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Macrophage phenotypes in the collagen-induced foreign body reaction in rats

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ABSTRACT

Implantation of biomaterials into the body elicits a material-dependent inflammatory response called the foreign body reaction (FBR). Macrophages play a pivotal role in the FBR by orchestrating the pro-inflammatory microenvironment around the biomaterials by secreting cytokines, chemokines and growth factors. When the biomaterial is porous or degradable, macrophages can migrate into the material and continue the generation of a pro-inflammatory microenvironment inside the materials. They also regulate the degradation of biomaterials by secreting proteolytic enzymes and by phagocytosis. We hypothesize that macrophages present in the different microenvironments of the FBR have different phenotypes. Fundamental knowledge of the phenotypes of macrophages and their dynamics during the FBR will contribute to our overall understanding of the mechanisms involved in the FBR, and may provide us with additional tools to modulate the FBR. To investigate the phenotype of macrophages in the FBR, we validated phenotype-specific markers for rat macrophages in vitro by stimulating them with IFNγ/LPS, IL4/IL13 or IL4/dexamethasone to induce classically activated macrophages (M1φ) or alternatively activated macrophages (M2φ). Gene expression analysis, Western blot and immunohistochemistry revealed that iNOS and CD206 are specifically expressed by M1φ and M2φ, respectively. Using these markers, we investigated the distribution of M1φ and M2φ in the FBR induced by subcutaneously implanted hexamethylenediisocyanate cross-linked dermal sheep collagen (HDSC) disks in AO rats. We found that part of the macrophages display an M2 phenotype, whereas the M1phenotype was not detected. Our data suggest that many macrophages in the FBR induced by HDSC do not fit into the classical M1 or M2 dichotomy.

1. Introduction

Within the field of tissue engineering, biomaterial scaffolds are often employed at sites that require functional restoration in order to provide structural support, to deliver cells and/or to deliver soluble mediators. Introduction of biomaterial scaffolds into the body elicits an inflammatory reaction called the foreign body reaction (FBR) (reviewed in Refs. [1–3]). The FBR is commonly subdivided into onset, progression and resolution phases. During the progression phase of the FBR, a transition occurs from an acute to a chronic inflammation. In this phase two separate microenvironments can be distinguished. Inside the implanted material macrophages display at least three different functionalities, influenced by the size, surface geometry and physicochemical properties of the material. Firstly, the macrophages secrete pro-inflammatory cytokines and chemokines, which orchestrate the pro-inflammatory microenvironment which by itself further attracts more monocytes and macrophages [4]. Secondly, the macrophages produce proteolytic enzymes, such as matrix metalloproteinases (MMPs), that degrade the biomaterial [5]. Thirdly, activated macrophages fuse into multinucleated giant cells, which also contribute to the maintenance of the pro-inflammatory state through secretion of pro-inflammatory chemokines, cytokines and MMPs. The secreted MMPs (pre)degrade the biomaterial, while macrophages and giant cells phagocytose the resultant biomaterial particles.

In general, the microenvironment inside the implanted material is aimed at inducing and maintaining inflammation and degradation of the material. Outside of the implanted material, a mature fibrous capsule is formed, consisting predominantly of fibroblasts and a few macrophages [1–3]. Here, the fibroblasts proliferate, align and secrete extracellular matrix (ECM) which physically shields the host from the implanted material. Because of the fundamental differences in these two microenvironments, we speculate that the macrophages present inside and outside the implanted material may display different phenotypes. Most literature distinguishes two general categories of macrophage phenotypes: classically activated macrophages (M1φ) and alternatively activated macrophages (M2φ) [6–9]. M1φ play important roles in microbiocidal activity, cellular immunity and tissue damage, whereas M2φ

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play important roles in humoral immunity, allergic and anti-parasite responses, and tissue repair. Because M1φ and M2φ are important in tissue damage and tissue repair, respectively, we hypothesize that M1φ are predominantly present inside the implanted material, which is aimed at the destruction of the material, and that M2φ are predominantly present in the capsule, which is aimed at the generation of fibrous tissue.

In vitro, unstimulated human or murine macrophages adopt an M1 phenotype when stimulated with lipopolysaccharides (LPSs) and interferon gamma (IFNγ). These M1φ have been reported to express increased levels of C–C chemokine receptor type 7 (CCR7), CD80, CD86, interleukin 1β (IL1β), interleukin 12 (IL12), inducible nitric oxide synthase (iNOS, only murine macrophages), and tumor necrosis factor alpha (TNFα) [6–11]. Similarly, unstimulated human and murine macrophages adopt an M2 phenotype when stimulated with interleukin 4 (IL4) and interleukin 13 (IL13), or with IL4 and dexamethasone (Dexa). These M2φ have been reported to express increased levels of CD163, CD206, C-type lectin domain family 10 member A (Clec10a), E-cadherin (Ecad), macrophage scavenger receptor 1 (MSR1), and stabilin 1 (Stab1) [6–17].

Unfortunately, very few studies have been published in which phenotypes of rat macrophages have been described or validated. We therefore stimulated macrophages from rat bone marrow with LPS/IFNγ, IL4/IL13 or IL4/Dexa to induce M1 and M2 phenotypes. These phenotypes were confirmed using quantitative real-time polymerase chain reaction (qPCR) and the phenotype-specific expression of two markers was confirmed at the protein level using Western blot and immunohistochemistry (IHC). Using these validated phenotype-specific markers, the localization and dynamics of M1φ and M2φ were studied in our model for the FBR, namely the subcutaneous implantation of hexamethylenediisocyanate-crosslinked dermal sheep collagen (HDSC) disks in AO rats.

2. Materials and methods

2.1. Generation and stimulation of bone marrow-derived macrophages in vitro

Femurs of healthy male rats ($n = 3$) were flushed using sterile phosphate-buffered saline (PBS). The bone marrow cells were centrifuged at 300*g* at 4 °C for 10 min. Erythrocytes were lysed for 5 min on ice in erythrocyte lysis buffer, containing 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.4). Afterwards, the remaining leukocytes were washed in PBS, centrifuged at 300*g* at 4 °C for 10 min, and resuspended in DMEM medium containing 1 g l^{-1} glucose, 10% fetal calf serum (FCS), 2 mM L-glutamine (Sigma–Aldrich), and 1% penicillin/streptomycin (Sigma–Aldrich). The medium was supplemented with 10 ng ml^{-1} macrophage colony-stimulating factor (M-CSF) (Peprotech) to promote the outgrowth of macrophages. After 3 and 5 days medium was replenished. After 7 days the medium was replaced with medium containing LPS (1 μg ml^{-1}) and IFNγ (10 ng ml^{-1}), or IL4 (10 ng ml^{-1})/IL13 (10 ng ml^{-1}), or IL4 (10 ng ml^{-1})/Dexa (100 nM), or with medium without additions.

2.2. RNA isolation, cDNA synthesis and qPCR

After 24 h of stimulation the cells were lysed in RLT buffer (Qiagen) and RNA was isolated using a RNeasy Micro Kit (Qiagen) according to the instructions of the manufacturer. Total RNA was reverse transcribed using a First Strand cDNA synthesis kit (Fermentas). Gene expression was quantified using quantitative polymerase chain reaction (qPCR) in a final reaction volume of 10 μl, consisting of 1× Sybr green Supermix (Bio-Rad), 6 μM of forward and reverse primer (Table 1, developed using the Roche assay design center), and 5 ng cDNA. The reactions were performed at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s for 40 cycles in a TaqMan ABI7900HT cycler (Applied Biosystems). Analysis of the data was performed using Science Detection Software 2.2.2. Relative expression was calculated as $2^{-\Delta CT}$ using β-actin as a reference gene, as it was the most stable gene out of three reference genes.

2.3. Protein expression analysis on cultured macrophages

Flow-through of RNA isolations was treated as described by the manufacturer of the RNA isolation kits, in order to allow both RNA and protein analysis on the same samples. In short, flow-though (containing mainly proteins and some genomic DNA in RLT buffer) was treated with 4 volumes of ice-cold acetone to precipitate the proteins in the sample. Precipitated proteins were pelleted by centrifugation and air-dried. Subsequently the pellets were redissolved in 2-D buffer [18], consisting of 30 mM Tris–HCl, 7 M urea, 2 M thiourea and 4% CHAPS (all from Sigma–Aldrich), and loaded on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gels. Proteins were separated at 200 V for 1 h and transferred onto nitrocellulose membranes (Millipore) at 100 V for 1 h. Both procedures were performed under reducing conditions. The blots were incubated overnight in 5% milk powder (Campina) in PBS. Next, the blots were probed with antibodies detecting iNOS (Abcam, Ab15323, 1:1000), CD206 (Abcam, Ab64693, 1:1000) and β-actin (Abcam, Ab8227, 1:1000). Primary antibodies were detected using horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies (Abcam, 1:1000). Detected proteins were visualized using ECL substrate (Promega) according to the

Table 1
Overview of the primers used for qPCR.

	Gene	Forward primer	Reverse primer
M1 genes	iNOS	CCTGGTGCAAGGGATCTTGG	GAGGGCTTGCCTGAGTGAGC
	IL1β	TCCAGGATGAGGACCCAAGC	TCGTCATCATCCCACGAGTCA
	IL12	GGAGGCCCAGCAGCAGAATA	TTCTTGGGTGGGTCCGGTTT
	TNFα	TTCCCAAATGGGCTCCCTCT	GTGGGCTACGGGCTTGTCAC
	CCR7	TGGCTCTCCTGGTCATTTTC	GCCGATGTAGTCGTCTGTGA
	CD80	TGCTGGTTGGTCTTTTCCA	TGACTGCTCTTCAGAACAAAA
	CD86	TCCTCCAGCAGTGGGAAACA	TTTGTAGGTTTCGGGTATCCTTGC
M2 genes	CD206	GGTTCCGGTTTGTGGAGCAG	TCCGTTTGCATTGCCCAGTA
	CD163	CTCAGCGTCTCTGCTGTCAC	GGCCAGTCTCAGTTCCTTCTT
	Clec10a	GAGAAAAACCAAGAGGCTGGT	CTAAGGCCCAGGGAGAACA
	IL10	CAAGGCAGTGGAGCAGGTGA	CCGGGTGGTTCAATTTTTCATT
	Ecad	TGGTGTGGGTCTGGAGATCG	CAGCCCGAGTGGAAATGACC
	Stab1	CCATGGAGAGAAGTCATCATCA	GTGACACAGCTCCTGACAACAT
	MSR1	CTGGTGTTCCAGGTGCAAG	AAGCCAACTGGTCCCTGAT
Reference gene	ActB	TGGGACGATATGGAGAAGAT	TGTTGAAGGTCTCAAACATGA