Immunobiology 222 (2017) 847–856

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Transcriptome analysis of IL-10-stimulated (M2c) macrophages by next-generation sequencing

Emily B. Lurier[a], Donald Dalton[a], Will Dampier[b], Pichai Raman[a,c,d], Sina Nassiri[a], Nicole M. Ferraro[a], Ramakrishan Rajagopalan[c], Mahdi Sarmady[e], Kara L. Spiller[a,*]

[a] School of Biomedical Engineering, Scienc PA, 19104, USA
[b] Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N Broad St. Philadelphia, PA, 19107, USA
[c] Deparment of Biomedical and Health Informatics, Children's Hospital of Philadelphia, Philadelphia, PA, 19104, USA
[d] Center for Data-Driven Discovery in Biomedicine, Children's Hospital of Philadelphia, Philadelphia, PA, USA
[e] Division of Genomic Diagnostics, Children's Hospital of Philadelphia, Philadelphia, PA, 19104, USA

ARTICLE INFO

Article history:
Received 15 September 2016
Accepted 15 February 2017
Available online 20 February 2017

Keywords:
Macrophage
Phenotype
Gene expression
Next generation sequencing
IL-10
Wound healing

ABSTRACT

Alternatively activated "M2" macrophages are believed to function during late stages of wound healing, behaving in an anti-inflammatory manner to mediate the resolution of the pro-inflammatory response caused by "M1" macrophages. However, the differences between two main subtypes of M2 macrophages, namely interleukin-4 (IL-4)-stimulated "M2a" macrophages and IL-10-stimulated "M2c" macrophages, are not well understood. M2a macrophages are characterized by their ability to inhibit inflammation and contribute to the stabilization of angiogenesis. However, the role and temporal profile of M2c macrophages in wound healing are not known. Therefore, we performed next generation sequencing (RNA-seq) to identify biological functions and gene expression signatures of macrophages polarized *in vitro* with IL-10 to the M2c phenotype in comparison to M1 and M2a macrophages and an unactivated control (M0). We then explored the expression of these gene signatures in a publicly available data set of human wound healing. RNA-seq analysis showed that hundreds of genes were upregulated in M2c macrophages compared to the M0 control, with thousands of alternative splicing events. Following validation by Nanostring, 39 genes were found to be upregulated by M2c macrophages compared to the M0 control, and 17 genes were significantly upregulated relative to the M0, M1, and M2a phenotypes (using an adjusted p-value cutoff of 0.05 and fold change cutoff of 1.5). Many of the identified M2c-specific genes are associated with angiogenesis, matrix remodeling, and phagocytosis, including *CD163, MMP8, TIMP1, VCAN, SERPINA1, MARCO, PLOD2, PCOCLE2 and F5*. Analysis of the macrophage-conditioned media for secretion of matrix-remodeling proteins showed that M2c macrophages secreted higher levels of MMP7, MMP8, and TIMP1 compared to the other phenotypes. Interestingly, temporal gene expression analysis of a publicly available microarray data set of human wound healing showed that M2c-related genes were upregulated at early times after injury, similar to M1-related genes, while M2a-related genes appeared at later stages or were downregulated after injury. While further studies are required to confirm the timing and role of M2c macrophages *in vivo*, these results suggest that M2c macrophages may function at early stages of wound healing. Identification of markers of the M2c phenotype will allow more detailed investigations into the role of M2c macrophages *in vivo*.

1. Introduction

Macrophages exist on a spectrum of phenotypes that range from pro-inflammatory to anti-inflammatory depending on microenvironmental conditions. In response to injury, macrophages rapidly switch their behavior from pro-inflammatory (often called M1) in the early stages of healing to a state that promotes resolution of inflammation and healing (often called M2) at later stages. This M1-to-M2 transition has been described during wound repair in a diverse array of tissues, including heart, lung, muscle, skin, and bone (Schlundt et al., 2015; Troidl et al., 2009; Johnston et al.,

Abbreviations: RNA-seq, RNA-sequencing; ERCC, external RNA control consortium; FDR, false discovery rate; GO, gene ontology; QC, quality control; IFNγ, interferon-γ; LPS, lipopolysaccharide; IL-4, interleukin-4; ELISA, enzyme-linked immunosorbent assay; MCSF, macrophage colony stimulating factor; qRTPCR, quantitative reverse transcription polymerase chain reaction; MMPs, matrix metalloproteases; TIMP1, tissue inhibitor of metalloprotease 1; LFC, log fold change; TMM, Trimmed Mean of M-values; cpm, counts per million; SE, skipped exons; A5SS, alternative 5′ splice sites; A3SS, alternative 3′ splice sites; MEX, mutually exclusive exons; RI, retained intron; EBI, European Bioinformatics Institute.

* Corresponding author.
E-mail address: spiller@drexel.edu (K.L. Spiller).

http://dx.doi.org/10.1016/j.imbio.2017.02.006
0171-2985/

2012; Arnold et al., 2007; Mirza and Koh, 2011). Impairment of the M1-to-M2 transition is associated with defective wound healing (Mirza and Koh, 2011; Nassiri, 2015), leading to recognition of M2 macrophages as "pro-healing" or "pro-regenerative" (Mirza and Koh, 2011; Zhang et al., 2009), although M1 macrophages are also critical for healing (Lucas et al., 2010; Willenborg et al., 2012). The mechanisms behind how macrophages of different phenotypes orchestrate tissue repair are not well understood.

Two main subtypes of M2 macrophages have been implicated in wound healing, namely those stimulated (at least *in vitro*) by interleukin-4 (IL-4) (called M2a) and those stimulated by IL-10 (called M2c). Previously, we and others have shown that IL-10-stimulated M2c macrophages promoted more angiogenesis *in vitro* and *in vivo* compared to the M1 and M2a phenotypes (Spiller et al., 2014; Jetten et al., 2014). Zizzo et al. showed that IL-10-stimulated M2c macrophages have a higher phagocytic capacity for apoptotic cells *in vitro*, a critical process in wound healing (Zizzo et al., 2012). Lolmede et al. showed that M2c macrophages recruit blood vessel-associated stem cells *in vitro* to the same extent but through different mechanisms compared to M1 and M2a macrophages (Lolmede et al., 2009). Finally, M2c macrophages express high levels of the surface marker CD163 (Spiller et al., 2014; Zizzo et al., 2012), and CD163+ macrophages have been shown to infiltrate wound sites during early phases (1–2 days) of wound healing in humans (Evans et al., 2013; Philippidis et al., 2004). These studies suggest that M2c macrophages play important roles in wound healing, but the mechanisms behind their actions are poorly understood.

To this end, several groups have performed microarray analysis of human M2c macrophages *in vitro* to identify a gene expression signature that can be used to perform mechanistic studies and to explore the timing of M2c activation *in vivo*, with inconclusive results. While Williams et al. (2002) found that just 19 genes were upregulated in M2c macrophages after 3 h of IL-10 stimulation, Derlindati et al. (2015) reported that no genes were upregulated after 6, 12, and 24 h of polarization. Therefore, the purpose of this study was to identify a gene expression signature for M2c macrophages using whole transcriptome shotgun sequencing, or RNA-seq, a more sensitive method of gene expression analysis. Identified genes were validated using Nanostring, a highly sensitive gene expression array for custom-designed gene sets. We also validated genes related to extracellular matrix remodeling on the protein level using enzyme-linked immunosorbent assay (ELISA). Finally, changes in expression levels of the validated M2c markers over time during human wound healing were identified using a publicly available data set as a preliminary analysis of the timing of M2c activation in wound healing. Identification of an M2c macrophage gene expression signature will allow further investigation into the role of these macrophages *in vitro* and *in vivo*.

2. Materials and methods

2.1. Culture and polarization of primary human macrophages

For RNA-seq analysis, human blood-derived monocytes were isolated from peripheral blood mononuclear cells obtained from buffy coats from n = 4 donors (New York Blood center) via sequential gradient centrifugation (Spiller et al., 2014). For follow up analysis using Nanostring, quantitative reverse transcription polymerase chain reaction (qRTPCR), and protein secretion, CD14+ monocytes were purchased from the human immunology core at the University of Pennsylvania (n = 6 donors) (Philadelphia, PA) following their negative selection from peripheral blood mononuclear cells (Witherel et al., 2016; Graney et al., 2016). Monocytes were differentiated into macrophages and polarized to the M1, M2a, and M2c phenotypes following previously established methods (Spiller et al., 2014). Briefly, monocytes were cultured in ultra-low tissue culture plates and differentiated to unactivated macrophages (M0) using 20 ng/mL macrophage colony stimulating factor (MCSF, Peprotech) in complete medium (RPMI 1640 +10% human serum +1% penicillin streptomycin) for five days at 1×10^6 cells/mL with a media change at day 3. On day 5, M0 macrophages were stimulated for 48 h with 20 ng/mL of MCSF plus 100 ng/mL lipopolysaccharide (LPS, Sigma Aldrich) and 100 ng/mL interferon-γ (IFN-γ, Peprotech) for M1; 40 ng/mL IL-4 (Peprotech) and 20 ng/mL IL-13 (Peprotech) for M2a; and 40 ng/mL of IL-10 (Peprotech) for M2c. Macrophages were collected by gently scraping the cells and centrifuging at 400xg for 7 min. For gene expression analysis, media was removed and the cell pellets were mixed with lysis buffer (Thermofisher) and then stored at −80 °C for future analysis. To prepare conditioned media for protein secretion analysis, media was refreshed on day 7 (*i.e.* after 2 days of polarization) with basal media and the cells were placed back in culture for 24 h, after which the macrophage-conditioned media was collected and frozen at −80 °C until analysis.

2.2. RNA extraction

Total RNA was extracted using the RNeasy kit (QIAGEN) or RNAqueous Micro kit (Ambion) following the manufacturers' instructions. The concentration of extracted RNA was measured using a Nanodrop ND1000 and was considered pure if the 260/280 and 260/230 ratios were ~2. Samples were then stored at −80 °C until gene expression analysis was conducted by RNA-seq, Nanostring, or qRTPCR.

2.3. RNA-seq library construction and sequencing

RNA-seq libraries were prepared using the Illumina TruSeq Stranded Total RNA Kit. 200 ng of total RNA from each sample (n = 4 human donors) were subjected to ribosomal RNA removal using RiboZero components. The rRNA-depleted fraction was then fragmented and annealed to random hexamers in preparation for first-strand cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Next, second strand cDNA synthesis was conducted and the blunt-end double-stranded material was purified with Ampure XP beads (Beckman Coulter, Indianapolis, IN). Subsequent steps included addition of an A-tail overhang at the 3′ end, ligation of the indexing-specific adaptor, and amplification of the library. Following each step, library material was purified using Agencourt Ampure XP beads and the number of amplification cycles was minimized (typically 15 cycles) to reduce the number of duplicate reads. Size and yield of the bar-coded libraries were assessed using the LabChip GX, with an expected distribution of 200–400 bp. The concentration of each library was then measured using qRTPCR via the Kapa Biosystems kit (Wilmington, MA). Pools of indexed samples were created in preparation for cluster generation and 100 bp × 100 bp paired end sequencing on the Illumina HiSeq 2500s. Four libraries were pooled per lane so that all phenotypes from each donor were run in the same lane to account for potential donor effects, with a target of 40 million reads per sample. Finally, raw sequences were received in FastQ format and initial quality control (QC) was performed using the FastQC toolkit (Andrews, 2016).

2.4. Overview of RNAseq analysis

RNA-seq data were analyzed using three different methods that are commonly used in RNA-seq analysis to obtain a consensus list for downstream validation by Nanostring and qRTPCR. In the first method (Method 1), reads were aligned to a reference genome using the STAR aligner followed by linear mixed effects model-