation around implants that aim to prevent unwanted pro-inflammatory activation of macrophages and stimulate wound healing [1]. For this purpose, anti-inflammatory cytokines and their combinations can be loaded on thin adhesive coatings. For example, local immunomodulation using well recognized anti-inflammatory factor IL4 alleviated biomaterial-induced osteolysis in mice [21]. Controlled release of IL4 from polyelectrolyte multilayer-based coatings and silk biomaterials was shown to promote differentiation of macrophages with anti-inflammatory M2 properties [16, 22]. However, IL4 alone may not be sufficient to sustain long-term anti-inflammatory macrophage phenotype and low responsiveness to inflammatory stimuli. In our study, production of pro-inflammatory cytokine IL6 upon LPS challenge was comparable in non-stimulated and IL4-stimulated macrophages. In addition, there was no significant difference in TNF-α production in response to LPS between non-stimulated and IL4 pre-differentiated macrophages on day 3 after cytokine withdrawal from culture medium. These data are supported by the fact that transfer of IL4 differentiated macrophages was not sufficient to protect mice from lethal endotoxemia [23]. Thus, we have hypothesized that a cytokine cocktail containing combination of anti-inflammatory factors will be more potent in terms of macrophage phenotype fixation and long-term phenotype control. The use of multiple factors for macrophage polarization is also physiologically relevant since the cells in *in vivo* conditions are always influenced by complex cytokine milieu. Double and triple combinations of anti-inflammatory factors IL4, IL10 and TGFβ1 were previously shown to be more efficient than single cytokines, and induced robust tolerogenic phenotype in mouse and human macrophages [8, 9, 12]. In addition, macrophages stimulated by such cytokine combinations partially retained anti-inflammatory phenotype *in vivo* and their adoptive transfer protected mice from chronic inflammatory disorders including autoimmune type 1 diabetes and adriamycin nephrosis [8, 9]. In our study, we have utilized triple combination of IL4, IL10 and TGFβ1 (named M2Ct) in order to generate macrophages with prolonged anti-inflammatory phenotype. We have

further optimized concentrations of individual cytokines to diminish pro-inflammatory cytokine production by macrophages and used serum free medium to exclude impact of undefined growth factors on macrophage phenotype. In these conditions M2Ct cocktail was superior to IL4 in terms of anti-inflammatory potential and induced long-term inhibition of TNF-α production that was pronounced for 12 days after single cytokine stimulation. Importantly, macrophages differentiated in the presence of M2Ct retained their anti-inflammatory properties for 6 days after cytokine withdrawal indicating partial fixation of phenotype and formation of innate immune memory.

Adequate wound healing response is an important event upon biomaterials implantation which facilitates tissue integration of implanted biomedical devices [24, 25]. M2-like polarized macrophages are known to favor healing events following injury [26, 27]. In our study, M2Ct-differentiated macrophages markedly enhanced fibroblast-mediated wound closure *in vitro* indicating favorable pro-healing phenotype that is necessary for successful implant integration *in vivo*. Improved wound healing in the presence of M2Ct-differentiated macrophages was associated with elevated production of MMP-7. These results suggested the role of MMP-7 in wound closure in our system. Previous studies revealed complex role of MMP-7 in wound healing. For example, elevated presence of MMP-7 in the tissue and plasma was associated with impaired wound healing in patients with periodontitis and combat wounds [22, 23]. At the same time MMPs are necessary for matrix remodeling and cell migration during wound closure [28]. MMP-7 was shown to be involved in re-epithelization after injury and facilitated cell migration through matrix degradation. MMP-7 deficient mice showed strong impairment in wound healing [29]. These studies indicated that balanced and timely production of MMPs is required for efficient healing. Our results revealed that elevated production of MMP-7 in the context of prevailing anti-inflammatory cytokine milieu generated by M2Ct macrophages is associated with enhanced fibroblast-mediated wound closure. In addition, these results indicate that M2Ct macrophages not only acquire an M2-like cytokine secretion profile but they are also capable of controlling other cell types involved in wound healing such as fibroblasts.

Macrophages are innate immune cells with extreme phenotypic plasticity due to involvement in both induction and resolution of inflammation. Due to this dual function, permanent fixation of

macrophage phenotype cannot be achieved. Although M2Ct in our study mediated long-term anti-inflammatory polarization of macrophages, its withdrawal from culture medium with simultaneous addition of IFNg completely restored TNF-α production by macrophages upon LPS challenge indicating capability of M2-like polarized macrophages for adequate response to exogenous danger stimuli in dominating pro-inflammatory environment [17]. This result also indicated that the presence of M2Ct is necessary for the support of anti-inflammatory macrophage phenotype and its prolonged local availability can be provided by self-standing controlled release systems. The system based on tyraminated gelatin/hyaluronic acid (HA) was designed with the purpose to retain loaded cytokines and could be transferred to target environment owing to incorporation of sacrificial layer. Gelatin and hyaluronic acid have been selected as constituents of adhesive films due to the cytokine and growth factor retaining capacities of ECM components. HA as a highly swollen, viscous ECM component that directly affects the distribution and release of growth factors/cytokines via molecular exclusion and resistance to flow [30]. Utilization of a double enzyme mediated crosslinking process resulted in an interpenetrating network that was stable in culture conditions up to 21 days. In comparison, our previous gelatin based system can only survive in culture conditions for 7 days [20]. The loading efficacy of cytokines on adhesive coatings was previously assessed by us using capillary electrophoresis-tandem mass spectrometry analysis and was estimated as 10% [20]. This was the basis for the selection of 10X concentrated amounts of all cytokines for loading in the current study. Interestingly, Gelatin/HA coating alone induced M2-like macrophage phenotype which was characterized by dramatic decrease in TNF-α production in response to LPS as well as moderate increase in IL1Ra, CCL18 and IL1Ra/IL1β ratio. This anti-inflammatory effect of the coating could be explained by the presence of HA that is known to reduce macrophage activation and alleviate inflammation [31, 32]. Anti-inflammatory cytokine IL1Ra is known to neutralize pro-inflammatory effects of IL1β by binding non-productively to IL1 receptor on target cells [33, 34]. Thus, measurement of IL1Ra concentration and IL1Ra/IL1β ratio provides important information about pro- and anti-inflammatory properties of biomaterials. Macrophage culture on Gelatin/HA coatings loaded with M2Ct for 6 days resulted in apparent increase in IL1Ra production and IL1Ra/IL1β ratio indicating favorable effects of released M2Ct components on M2-like macrophage polarization and providing proof for functionality of controlled release system. Another advantage of

multicomponent release systems is the ability to differentially regulate the release of cytokines which can provide more biomimetic gradients and an additional control over macrophage polarization beyond cytokine concentrations.

5. Conclusions

Control of macrophage phenotype around implanted biomaterials can provide a pro-healing microenvironment that can facilitate the integration of biomaterials and prolong their functional lifetime. Here we reported a potent cytokine cocktail that can significantly increase the differentiation of monocytes to M2 macrophages with long term stability. The cocktail increases anti-inflammatory cytokine production and considerably decreases the pro-inflammatory cytokine release. The functional effect of the phenotype control was shown in a wound healing model where the presence of M2-like macrophages obtained via stimulation with the cocktail significantly increased the rate of wound closure. In order to show the applicability of the cocktail *in situ* by a controlled release system, a self-standing gelatin/HA based double crosslinked film was developed and shown to be effective in controlling macrophage phenotype via delivering the cocktail formulation. Controlled delivery of macrophage phenotype controlling cytokine cocktails can be used for effective, long term immunomodulation around implanted materials. Our future research will focus on the further elaboration of the cocktail formula and determination of the underlying mechanism for phenotype fixation.

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Conflict of interest: No

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Figure legends:

Figure 1. Anti-inflammatory properties of M2Ct. Monocytes were cultured for 6 days with IFNg, IL4, M2Ct or left untreated (ns). Concentrations of TNF-α (n=14) **(A)** and IL6 (n=5) **(B)** in supernatants were measured by ELISA after stimulation of the cells with 1 μg/ml LPS for 24h. Concentrations of CCL18 (n=9) **(C)** and IL1Ra (n=7) **(D)** were assessed in supernatants by ELISA before LPS treatment. *p<0.05, ****p<0.0001, ANOVA with Tukey's multiple comparisons, nd – no statistical difference.

Figure 2. Macrophage phenotype during long term culture. (A) Monocytes of 7 donors were cultured for 12 days in the presence of IFNg, IL4, M2Ct or left untreated (ns). On days 6, 9, and 12 of culture

cells were stimulated with 1 μg/ml LPS for 24h and concentrations of TNF-α in supernatants were assessed by ELISA. *p<0.05, ***p<0.001, ANOVA with Tukey's multiple comparisons. **(B)** Monocytes of 5 donors were cultured for 6 days in the presence of IL4, M2Ct or left untreated (ns) followed by replacement of culture medium for complete medium containing 10 ng/ml M-CSF and polarizing cytokines or basal medium containing 1 ng/ml M-CSF and 10^{-8}M dexamethasone. On days 3 and 6 after medium change macrophages were stimulated using 1 μg/ml LPS for 24h and concentrations of TNF-α in supernatants were assessed by ELISA. *p<0.05, Friedman test with Dunn's multiple comparisons. **(C)** Monocytes of 3 donors were cultured for 6 days in the presence of IL4, M2Ct or left untreated (ns) followed by removal of conditioned medium and addition of fresh medium containing IFNg (100 ng/ml) for 24h followed by challenge with LPS (1 μg/ml). TNF-α concentration in supernatants was assessed by ELISA. Data are mean ± SD, Nd – no difference, Friedman test with Dunn's multiple comparisons.

Figure 3. Wound closure dynamics and MMP-7 levels during fibroblasts co-culture with differentially polarized macrophages. (A) Human fibroblasts were co-cultured with different cytokine-polarized macrophages right after inflicting a wound. Fibroblasts were pre-labelled with a fluorescent cell tracer (25μM CFDA-SE, green fluorescence). Healing was evaluated after 0h, 24h and 48h under a fluorescence microscope with 2.5x objective. Representative images from two independent experiments are presented. Scale bar = 500μm **(B)** Quantification of fibroblast wound healing was done after 24h and 48h co-culture with diverse cytokine-polarized macrophages. Fluorescence intensity of wound area was quantified using Image J software and data was expressed as a percentage of wound closure at specific time point. **(C)** MMP-7 levels were measured in macrophage/fibroblast co-culture supernatants by ELISA at 24h and 48h after inflicting the wounds, n=2. ***p<0.01 ****p<0.001, Data are mean ± SEMs, two way ANOVA test.

Figure 4. Characterization of self-standing release system. (A) Schematic illustration of the double crosslinking process using transglutaminase for gelatin (formation of amide bond between glutamine and lysine residues) and horseradish peroxidase for HA-tyramine (dimerization of tyramine to dityramine). **(B)** SEM picture of the film (cross section). **(C)** BSA real-time adsorption on gelatin/HA-

tyramine film using XNano II instrument (Insplorer®). Thickness and homogeneity of the film determined by confocal microscope either with 2D **(E)** or 3D **(D)** pictures using BSA^{FITC} as fluorescent labelling agent. **(F)** A representative photograph of the self-standing film.

Figure 5. The effect of adhesive coatings with controlled cytokine release on macrophage phenotype. Monocytes of 4 donors were cultured for 6 days without coatings (control), and in the presence of coatings without or with controlled cytokine release. For the cells in control plates and on coatings without controlled release system cytokines were added in culture medium on day 0. Concentrations of TNF-α **(A)**, IL6 **(B)** and IL1β **(C)** in supernatants were measured by ELISA after stimulation of the cells with 1 μg/ml LPS for 24h. **(D)** Concentration of IL1Ra was assessed in supernatants by ELISA before LPS treatment. **(E)** IL1Ra/IL1β ratio was calculated by dividing concentration of IL1Ra to corresponding concentration of IL1β in the supernatant of the same donor. **(F)** Concentration of CCL18 was assessed in supernatants by ELISA before LPS treatment. Data are combined for 4 donors and presented as mean ± SD; *$p<0.05$, **$p<0.01$, Friedman test with Dunn's multiple comparisons.