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Macrophage-specific nanotechnology-driven CD163 overexpression in human macrophages results in an M2 phenotype under inflammatory conditions

Perla Abigail Alvarado-Vazquez[a,1], Laura Bernal[a,b,1], Candler A. Paige[a,c], Rachel L. Grosick[a], Carolina Moracho Vilrriales[a], David Wilson Ferreira[a,d], Cristina Ulecia-Morón[a,e], E. Alfonso Romero-Sandoval[a,*]

[a] Department of Pharmaceutical and Administrative Sciences, Presbyterian College School of Pharmacy, 307 N. Broad St, Clinton, SC 29325, USA
[b] Department of Systems' Biology, School of Medicine, University of Alcala Campus Universitario – C/19, Carretera Madrid-Barcelona, Km 33,600, 28871 Alcalá de Henares, Madrid, Spain
[c] Department of Brain and Behavioral Sciences, Systems Neuroscience, University of Texas at Dallas, 800W Campbell Road, Richardson, TX 75080, USA
[d] Department of Pharmacology, Ribeirao Preto Medical School – University of Sao Paulo, 3900 Bandeirantes Avenue, Ribeirão Preto SP 14049-900, Brazil
[e] Department of Pharmacology, Faculty of Medicine, University Complutense. Pza. Ramón y Cajal, s/n, Ciudad Universitaria., 28040 Madrid, Spain

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ABSTRACT

M1 macrophages release proinflammatory factors during inflammation. They transit to an M2 phenotype and release anti-inflammatory factors to resolve inflammation. An imbalance in the transition from M1 to M2 phenotype in macrophages contributes to the development of persistent inflammation. CD163, a member of the scavenger receptor cysteine-rich family, is an M2 macrophage marker. The functional role of CD163 during the resolution of inflammation is not completely known. We postulate that CD163 contributes to the transition from M1 to M2 phenotype in macrophages. We induced CD163 gene in THP-1 and primary human macrophages using polyethylenimine nanoparticles grafted with a mannose ligand (Man-PEI). This nanoparticle specifically targets cells of monocytic origin via mannose receptors. Cells were challenged with a single or a double stimulation of lipopolysaccharide (LPS). A CD163 or empty plasmid was complexed with Man-PEI nanoparticles for cell transfections. Quantitative RT-PCR, immunocytochemistry, and ELISAs were used for molecular assessments. CD163-overexpressing macrophages displayed reduced levels of tumor necrosis factor-alpha (TNF)-α and monocytes chemoattractant protein (MCP)-1 after a single stimulation with LPS. Following a double stimulation paradigm, CD163-overexpressing macrophages showed an increase of interleukin (IL)-10 and IL-1ra and a reduction of MCP-1. This anti-inflammatory phenotype was partially blocked by an anti-CD163 antibody (effects on IL-10 and IL-1ra). A decrease in the release of TNF-α, IL-1β, and IL-6 was observed in CD163-overexpressing human primary macrophages. The release of IL-6 was blocked by an anti-CD163 antibody in the CD163-overexpressing group. Our data show that the induction of the CD163 gene in human macrophages under inflammatory conditions produces changes in cytokine secretion in favor of an anti-inflammatory phenotype. Targeting macrophages to induce CD163 using cell-directed nanotechnology is an attractive and practical approach for inflammatory conditions that could lead to persistent pain, i.e. major surgeries, burns, rheumatoid arthritis, etc.

1. Introduction

Persistent inflammation can promote the development of chronic diseases, such as rheumatoid arthritis, osteoarthritis, metabolic syndrome-associated disorders (including type 2 diabetes and atherosclerosis), Alzheimer's disease, asthma, or Crohn's disease (Wong and Lord, 2004; Libby, 2006; Okin and Medzhitov, 2012). Most of these chronic inflammatory diseases result in persistent pain due to the sensitization of peripheral terminals and a subsequent central sensitization. The research in this field has been mainly focussed on reducing proin-

* Corresponding author. Current address: Department of Anesthesiology, Wake Forest University School of Health Sciences, Medical Center Blvd, Winston-Salem, NC 27157, USA. Tel.: +1 864 938 3833; fax: +1 864 938 3903.
E-mail address: eromeros@wakehealth.edu (E.A. Romero-Sandoval).
[1] These authors contributed equally to this article.

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flammatory final effectors (cytokines, prostaglandins, etc.), and, therefore, the current therapies (i.e. glucocorticoids, antibodies against cytokines, COX inhibitors, etc.) are based on the inhibition, specifically or globally, of acute proinflammatory mediators. These therapies have been shown to improve the quality of life of patients suffering from chronic inflammatory diseases, but they have many downfalls. For example, they produce multiple side effects, are not effective for all patients, and, most importantly, do not target the underlying causes of the disorder. Even though there have been significant advances in the identification of anti-inflammatory effectors, our understanding of the upstream molecular signaling that drives, promotes, or alters the normal resolution of inflammation is not complete. Therefore, the elucidation of the molecular mechanisms that drive the normal resolution of inflammation will provide the foundation to develop a novel and promising therapeutic approach aiming to restore tissue homeostasis.

Resolution of inflammation is an active and coordinated process preceded by an early cytodestructive phase (Serhan et al., 2007; Serhan, 2011). Cells of the monocyte/macrophage lineage have been recognized as essential in both phases (Wynn et al., 2013; Dunster, 2016). Usually, macrophages are divided into two categories (M1 and M2), each of which is predominant in the initiation or resolution of inflammation (Martinez and Gordon, 2014). The M1/M2 dichotomy is based on the response of macrophages to an external stimuli (Martinez and Gordon, 2014). For example, the presence of lipopolysaccharide (LPS), interferon-gamma, or tumor necrosis factor-alpha (TNF-α) polarize macrophages toward and M1 phenotype. This macrophage phenotype produces proinflammatory cytokines (interleukin [IL]-1, IL-6, IL-12, TNF-α, etc.) and oxidative metabolites (nitric oxide and superoxide) that promote host defense and removal of damaged tissue (Gordon, 2003; Gordon and Taylor, 2005; Roszer, 2015). In contrast, macrophages with an M2 phenotype (usually labeled as anti-inflammatory) represent a heterogeneous population of macrophages composed by multiple sub-groups or sub-phenotypes (Martinez and Gordon, 2014). For instance, the M2a macrophage phenotype is characterized by the cellular surface expression of CD163/CD206 (Martinez and Gordon, 2014). Macrophages that adopt an M2b phenotype express markers such as CD86 and class II major histocompatibility complex (MHCII), and macrophages that adopt an M2c phenotype express signaling lymphocyte-activation molecule (SLAM) (Martinez and Gordon, 2014). All these M1/M2 hallmarks do not necessarily exclude each other and usually coexist and adapt depending of the stimulus or the conditions of the system (Davis et al., 2013). Until date, all functions of these markers and phenotypes are not thoroughly understood. When macrophages are unable to change their phenotype from M1 to M2, inflammation does not resolve, becoming persistent or chronic (Nathan and Ding, 2010). Therefore, therapies that specifically target macrophages could result in the resolution of inflammation or pain. In fact, promoting an M2 phenotype has been shown to decrease pain-related behaviors in murine models of pain (Hasegawa-Moriyama et al., 2012; Kiguchi et al., 2015). This study focusses on exploring the functions of CD163 in human macrophages phenotype beyond its use as a phenotype marker. To our knowledge, the functional capabilities of CD163 in the acquisition of an M2 phenotype in macrophages have not been explored.

CD163 was first described as a cysteine-rich scavenger receptor exclusively expressed in M2 macrophages (Zwadlo et al., 1987). In fact, CD163 binds to the hemoglobin–haptoglobin complex (Hb–Hp) (Kristiansen et al., 2001), degradation of which causes the release of compounds with anti-oxidative and anti-inflammatory effects (i.e. IL-10, free iron, biliverdine, and carbon monoxide) (Stocker et al., 1987; Otterbein et al., 2000; Otterbein et al., 2003). Moreover, the expression of CD163 in circulating human monocytes is associated with the resolution phase of inflammation following major surgeries (Philippidis et al., 2004). Similarly, CD163-positive macrophages are a hallmark of wound healing in humans (Evans et al., 2013). Although this evidence shows that CD163 is a definitive marker of an M2 macrophage phenotype associated with the resolution of inflammation, the cause–effect relationship between CD163 and the resolution of inflammation has not yet been explored. Thus, we hypothesize that the specific induction of CD163 gene expression in human macrophages promotes a transition from an M1 to M2 macrophage phenotype. To test our hypothesis, we used THP-1 and primary human macrophages stimulated with LPS. First, we investigated whether the specific blockade of CD163, using a specific antibody, delayed the reduction of proinflammatory cytokines or the increase of anti-inflammatory cytokines. Secondly, we overexpressed the CD163 gene using Man-PEI nanoparticles and measured cytokine secretion as functional outcomes to determine M1 and M2 phenotypes. Thirdly, we confirmed the CD163 dependence of the observed effects by blocking the CD163 receptor using a specific antibody.

One of the translational and clinically relevant features of our study is the utilization of a novel cell-directed gene induction technique using nanotechnology. Specifically, we used polyethyleneimine (PEI) grafted with a mannose receptor ligand (Man-PEI). This feature makes Man-PEI a powerful tool to develop a specific cell (macrophage)-directed gene therapy. Man-PEI preferentially targets monocytes, since these are the only cells that express mannose receptors. In addition to this clear advantage of our approach, Man-PEI has other superior features when compared to other techniques, such as naked DNA, electroporation, lipoplexes, diethylaminoethyl-dextran, or viruses. These alternative techniques produce low transfection efficiencies, short-lasting transfections, cytotoxicity, and immunogenic responses. We have previously demonstrated that Man-PEI efficiently induces different genes in human macrophages under inflammatory conditions without inducing cytotoxicity or significant immunogenicity (Bernal et al., 2016). Furthermore, this nanoparticle possesses a strong clinical background, since it has been successfully used to induce HIV gene antigens, which activate monocytes from HIV-positive patients, improving clinical outcomes (Lisziewicz et al., 2012; Rodriguez et al., 2013).

2. Material and methods

2.1. THP-1 cell culture conditions, cell differentiation, and lipopolysaccharide (LPS) stimulation

Immortalized human acute monocytic leukemia cell line (THP-1) was cultured in Roswell Park Memorial Institute 1640 media (RPMI 1640. Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were plated in 75 cm^2 flasks at 8×10^4 cells/cm^2 and incubated at 37 °C in a 5% CO_2 atmosphere. The viability of THP-1 cells was monitored in each experiment using the Trypan blue assay (dye exclusion method). Briefly, the cell suspension was diluted at a 1:1 ratio and incubated at room temperature with a Trypan blue dye (Life Technologies, Grand Island, NY, USA) for 1 min. Dead cells were identified as Trypan blue-positive cells. Cells were counted using a hemocytometer (VWR International, Wayne, PA, USA).

THP-1 monocytes were differentiated into macrophages by incubating them with 60 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma, St Louis, MO, USA) for 36 h (37 °C, 5% CO_2) (Landry et al., 2012). Cells were seeded on 24-well plates (250,000 cells/mL per well) for 1 h and then stimulated with 5 μg/mL of LPS (*Escherichia coli* O111:B4, Sigma).

Based on our previous work, two stimulation paradigms were utilized: single stimulation (acute inflammation paradigm) and double stimulation (sub-acute stimulation paradigm) (Bernal et al., 2016). For the single stimulation experiments, THP-1 macrophages were incubated from 24 to 96 h after a single LPS stimulation (5 μg/mL). For double stimulation experiments, THP-1 macrophages were incubated for 48 h after a single LPS stimulation (5 μg/mL). Then, supernatants were removed and fresh media was added before the second challenge with LPS (5 μg/mL). The time point for a second stimulation (48 h) was chosen based on the time point in which gene overexpression is