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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* applications 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.

Statement of Significance

Uncontrollable activation of macrophages in the microenvironment of implants and engineered tissues is a significant problem leading to poor integration of implants and artificial tissues. In the current manuscript we demonstrate that self-standing, transferable gelatin/tyraminated hyaluronic acid based thin films are perspective tools for controlled release of anti-inflammatory cytokine combinations and can be used to down-modulate macrophage activation on implant surfaces. We also show that optimized cytokine cocktail consisting of IL4/IL10/TGFβ1 (M2Ct) induces long-term anti-inflammatory and pro-healing phenotype in human primary monocyte-derived macrophages. This cocktail formulation could be loaded on gelatin/tyraminated films and promoted favorable M2-like macrophage phenotype with

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low responsiveness to pro-inflammatory stimuli. Such self-standing release systems can be used for prolonged local control of macrophage phenotype upon implantation.

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 24 h 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