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Modulation of Mouse Macrophage Polarization *in vitro* Using IL-4 Delivery by Osmotic Pumps

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Abstract

Modulation of macrophage polarization is emerging as promising means to mitigate wear particle-induced inflammation and periprosthetic osteolysis. As a model for continuous local drug delivery, we used miniature osmotic pumps to deliver IL-4 in order to modulate macrophage polarization *in vitro* from non-activated M0 and inflammatory M1 phenotypes towards a tissue regenerative M2 phenotype. Pumps delivered IL-4 into vials containing mouse bone marrow macrophage (mBMM) media. This conditioned media (CM) was collected at seven day intervals up to four weeks (week 1 to week 4 samples). IL-4 concentration in the CM was determined by ELISA and its biological activity was assayed by exposing M0 and M1 mBMMs to week 1 or week 4 CM. The IL-4 concentration in the CM approximated the mathematically calculated amount, and its biological activity was well retained, as both M0 and M1 macrophages exposed to either the week 1 or week 4 CM assumed M2-like phenotype as determined by qRT-PCR, ELISA and immunocytochemistry. The results show that IL-4 can be delivered using osmotic pumps and that IL-4 delivered can modulate macrophage phenotype. Results build a foundation for *in vivo* studies using our previously validated animal models and provide possible strategies to locally mitigate wear particle-induced macrophage activation and periprosthetic osteolysis.

Keywords

total joint replacement; inflammation; osteolysis; macrophage polarization; interleukin-4; osmotic pumps

Introduction

Aseptic loosening remains one of the main long-term complications of total joint replacement surgery. Periprosthetic osteolysis and implant loosening are mainly driven by

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chronic, low-grade inflammation that is caused by biomaterial wear-debris released from implant bearing surfaces [1, 2]. For a long time, it has been recognized that macrophages are the key mediators of this wear debris-induced inflammation [3, 4]. Wear-debris activated macrophages secrete chemokines and pro-inflammatory cytokines that lead to further macrophage recruitment, increased osteoclastogenesis, and suppression of osteoblast formation and function. Eventually the balance of local bone remodeling shifts from bone formation to resorption, ultimately leading to peri-implant osteolysis and implant loosening.

Currently the treatment modalities for aseptic loosening are limited to implant revision surgeries that are costly, technically demanding and the source of significant patient discomfort. Novel treatment strategies, targeted especially against developing osteolytic lesions around implants that are otherwise well-functioning and potentially salvageable, are called for. One such emerging strategy to mitigate wear-debris-induced osteolysis is local modulation of macrophage polarization [5].

Macrophages form a dynamic and adaptive population of cells that can assume various functional phenotypes as instructed by the local microenvironment [6–9]. Macrophages that are exposed to interferon gamma (IFN- γ) and/or lipopolysaccharide (LPS) assume a pro-inflammatory phenotype known as classical macrophage activation or M1 macrophage polarization. M1 macrophages are effector cells of the cell mediated immunity and T helper (Th) cell type 1 immune response; they are effective in antigen presentation and support Th1 polarized immune response by secreting interleukin-12 (IL-12). Additionally, M1 macrophages are effective in the killing of phagocytosed microbes via upregulation of inducible nitric oxide synthase (iNOS) and subsequent production of nitrogen and oxygen radicals. M1 macrophages produce pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α) and secrete a distinct set of inflammatory chemokines including CCL2, CCL3, CCL4 and CXCL9 [10]. Key transcription factors that are responsible for the induction of M1 phenotype include STAT1 and IRF5 [11, 12].

IL-4 and IL-13 drive alternative macrophage activation, also known as M2 polarization [6, 8, 13]. M2 macrophages participate in a wide variety of physiological and pathophysiological processes including tissue regeneration, fibrosis and Th2 polarized immune response. M2 macrophages upregulate scavenger receptors, such as mannose receptor (CD206), and secrete various growth factors and anti-inflammatory mediators such as IL-1Ra. In addition, M2 macrophages participate in the production of extracellular matrix components via upregulation of arginase 1 (Arg1). Key transcription factors that are responsible for the induction of M2 phenotype include STAT6 and IRF4; these transcription factors also exert an inhibitory effect on M1-related inflammatory responses [9, 11, 14].

In vitro and *in vivo* studies have shown that induction of M2 macrophage polarization by IL-4 treatment mitigates biomaterial particle-induced and macrophage-mediated chronic inflammatory reaction [15–20]. Indeed, in the field of biomaterial research in general, there has been a growing interest towards the modulation of macrophage phenotype as a means to limit biomaterial-induced adverse effects and to improve tissue integration [21]. As a model for continuous local drug delivery, we used miniature osmotic pumps to deliver IL-4 in order to modulate macrophage polarization *in vitro* from non-activated M0 and

inflammatory M1 phenotypes towards an anti-inflammatory and tissue regenerative M2 phenotype.

Materials and Methods

IL-4 delivery by osmotic pumps

16 Alzet, model 2006 miniature osmotic pumps (Durect Corporation, Cupertino, CA) were loaded with mouse recombinant IL-4 (R&D Systems, Minneapolis, MN) at a concentration of 2 µg/ml diluted in carrier solution comprising of 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) and phosphate buffered saline (PBS). 5 additional pumps were loaded with IL-4 with carrier solution and 15 mg/ml UHMWPE particles (mean diameter $1\mu\text{m} \pm 0.1\mu\text{m}$ as assessed by electron microscopy) isolated from knee replacement simulators as previously described [22]. The particles were tested negative for endotoxin using Limulus Amebocyte Lysate Kit (BioWhittaker, Walkersville, MD). Pumps were connected via 6cm long vinyl tubing (Durect Corporation), pre-filled with their corresponding solutions, to a collection vessel containing 500µl of a mouse bone marrow macrophage (mBMM) augmented media. The whole assembly was placed inside 50ml centrifuge tube partially filled with PBS, and then incubated at +37°C for 4 weeks. The conditioned mBMM media was initially collected at day 3, 5 and 7 after the beginning of the infusion and then at seven day intervals (week 1, 2, 3 and 4 samples) with fresh media added to the collection vessel after each sample collection. Conditioned media was stored at -80°C until used for subsequent experiments.

Theoretical IL-4 accumulation

Theoretical IL-4 accumulation to collection vessel was calculated using equation $c_2 = (c_1at) / (V_2 + at)$ where c_1 is the IL-4 concentration inside the pump, a is the delivery rate of the pump, t the time of the infusion, and V_2 the volume of the medium in the collection tube.

Mouse bone marrow macrophage (mBMM) isolation

The use of animals in the study was approved by our institutional ethics committee. mBMMs were collected from the femora and tibiae of 10 male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) aged 8 to 10 weeks using an established protocol [23, 24]. Briefly, the mice were sacrificed with CO₂ followed by cervical dislocation and then sterilized by dipping in 70% ethanol for 3 × 5min. The femora and tibiae were surgically removed and the bones transversally cut at their proximal and distal ends. The bone marrow was flushed out by injecting 5 ml of mBMM basal medium comprising of Gibco RPMI 1640 (Life Technologies, Pleasanton, CA) supplemented with 10% heat inactivated Gibco fetal bovine serum (FBS, Life Technologies) and 1% Gibco Antibiotic-Antimycotic (Life Technologies) into the medullary canal with a syringe and 25-gauge needle. The cells were filtered through a 100 µm cell strainer, centrifuged and resuspended in ice cold red blood cell lysis buffer (Sigma-Aldrich). After 2min incubation at +4°C, mBMM basal media was added, cells centrifuged, and resuspended in augmented medium comprised of mBMM basal medium (RPMI-1640 with 10% FBS and antibiotics) supplemented with 30% L929 cell-conditioned medium and 10 ng/mL macrophage colony-stimulating factor (M-CSF, R&D Systems). The cells were counted using a hemocytometer, and plated into 175 cm² culture

flasks (BD, Franklin Lakes, NJ) at a concentration of 5×10^7 cells per flask. Cells were cultured for 7 days during which culture medium was changed every 2 to 3 days to remove non-adherent cells. After reaching confluence cells were lifted with Gibco 0.25% Trypsin-EDTA (Life Technologies) and gentle scraping, centrifuged and resuspended in a cryoprotectant medium (10% dimethyl sulfoxide, 90% FBS). Cells were frozen using slow-freeze method and stored in liquid nitrogen until used.

mBMM polarization and exposure to conditioned media

Frozen mBMM were rapidly thawed, resuspended in augmented medium (mBMM basal medium supplemented with 30% L929-cell conditioned medium and 10ng/ml M-CSF) and plated to 175 cm² culture flasks at a density of 5×10^6 cells per flasks. mBMMs were allowed to recover for 7 days, during which culture media was changed every 2 to 3 days. Cells were then lifted and plated to 24 well plates (BD), 1×10^5 cells per well, in 500 µl of augmented media or to 8-well Lab-Tek II Chamber Slides (Thermo Scientific), 4×10^4 cells per chamber, in 200µl of augmented media. After 24 hours of culture mBMMs were either polarized to M1 phenotype by 24 hour treatment with lipopolysaccharide (*Escherichia coli* 0127:B8, Sigma-Aldrich) 100 ng /ml in augmented medium without M-CSF or received just augmented medium without M-CSF (M0 macrophages). Both M0 and M1 macrophages were then exposed either to fresh IL-4 at various concentrations (0.5, 1, 10, 20 or 40 ng/ml in 500 µl of augmented media without M-CSF, positive control) to 500µl of one of the week 1, 2, 3 or 4 conditioned media (augmented media without M-CSF with IL-4 infusion, experimental groups) or received only 500 µl of augmented medium without M-CSF (negative controls). After 3 days cell culture supernatants were collected and cells lysed for total RNA extraction with lysis buffer of the RNeasy kit (Qiagen, Venlo, Limburg, Netherlands). Both the culture supernatants and cell lysates were stored in -80°C until used. Chamber slides were washed with PBS followed by removal of the chamber and fixation in ice-cold acetone for 5min at -20°C and washing in PBS. Slides were stored at -80°C until stained.

Cell viability

The effect of various treatments (exposure to LPS, the various concentrations of IL-4 and the week 1, 2, 3 and 4 conditioned media) on M0 and M1 macrophage viability was assessed by measuring the lactate dehydrogenase (LDH) activity from cell culture supernatants using a commercial kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI) following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using RNeasy Mini kit following manufacturer's instructions. The quality and the amount of the total RNA were measured with a NanoDrop 1000 spectrophotometer (Thermo scientific). Complementary DNA (cDNA) was synthesized using an equal amount of total RNA from each sample and a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). For quantitative real-time PCR, a reaction mix was prepared containing sample cDNA, one of 18S, TNF- α , IL-1Ra, CD206, Arg1, IRF4, IRF5, iNOS or CXCL9 TaqMan primer-probes and TaqMan Gene Expression Master Mix

(all from Applied Biosystems). qRT-PCR was performed with ABI 7900HT Sequencing Detection System (Applied Biosystems) using 18S rRNA as an internal control. The final results were obtained by use of the comparative Ct method [25].

Enzyme-linked immunosorbent assay (ELISA)

IL-4 concentration in the conditioned media was measured using commercial ELISA kit (with a detection limit of 4 pg/ml, eBioscience, San Diego, CA) following manufacturer's instructions. Production of TNF- α (detection limit 8 pg/ml, eBioscience) and IL-1Ra (detection limit 2 pg/ml, Sigma-Aldrich) was assayed from mBMM culture supernatants using commercial ELISA kits following manufacturer's instructions.

Immunofluorescence staining

Chamber culture slides were allowed to reach room temperature, washed with PBS 2 $\times$ 5min and blocked with 1% BSA-PBS for 30min. Primary antibodies against CD11b (2.5 μ g/ml, FITC-conjugated rat anti-mouse IgG2b κ , eBioscience), CD206 (1 μ g/ml, PE-conjugated, monoclonal rat anti-mouse IgG2a κ ; BioLegend, San Diego, CA), Arg1 (5 μ g/ml, unconjugated, polyclonal rabbit anti-mouse IgG; Abcam, Cambridge, United Kingdom) or iNOS (1 μ g/ml, FITC-conjugated monoclonal mouse anti-mouse IgG2a; BD) were diluted in 1% BSA-PBS to the indicated concentrations and then applied to the cells followed by incubation for 1h at room temperature protected from light. Slides were then washed in PBS 3 $\times$ 5min, after which CD11b, CD206 or iNOS stained slides were mounted with ProLong Glod with DAPI (Life Technologies). For Arg1 staining secondary antibody (Alexa Fluor 488; polyclonal Anti-rabbit IgG; Life Technologies) diluted 1/200 in 1% BSA-PBS was applied to the cells and incubated for 1h at room temperature protected from light, followed by washing with PBS 3 $\times$ 5min and mounting. Slides were imaged (Axio Observer 3.1; Zeiss, Oberkochen, Germany) and the mean fluorescence intensity per cell quantified from 3 representative fields of view using ImageJ (National Institute of Health).

Statistics

All cell culture experiments were done in triplicate. Data are presented as mean $\pm$ standard error of the mean. Statistical analyses were conducted using Graphpad Prism version 6.03 (GraphPad Software, La Jolla, CA). Student t-test was used for pair-wise comparison of variables and one-way ANOVA with post hoc Dunnett's multiple comparison test for the group analyses. Two-sided p values less than 0.05 were considered significant.

Results

mBMMs and M1 polarization

Isolated mBMMs were positive for the macrophage marker CD11b as determined by immunocytochemistry (Figure 1a). mBMMs treated with LPS for 24 hours assumed M1-like phenotype as indicated by upregulation of M1-related genes TNF- α , iNOS, CXCL9 and IRF5 using qRT-PCR (Figure 1b). Corresponding changes were seen also in TNF- α and iNOS protein production (Figure 1c, 1d). The LPS treatment did not have an effect on macrophage viability as assessed by LDH activity from cell culture supernatants (data not shown).

Determining optimal IL-4 dosage to induce M2-polarization

The smallest IL-4 concentration tested (0.5ng/ml) already had a clear impact on M0 macrophage phenotype with a significant downregulation of TNF- α , and upregulation of CD206, Arg1 and IRF4 (Figure 2). Increasing IL-4 dosage above 0.5 ng/ml had a relatively minor effect on M2 marker expression in M0 macrophages; M1 macrophages appeared to be more resistant to the smaller IL-4 concentrations but a more clear-cut dose-dependent effect was observed. The maximal effect on M2 marker expression both in M0 and M1 macrophages was generally observed at IL-4 dosages of 10ng/ml while increasing dosage above 20ng/ml had little additional effect on the marker expression. Somewhat paradoxically IL-4 treatment had little effect on IL-1Ra expression in M0 macrophages whereas in IL-4 treated M1 macrophages IL-1Ra was slightly but statistically significantly upregulated. None of the tested IL-4 concentrations caused cytotoxicity in M0 or M1 macrophages (data not shown).

IL-4 in conditioned media

During the first 7 days of IL-4 delivery the IL-4 concentration in the conditioned media increased steadily, approximating the expected rate of IL-4 accumulation (Figure 3a). Based on the pump flow rate, the concentration of IL-4 inside the pump and the volume of the medium in the collection vessel, the theoretical IL-4 concentration in the conditioned media after 7 day of delivery was calculated to be 96ng/ml. After the first week of IL-4 delivery, the measured IL-4 concentration was approximately 75% of the calculated value (Figure 3b). During the subsequent weeks the dose of the IL-4 delivered during the 7 day infusion period was steadily reduced reaching a level of about 50% of the theoretical value. Addition of UHMWPE particles into the pump reduced the IL-4 delivery in all time points to about 25% of the calculated one (Figure 3b).

Exposure of M0 and M1 macrophages to conditioned media

In both M0 and M1 macrophages exposed to one of the week 1 to week 4 conditioned media downregulation of TNF- α mRNA and up-regulation of CD206 and Arg1 was detected using qRT PCR (Figure 4a). Additionally, in M0, but not in M1, macrophages upregulation of IRF4 was detected both in positive controls and in the cells exposed either to week 1 or to week 4 conditioned media. IL-1Ra was upregulated in IL-4 treated M1 macrophages but again somewhat paradoxically slightly downregulated in M0 macrophages. These phenotype changes were generally similar in positive controls (20 ng/ml of fresh IL-4) and between week 1 to week 4 conditioned media. The week 2 and week 3 results were similar to the results of week 1 and week 4 (data not shown).

The amount of TNF- α protein was reduced and the amount of IL-1Ra generally increased in the cell culture media of both M0 and M1 macrophages exposed to week 1 to week 4 conditioned media or to fresh IL-4 as assayed by ELISA (Figure 4b). Similarly, in immunofluorescence staining, increased production of CD206 and Ag1 was generally detected in both M0 and M1 macrophages exposed either to fresh IL-4 or to one of the week 1 to week 4 conditioned media (Figure 4c). The magnitude of these changes in protein production was generally similar in positive controls and between week 1 to week 4

conditioned media, although not all of the changes reached statistical significance. The results of week 2 and 3 were similar to weeks 1 and 4 (data not shown).

The exposure of M0 or M1 macrophages to any of the pump conditioned media did not have an effect on macrophage viability (data not shown).

Discussion

Currently, treatment options for developing aseptic osteolysis are limited to costly and demanding revision surgeries; effective non-operative biological treatment options are still to emerge. Clinical trials targeting a single inflammatory mediator e.g. TNF- α have not been successful, perhaps due to the complexity and the redundancy of the cytokine networks involved in the process of periprosthetic osteolysis [26]. Furthermore the systemic interventions carry the risk of unacceptable side-effects. More comprehensive biological intervention strategies that take into the account both the local nature and the complexity of the osteolytic process are called for.

One such emerging strategy is the local modulation of macrophage polarization [5]. *In vitro* and *in vivo* studies have shown that modulation of macrophage polarization by IL-4 treatment suppresses both wear debris induced inflammation and subsequent osteolysis [15–20]. As a model for continuous and local drug delivery system, we investigated delivery of IL-4 and modulation of macrophage polarization *in vitro* from non-activated M0 and inflammatory M1 phenotypes towards anti-inflammatory and tissue regenerative M2 phenotype using miniature osmotic pumps.

During the 4-week experiment, osmotic pumps delivered IL-4 at a rate of 50–75% of the theoretical one. In addition to possible imprecisions in the dosing of IL-4 and in the measurement of its concentration, the discrepancy between the theoretical and observed IL-4 delivery rate is likely due to binding of the IL-4 to the walls of the pump and tubing. The assumption is supported by the fact that addition of UHMWPE particles to the system reduced the availability of the IL-4 to 25% of the theoretical value, possibly due to binding of IL-4 to the high surface area of the UHMWPE particles. In contrast, degradation of the IL-4 in the collection vessel during the 7 day infusion period did not seem to be a major issue as the IL-4 concentration in the conditioned media increased steadily during the first 7 days of infusion, well approximating the expected rate of IL-4 accumulation. Despite the binding of IL-4 to the walls of the delivery system and to the particles the amount of IL-4 delivered using the pumps was still more than 20-times higher than the 0.5 ng/ml that already had a clearly detectable effect on both M0 and M1 macrophages and well in the concentration range (20 to 40ng/ml) that had the most clear cut effect to macrophage phenotype.

Both M0 and M1 macrophages exposed to the IL-4 containing conditioned media or fresh IL-4 assumed M2-like phenotype as determined by up-regulation of several M2 markers including CD206 and Arg1 as well as corresponding downregulation of TNF- α and increase in IL-1Ra production. The magnitude of these phenotypic changes was similar in positive controls and both in macrophages exposed to week 1 and week 4 conditioned media,

indicating that the biological activity of the IL-4 was well preserved during the 4-week delivery period. Furthermore, these phenotype changes were generally observed both at the level of mRNA and protein production. However, despite clear trends, not all of these changes reached statistical significance likely due to limited number of parallel experiments performed.

Slight differences in subsequent M2 macrophage phenotype were observed depending on the initial polarization of the macrophage. For example, M1 macrophages exposed to IL-4 or IL-4 containing conditioned media produced almost ten times more IL-1Ra than corresponding M0 macrophages. The results are in agreement with previous reports that the characteristics of M2 polarization are more evident if the cells have first passed through an M1 phenotype [17, 27], possibly reflecting the physiological transition from inflammation to tissue regeneration. In contrast, IRF4, a transcription factor of which production has been described to characterize M2 polarization [14] was strongly upregulated in M0 macrophages exposed to IL-4 but much less so in M1 macrophages suggesting the action of some, thus far uncharacterized inhibitory mechanism. Also the down-regulation of TNF- α production was more subtle in M1 macrophages than in M0 macrophages exposed to IL-4 or the IL-4 containing conditioned media.

Our results show that IL-4 can be locally delivered using osmotic pumps, and that IL-4 so delivered can modulate macrophage phenotype both from M0 and M1 phenotypes towards an M2 phenotype. Results build a foundation for further *in vivo* studies using our murine continuous femoral intramedullary particle infusion model [22, 28] with the ultimate goal being development of treatment for developing osteolytic lesions by local modulation of macrophage phenotype.

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Figure 1. LPS treated M0 macrophages assumed M1-like phenotype

(a) Isolated mouse bone marrow macrophages (mBMMs) were positive for macrophage maker CD11b. When treated with LPS for 24 hours these M0 macrophages assumed a M1-like phenotype as indicated by (b) upregulation of TNF- α , iNOS, CXCL9 and IRF5 mRNA in qRT-PCR (c) increased secretion of TNF- α protein to culture media as assayed by ELISA and (d) increased intensity of iNOS immunoreactivity as assayed by immunofluorescence staining. Results expressed either as (b) relative expression to an arbitrary external value, (c) ng/ml or (d) percent change in the fluorescence intensity between M0 and M1 macrophages. ***=p<0.001 M0 vs. M1 macrophages.

Figure 2. Low concentrations of IL-4 induced M2-like phenotype

M0 and M1 mouse macrophages were exposed to various concentrations of IL-4 (from 0.5 to 40ng/ml) for 3 days after which relative expression of indicated M1 and M2 markers was evaluated using qRT PCR. Results are expressed as common logarithm-transformed fold change (fc) to corresponding untreated cells. ns=not statistically significant; *=p<0.05; **=p<0.01; ***=p<0.001 compared to the corresponding non-treated M0 or M1 macrophages.

Figure 3. Osmotic pumps delivered IL-4 at a rate that closely followed the expected delivery rate IL-4 with or without UHMWPE particles was delivered into collection vessels containing macrophage culture media for 4 weeks using osmotic pumps. Samples of this conditioned media were initially collected at days 3, 5, and 7 (a) and then at 7 day intervals (b). The IL-4 concentrations in conditioned media were measured using ELISA. Grey bars = IL-4 concentration in samples without UHMWPE particles; white bars = IL-4 concentration in samples with UHMWPE particles. Dashed lines indicate the calculated IL-4 concentration at each time point.

Figure 4. M0 and M1 macrophages exposed to IL-4 in conditioned media assumed M2-like phenotype

M0 and M1 mouse macrophages were exposed to fresh IL-4 (20 ng/ml, positive controls, black bars) or one of week 1 to week 4 pump conditioned media for 3 days after which **a)** relative expression of indicated M1 and M2 markers were evaluated using qRT PCR; **b)** production of TNF- α and IL-1Ra protein was assayed from cell culture supernatants using ELISA; and **c)** CD206 and Arg1 were stained using immunocytochemistry followed by quantification of fluorescence intensity using image analysis. Results are expressed either as **(a)** common logarithm-transformed fold change (fc) to, or **(c)** percent of corresponding untreated cells. For simplicity only results for week 1 and week 4 are shown. ns= not statistically significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ compared to the corresponding non-treated M0 or M1 macrophages.