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Research Article

Engineering macrophages to control the inflammatory response and angiogenesis

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ABSTRACT

Macrophage (MΦ) dysregulation is increasingly becoming recognized as a risk factor for a number of inflammatory complications including atherosclerosis, cancer, and the host response elicited by biomedical devices. It is still unclear what roles the pro-inflammatory (M1) MΦ and pro-healing (M2) MΦ phenotypes play during the healing process. However, it has been shown that a local overabundance of M1 MΦs can potentially lead to a chronically inflamed state of the tissue; while a local over-exuberant M2 MΦ response can lead to tissue fibrosis and even promote tumorigenesis. These notions strengthen the argument that the tight temporal regulation of this phenotype balance is necessary to promote inflammatory resolution that leads to tissue homeostasis. In this study, we have engineered pro-inflammatory MΦs, MΦ-cTLR4 cells, which can be activated to a M1-like MΦ phenotype with a small molecule, the chemical inducer of dimerization (CID) drug. The MΦ-cTLR4 cells when activated with the CID drug, express increased levels of TNFα, IL-6, and iNOS. Activated MΦ-cTLR4 cells stay stimulated for at least 48 h; once the CID drug is withdrawn, the MΦ-cTLR4 cells return to baseline state within 18 h. Further, *in vitro* CID-activated MΦ-cTLR4 cells induce upregulation of VCAM-1 and ICAM-1 on endothelial cells (EC) in a TNFα-dependent manner. With the ability to specifically modulate the MΦ-cTLR4 cells with the presence or absence of a small molecule, we now have the tool necessary to observe a primarily M1 MΦ response during inflammation. By isolating this phase of the wound healing response, it may be possible to determine conditions for ideal healing.

1. Introduction

The physiological innate inflammatory response requires a highly orchestrated series of events characterized by four basic phases: reaction, regrowth, remodeling, and resolution [1]. During the course of this healing process, MΦs play an active role in secreting chemokines and cytokines that direct the recruitment and egress of various immune cell types at the injured site. The functional MΦ phenotype depends on the microenvironment of the injured site and alters accordingly during the normal process of healing [2]. However, dysregulation of the MΦ phenotype can lead to a non-ideal healing outcome.

Monocytes are the precursor cells to MΦs, which are a main inflammatory cell type and are known to be key players in the inflammatory response. When activated, MΦs are classified into two major phenotypes that can be broadly defined as: pro-inflammatory MΦs and pro-healing MΦs. In literature, pro-inflammatory MΦs are often denoted as “classically activated” or “M1” and pro-healing MΦs are denoted as “alternatively activated” or “M2.” However, these two major MΦ phenotypes are the two extremes on the phenotype scale, as intermediate MΦ types also exist [3]. During the inflammatory reaction, M1 MΦs are the first to arrive at the inflammation site and this MΦ population subsequently shifts to a less inflammatory pro-healing M2 MΦ population during the repair phase. Pro-inflammatory MΦs release inflammatory cytokines, such as TNFα and IL-6 as well as produce reactive oxygen species (ROS) [4,5]. In comparison, the pro-healing MΦ phenotype has been shown to produce cytokines, such as IL-10 and TGFβ1, which are markers that can decrease the pro-inflammatory response and promote healing and fibrosis [3].

Angiogenesis, or the formation of new blood vessels, is a critical

Abbreviations: MΦ/s, Macrophage/s; pro-inflammatory MΦ, M1 MΦ; pro-healing MΦ, M2 MΦ; CID, chemical inducer of dimerization; cTLR4, cytoplasmic portion of TLR4; MΦ-cTLR4, engineered pro-inflammatory MΦ; MΦ-T2A, engineered control MΦ; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; ROS, reactive oxygen species; TNFα, tumor necrosis factor alpha; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; EC, endothelial cell/s; LPS, lipopolysaccharides; F36V, engineered dimerization domain; RE, restriction enzyme

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step in the wound healing process. In the course of the inflammation response, pro-inflammatory MΦs secrete TNFα and IFN-γ, which regulate expression of adhesion molecules on endothelial cells (EC). These cytokines promote leukocyte adhesion to EC and extravasation into tissues by increasing expression of both cell surface and soluble forms of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) [6–9]. These two adhesion molecules belong to the immunoglobulin superfamily group and have been implicated, along with integrins, in pro-angiogenic processes [10,11].

This study utilizes engineered pro-inflammatory M1-like cells to investigate EC activation. The engineered cells were designed by using the chemical inducer of dimerization (CID) technology to induce activation of the TLR4 receptor independent of the lipopolysaccharides (LPS) exogenous ligand, which is a well-established inducer of the M1 MΦ phenotype [12]. The CID technology has been used in several other groups to control a variety of cell signaling pathways [13–17]. For this system to function, the intracellular domain of a desired receptor is fused to a F36V protein, which is a mutated version of the FKBP12 protein. This F36V version has a binding site for a cell permeable CID drug. When CID drug is present, two F36V proteins will dimerize, bringing the desired intracellular domains in close enough proximity to activate receptor-specific pathways. These cells can be activated by the exposure to CID drug and be deactivated by the withdrawal of CID drug. Currently in the literature there have been no cellular engineering approaches to control or modulate MΦ polarization to determine ideal healing conditions. Having the ability to control MΦ polarization during and after an inflammatory response could potentially allow for the manipulation of the host response and the optimal healing of multiple inflammatory conditions.

2. Methods and materials

2.1. Reagents and antibodies

The monoclonal anti-human/mouse/rat FKBP12 antibody was purchased from Thermo Scientific. The following antibodies were purchased from Cell Signaling: p44/42 MAPK, Phospho-p44/42 MAPK, IRF3 and Phospho-IRF3. The anti-iNOS/NOS type II antibody was purchased from BD Biosciences. The anti-mouse CD106 (VCAM-1) PE, the anti-mouse CD54 (ICAM-1) PE, the rat IgG2b isotype, and the anti-mouse TNFα antibodies were purchased from eBioscience. The HRP-conjugated goat-anti-rabbit antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. and the HRP-conjugated goat-anti-mouse antibody was obtained from Life Technologies. LPS and recombinant mouse TNFα was purchased from Sigma and recombinant mouse IL-4 was purchased from eBioscience. AP20187 (CID drug) was purchased from Clontech. Lipofectamine 2000 was purchased from Invitrogen. The Dual-Luciferase® reporter assay system was obtained from Promega Corporation.

2.2. Plasmid construction of cTLR4

The mouse Sport6-TLR4 vector was purchased from Open Biosystems. The cytoplasmic portion of TLR4 (cTLR4) was amplified (mRNA base pairs 2207-2748) and inserted into a pBluescript II KS+ vector with an existing myristolation domain and engineered F36V domain (pBluescript-Myr-F36V) (55) following BamHI and EcoRV restriction enzyme (RE) cuts. PCR products were gel purified using a QIAEX II gel extraction kit (Qiagen) before ligations were performed. This resulted in a pBluescript-Myr-F36V-cTLR4 construct. The pCDH-EF1α-MCS-T2A-copGFP lentiviral cDNA and expression vector was purchased from System Biosciences. This vector was cut in the MCS with both NheI and EcoRI, and a PCR amplified portion of the Myr-F36V-cTLR4 sequence was ligated into this site within the pCDH-EF1α-MCS-T2A-copGFP vector (7.26 kb). This resulted in the final cTLR4 lentiviral plasmid: pCDH-EF1α-Myr-F36V-cTLR4-T2A-copGFP (8.18 kb).

2.3. Cell transduction of cTLR4 lentiviral constructs

We utilized a 3rd generation lentiviral vector, pCDH (System Biosciences), carrying the cTLR4 gene under the control of the EF-1α promoter. For stable lentiviral transductions, 5×10^6 HEK293T packaging cells were seeded in 10-cm cell culture dishes that were previously coated with 50 ug/mL poly-D-lysine hydrobromide (Sigma). Culture medium was changed just prior to transduction. In total, 12 μg plasmid DNA was used for each 10-cm dish (2.8 μg transfer vector (cTLR4), 0.9 μg pSL3 (vesicular stomatitis virus G envelope), 5.4 μg pSL4 (HIV-1 gag/pol packing genes), and 2.8 μg pSL5 (rev gene required for HIV-1 envelope protein expression). DNA and Lipofectamine 2000™ (Life Technologies) were diluted in Opti-MEM® medium (Gibco) separately. After a 5 min incubation, DNA and lipofectamine were combined and incubated for 20 min at room temperature. The complexes were then added, drop-wise, to cell dishes with 8 mL growth medium and medium was replaced after 14–16 h. Virus supernatant was collected following an additional 48 h by filtering through a 0.45 μm filter. Filtered virus supernatant was then added either directly or in concentrated form to previously plated RAW264.7 cells (5×10^5 cells per well) in 6-well plates. Cells were then sorted for GFP expression to acquire $>$ 90% transduction efficiency.

2.4. Cell culture

RAW264.7 and bEnd.3 cells were obtained from ATCC. RAW264.7 and bEnd.3 cells were cultured in DMEM medium from Invitrogen containing 10% (v/v) heat-inactivated FBS and 100 U/ml pen/strep (Invitrogen) and incubated at 37 °C with 5% CO_2.

2.5. Western Blotting

Protein from RAW264.7, MΦ-T2A (vector control cells), and MΦ-cTLR4 cell monolayers were extracted by lysis in Laemmli buffer containing 1x Halt Protease Inhibitor cocktail (Thermo Scientific). Following lysis, samples were boiled and protein concentration was determined by performing a BCA assay from Thermo Scientific. Samples (10–30 μg of lysates) were run on 4-20% Mini-PROTEAN® TGX precast polyacrylamide gels (Bio-Rad). Protein from gels were transferred onto PVDF membranes and probed with the appropriate primary antibody overnight. Membranes were washed between each antibody incubation and subsequently probed with the appropriate HRP-conjugated secondary antibody (Life Technologies). The Clarity Western ECL Substrate (Bio-Rad) was used to detect bands.

2.6. Cytokine profile

We tested IL-6 and TNFα concentrations in supernatants of transduced RAW264.7 cells *in vitro*. Briefly, MΦ-cTLR4 cells (1×10^6) were plated in each well of a 6-well plate and treated with vehicle (100% EtOH), LPS (100 ng/mL), CID drug (50 nM), or left untreated in DMEM without serum. Supernatants were collected and tested using the mouse IL-6 ELISA Ready-SET-Go! and the mouse TNFα ELISA Ready-SET-Go! Kits (eBioscience) according to the manufacturer's instructions. Plates were read at 450 nM with a 570 nM wavelength subtraction, normalized to standard solutions, and concentrations (pg/mL) were calculated.