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

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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

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Conflict of interest

The authors declare that there is no conflict of interest.

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*.

Keywords

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

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., 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 (qRT-PCR), 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 (Witherell 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 $400 \times g$ 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 modeling using the voom-limma algorithm followed by adjustment for batch effects, and with relatively conservative thresholds of fold change of 2 and false discovery rate (FDR) of 0.01 to identify differentially expressed genes (Dobin et al., 2013; Law et al., 2014; Ritchie et al., 2015). In the second method (Method 2), potential donor or batch effects were directly incorporated into the voom-limma model followed by differential expression analysis using more lenient cutoff values (fold change > 1.5 and FDR < 0.1). Finally, the third method (Method 3) used pseudo-alignment instead of direct alignment through the BWA-mem algorithm to map genes on the transcript level to eliminate potential mapping errors (Bray et al., 2016), followed by differential expression analysis with relatively conservative cutoff values (fold change > 2 and FDR < 0.01). Any genes that were identified as upregulated in M2c macrophages relative to M0 macrophages in at least 2 of these 3 methods were selected for

downstream validation by Nanostring. In addition, all genes identified as upregulated using Method 3 were selected for validation because this method has been shown to outperform other methods in terms of accuracy (Bray et al., 2016). RNAseq data have been deposited with links to BioProject accession number PRJNA339309 in the NCBI BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject/>).

2.5. Detailed methods of RNA-seq analysis

In Method 1, raw sequences were mapped to human genome GRCh37.69 via the STAR aligner with a two-pass alignment scheme (Dobin et al., 2013). Initial QC analysis revealed an average of 49.9 million reads sequenced across samples with 88% of the reads uniquely mapped to the reference genome. The htseq-count function from the htseq library was then used to summarize read counts per gene transcript (Anders et al., 2014). Genes with read counts less than 20 in at least one library across all samples were filtered out, yielding 18117 genes with unique Ensemble gene IDs for downstream analysis. Following scale normalization using the Trimmed Mean of M-values (TMM) method as described by Robinson and Oshlack (2010), the voom transformation without quality weights using limma was performed on filtered genes to account for mean-variance trends in the data (Law et al., 2014; Ritchie et al., 2015). Donor batch effects were indirectly corrected for after voom transformation using the ComBat function in the SVA method (Leek et al., 2012). Differentially expressed genes in the M1, M2a and M2c phenotype relative to the M0 control, to allow for pooled donor level variation, were considered significant using a fold-change cutoff of 2 fold and an FDR of 0.01.

In Method 2, raw sequences were mapped as in Method 1. Only genes with counts per million (cpm) of at least 0.5 in at least 4 samples were included in downstream analysis, yielding 12481 unique gene Ensemble gene IDs (Law et al., 2014). The TMM method and voom transformation were used on filtered genes as in Method 1 but sample quality weights were directly included in the analysis (Law et al., 2014; Ritchie et al., 2015; Robinson and Oshlack, 2010). Potential batch or donor variability in gene expression were directly incorporated in the linear model describing the experimental design, and differential expression analysis was performed using limma's moderated t-statistic (Ritchie et al., 2015). This second list of significantly differentially expressed genes in the M1, M2a and M2c phenotypes relative to the M0 control were determined using a fold change cutoff of 1.5 and a FDR of 0.1.

Finally, Method 3 employed pseudo-alignment of paired end Illumina reads to the human genome version GRCh37.69 using the BWA-mem algorithm (Li and Durbin, 2009). Discordant reads and those with a mapping quality less than 60 were eliminated. Kallisto was used to quantify the number of observed transcripts per million utilizing the GRCh37 version Ensembl gene list (Bray et al., 2016). Transcript counts were again analyzed using the voom-limma algorithm (Law et al., 2014; Ritchie et al., 2015). Similar to Methods 1 and 2, gene expression in M1, M2a, and M2c macrophages was compared to M0 controls to allow for a pooled donor level variation. Differentially expressed genes were identified using a fold-change cutoff of 2 with a Benjamini-Hochberg adjusted p-value of 0.01. This third and final list of differentially expressed genes for each macrophage phenotype were then

tested for over- and under-enrichment in Gene Ontology (FatiGO) using the DAVID microarray tool (Huang da et al., 2009).

2.6. Alternative splicing analysis

Alternative splicing events were detected using the replicate multivariate analysis of transcript splicing (rMATS) program (Shen et al., 2014). Common alternative splicing events were identified including skipped exons (SE), alternative 5' splice sites (A5SS), alternative 3' splice sites (A3SS), mutually exclusive exons (MEX), and retained intron (RI). The occurrence of alternative splicing events was determined by comparing the M1, M2a and M2c phenotypes to the M0 control using paired end analysis by donor. Events were considered to be significant with an FDR < 0.01 and an inclusion level difference of 0.05, as suggested by Shen et al. (2014).

2.7. Nanostring gene expression array

Any genes that were identified as significantly upregulated in M2c macrophages relative to the M0 control in at least 2 of the 3 methods of analysis were chosen for downstream validation by Nanostring, a high throughput method for measuring gene expression of multiple target genes in a single sample. In addition, any genes identified using the more robust pseudo-alignment method were also selected for validation, for a combined list of 110 genes. Nanostring gene expression analysis was used to measure expression of these 110 genes as well as 6 housekeeping genes and 8 external RNA control consortium (ERCC) negative control transcripts following the manufacturer's instructions using 100 ng of RNA per sample (Nanostring Technologies, Seattle, WA). Gene expression of macrophages from $n = 6$ human donors and $n = 2$ experimental replicates per donor were analyzed.

Raw count data derived from the Nanostring data were pre-processed by averaging technical replicates according to phenotype, followed by normalization by library size using the voom transformation with sample-specific quality weighting (Law et al., 2014). QC was conducted by comparing gene count coefficient of variation distributions between samples pre- and post-normalization. Genes with low to undetectable expression were identified by comparison to the 8 ERCC negative control transcripts reported in each sample. Normalized counts for control probes were first averaged within samples, and a 1-tailed 2-sample Welch's t -test was performed for each gene against control values with an adjusted p -value < 0.01 taken as evidence of true expression. Genes with non-differential expression relative to the controls that were removed prior to downstream analysis included *AOX1*, *DNAH17*, *FCRLB*, *HS3STSA1*, *KALRN*, *KCNJ15*, *LRP6*, *NDP*, *PRRX1*, *SLC12A8*, *TMEM26*, *VWAI*.

Differential expression analysis across macrophage phenotype was performed using the limma algorithm with donor effects directly incorporated in the linear model (Law et al., 2014; Ritchie et al., 2015). Gene expression comparisons of M2c macrophages relative to M0, M1, and M2a phenotypes were performed with a global Benjamini-Hochberg adjusted multiple comparisons t -test and represented using complete agglomerative hierarchical clustering analysis using Euclidean distances with the complete linkage clustering method.

Genes with adjusted p-values ≤ 0.05 and log fold-changes ≥ 1.5 across all comparisons were considered significant.

Finally, validated lists of significantly upregulated M2c genes compared to M0 macrophages and the list of differentially expressed M2c genes compared to all other phenotypes were tested for over- and under-enrichment in Gene Ontology (FatiGO) using the DAVID microarray tool (Huang et al., 2009).

2.8. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Further validation of select genes resulting from the RNA-seq and Nanostring analyses was conducted using qRT-PCR. DNase treatment was conducted on thawed RNA using the DNase I removal kit following the manufacturer's instructions (ThermoFisher). cDNA synthesis was performed using the High Capacity cDNA Reverse Transcriptase kit according to the manufacturer's instructions (ThermoFisher). The SYBR[®] Green PCR Master Mix was used to perform qPCR to quantify the expression of the following genes using 20 ng cDNA per reaction: *CD163*, *MMP7*, *MMP8*, *VCAN*, and *MARCO*, as well as the housekeeping gene *GAPDH*. All primers were custom-designed and synthesized by ThermoFisher (Supplementary Table 1). The $\Delta\Delta C_t$ method was used to calculate relative gene expression of the target probes normalized to *GAPDH* expression (Schmittgen and Livak, 2008).

2.9. Protein secretion analysis

Enzyme-linked immunosorbent assays (ELISAs) were conducted according to the manufacturers' instructions to quantify secretion of select matrix metalloproteases (MMPs): MMP1 (Abcam), MMP2 (R&D systems), MMP3 (Abcam), MMP7 (R&D Systems), MMP8 (R&D Systems), MMP12 (Abnova), as well as tissue inhibitor of metalloprotease 1 (TIMP1) (Peprotech) in macrophage-conditioned media. A live-cell assay conducted on macrophages after 7 days of culture (2 days of polarization) was used to quantify secretion of MMP14, according to the manufacturer's instructions (Abcam).

2.10. Characterization of gene expression signatures during human wound healing using publicly available data

As a preliminary analysis of macrophage activation during human wound healing, publicly available data from microarray studies characterizing heterogeneous wound tissue were used because no studies have been conducted where macrophages were first isolated from human wounds and then profiled at the whole genome level. Three publicly available data sets of gene expression at various time points pre- and post-injury were identified using the Gene Expression Omnibus (GEO) and European Bioinformatics Institute (EBI) repositories (Bronneke et al., 2015; Greco et al., 2010; Gabriel et al., 2014). Only one of the three datasets, that produced by Gabriel et al. (2014), was determined to contain data of sufficient quality for further analysis, as the others either contained significant batch effects (Bronneke et al., 2015) or low numbers of replicates and time points (Greco et al., 2010). The selected data set was a burn wound model of healthy human skin undergoing aesthetic scarification, taking tissue biopsies immediately before injury, and then 1 h, 6 days, 25 days, 60 days and 80 days post-injury to analyze changes in gene expression over the course of wound healing (Gabriel et al., 2014). Raw intensity data from Illumina HumanWG-6 v3.0 Expression

BeadChips were downloaded from the European Bioinformatics Institute (EBI), and pre-processing was performed with the lumi package. Specifically, expression values were obtained by background correction, a variance-stabilizing and log base 2 transformation, and quantile normalization. QC checks were performed with replicate correlation and pairwise MA plots. Sample-specific quality weights were also quantified using limma's arrayWeights function and all samples were included in downstream analysis. Probe identifiers were annotated with the lumiHumanIDMapping file, and duplicate HUGO gene symbols were combined via a maximum mean method. In total, 48802 probes were mapped to 19344 unique gene symbols.

The top 100 enriched genes for the M1 and M2a phenotypes in terms of fold change over M0, determined from Method 3 of RNASeq data analysis discussed above, and all 17 validated M2c genes were used to create a 217 gene expression matrix from the wound healing dataset. Soft clustering was then performed with the mFuzz algorithm, which creates global clustering structures, to determine groups of genes that were expressed similarly over time (Futschik and Carlisle, 2005; Kumar and Futschik, 2007). The optimal number of clusters was determined to be four as this resulted in the greatest number of highly correlated genes or "core genes" in each cluster. Since genes are able to contribute to more than one cluster, a correlation coefficient threshold of 0.6 was used to identify the core genes in each cluster and the "fuzzifier" value, which prevents clustering of random data, was estimated via the Schwaemmle and Jensen relation (Schwämmle and Jensen, 2010). The relative proportions of macrophage phenotype-associated genes in each cluster were then calculated by dividing the number of genes in the cluster by the total number of genes that mapped to any cluster for each phenotype.

3. Results

3.1. RNA-Seq analysis

We aimed to identify genetic markers unique to the M2c macrophage phenotype compared to the M1 and M2a phenotypes to move towards elucidating the role of M2c macrophages in wound healing. RNA-seq analysis by three different methods showed that M1 and M2a macrophages upregulated hundreds to thousands of genes relative to the M0 control (results for pseudo-alignment analysis shown in Fig. 1a). In contrast, M2c macrophages upregulated just 90–130 genes compared to M0, depending on the method of analysis (Fig. 1b). Interestingly, however, gene ontology (GO) analysis showed that differentially upregulated M2c genes were associated with multiple pathways involved in regulation of platelet activity, the extracellular matrix, and the inflammatory response (Fig. 1c). Interestingly, thousands of alternative splicing events were detected in all three macrophage phenotypes compared to the M0 control (Fig. 2, Supplementary Fig. S1), suggesting that multiple proteins may be encoded by a single gene.

3.2. Validation of M2c markers with Nanostring

Hierarchical clustering of Nanostring gene expression data for the 110 putative M2c genes showed that the M2c macrophages clustered most closely to the M0 control, with M1 macrophages clustering furthest away from the other phenotypes (Fig. 3) (Warnes et al.,

2009). Of the 110 genes investigated, 39 genes were significantly upregulated in the M2c phenotype compared to the M0 control using a log fold change cutoff of 1.5 and an adjusted Benjamin-Hochberg p-value cutoff of 0.05 (Table 1). The most upregulated gene compared to M0 was *CD163*, a scavenger receptor involved in phagocytosis that has been previously associated with M2c macrophages (Spiller et al., 2014; Zizzo et al., 2012). Newly identified genes that are of interest to wound healing include genes involved in regulation of the extracellular matrix, including *VCAN*, *MMP7*, *MMP8*, *TIMP1*, *PLOD2*, and *COL22A1*. Indeed, GO analysis using the 39 genes upregulated by M2c macrophages compared to M0 identified multiple pathways related to regulation of the extracellular matrix (Supplementary Fig. S2).

Of the 39 genes upregulated by M2c macrophages compared to M0, only 17 were expressed at significantly higher levels when also compared to M1 and M2a macrophages (Supplementary Table 2). Interestingly, 9 of the 17 identified M2c genes were identified by all three methods of RNA-seq analysis, while only 1 gene was identified by Method 3 of RNA-seq analysis that was not also identified by Methods 1 and 2 (Supplementary Fig. S3). The 17 genes that emerged as M2c markers can be grouped based on function. Genes involved in regulation of extracellular matrix deposition and remodeling include *MMP8*, *TIMP1*, *PLOD2*, *PCOLCE2* and *VCAN* (Zimmermann et al., 1994; Gutiérrez-Fernández et al., 2007; Liu et al., 2014; Zheng et al., 2004; Liu et al., 2000). Genes involved in regulating clot formation and angiogenesis include *VCAN*, *SER-PIN1*, *SRPX2*, *F5*, and *MMP8* (Zheng et al., 2004; Liu et al., 2000; Fang et al., 2013; Villoutreix and Dahlbäck, 1998; Miljkovic-Licina et al., 2009). Genes involved in phagocytosis of damaged red blood cells and foreign organisms as well as other antibacterial behavior include *CD163*, *MARCO*, *FCRLA*, *FPR1* and *CD300E* (Mooney et al., 2014; Hussell and Bell, 2014; Shabo et al., 2009; Gemperle et al., 2012). Genes otherwise associated with wound healing and inflammatory resolution include *MMP8*, *CD163*, and *FPR1* (Philippidis et al., 2004; Gutiérrez-Fernández et al., 2007; Liu et al., 2014). The other identified M2c markers include *MCTP2*, which is involved in intracellular trafficking (Lalani et al., 2013); *GXYLT2*, which is a xylotransferase (Sethi et al., 2010); *LIN7A*, which is involved in epithelial and neuronal junction organization (Perego et al., 1999); and *SH3PXD2B*, which is involved in podosome formation (Lányi et al., 2011). Gene expression trends were further confirmed using qRT-PCR for *MMP8*, *MARCO*, *CD163*, and *VCAN* (Supplementary Fig. S4).

3.3. Protein secretion analysis

Of particular interest to us was the identification of genes involved in matrix remodeling (e.g. *MMP7*, *MMP8*, and *TIMP1*), because a fibrolytic role for macrophages in wound healing has been proposed but not thoroughly investigated (Anders and Ryu, 2011). Therefore, we next measured secretion of these proteins, as well as other important MMPs, using ELISA. M2c macrophages secreted higher levels of *MMP7*, *MMP8*, and *TIMP1* compared to M0, M1, and M2a macrophages, although only *MMP7* was statistically significant for the comparison to M0 (Fig. 4). Protein secretion levels of *MMP1*, *MMP2*, *MMP3*, *MMP12*, and *MMP14* were not significantly different between the macrophage phenotypes studied here (Fig. 4).

3.4. Longitudinal analysis of gene expression trends in human wound healing

Finally, in a preliminary analysis to identify the timing of M2c macrophage-related gene expression in human wound healing, we analyzed a publicly available data set of wound tissue following burn injury (Gabriel et al., 2014) for expression over time of the top ~100 M1 and M2a genes identified using RNA-seq as well as the 17 validated M2c genes. Of the 217 genes analyzed, 126 genes were assigned to at least one of four clusters, which emerged based on similarities in gene expression trends over time. Cluster 1 was composed of genes that increased at day 6 before rapidly returning to baseline (Fig. 5a). This cluster contained over 60% of the mapped M1- and M2c-related genes, and only 20% of M2a-related genes (Fig. 5b, Supplementary Table 3). Cluster 2 contained genes that peaked at day 6 and gradually declined thereafter (Fig. 5a). The composition of genes in this cluster was approximately equal for M1-, M2a-, and M2c-related genes (Fig. 5b, Supplementary Table 3). Cluster 3 contained genes that were primarily downregulated by day 6 following injury and then returned to baseline thereafter. The composition of this cluster was primarily M2a-related genes (Fig. 5b, Supplementary Table 3). Finally, Cluster 4 contained genes that were upregulated at day 25 after injury. M2a-related genes were the primary contributor to this cluster (Fig. 5b, Supplementary Table 3).

4. Discussion

Using next generation sequencing and subsequent validation by Nanostring, we identified 39 genes that were upregulated in the M2c phenotype relative to unactivated (M0) macrophages, and only 17 genes that were differentially upregulated in the M2c phenotype compared to M0, M1 and M2a macrophages. The identified M2c markers are involved in biological pathways necessary during the initiation of healing, and were found to be upregulated at early time points following injury in a human wound healing data set. While preliminary, these results suggest that M2c macrophages may play a role in the early stages of wound healing, in contrast to the characterization of “M2” macrophages as cells that act at later stages. With the identification of genetic markers specific for the M2c phenotype, future studies should focus on the role of these cells *in vitro* and *in vivo*.

Alternative splicing events have been previously shown to have effects on the process of macrophage polarization in M1 and M2a macrophages (Lin et al., 2016). However, the role of alternative splicing events in M2c macrophage polarization is largely unknown. Here we show that a high incidence rate of these events occur in M2c macrophages. Taken with the small number of differentially upregulated M2c genes determined herein, alternative splicing events may contribute largely to macrophage polarization towards the M2c phenotype.

Several studies have previously shown that M2c macrophages play a role in angiogenesis (Spiller et al., 2014; Jetten et al., 2014). For example, Jetten et al. showed that M2c macrophages caused increased endothelial cell migration and tube formation *in vitro* and *in vivo* compared to M0 and M1 macrophages, although there were no significant differences compared to M2a macrophages (Jetten et al., 2014). Spiller et al. cultured endothelial cells seeded on Matrigel in macrophage-derived conditioned media and found that M2c macrophages caused significantly higher sprouting compared to M1 and M2a macrophages, although these results were not different from M0 macrophages (Spiller et al., 2014).

Finally, macrophages positive for the M2c marker *CD163* have been associated with vascularization of wounds and biomaterials (Mayer et al., 2011; Gelati et al., 2008). In this study, M2c macrophages significantly upregulated important genes involved in angiogenesis, like *SERPINA1*, *SRPX2*, *MMP8*, and *VCAN*. Secretion of MMP8 was also confirmed on the protein level. These results point to potential mechanisms by which M2c macrophages promote angiogenesis, and future studies should be directed at testing these hypotheses.

In addition to angiogenesis, matrix remodeling is integral for the restoration of healthy tissue at a site of injury. M2c macrophages have previously been implicated in inhibiting fibrosis to promote healthy tissue remodeling. For example, Lu et al. showed that M2c macrophages reduced renal fibrosis more effectively than M2a macrophages by augmenting the behavior of regulatory T cells in a murine nephrotic injury model *in vivo*, although both reduced fibrosis (Lu et al., 2013). Additionally, IL-10 is known to mediate scarless wound healing observed in fetal tissue regeneration (Liechty et al., 2000). In this study, M2c macrophages were shown to secrete significantly higher quantities of the matrix remodeling enzymes MMP7, MMP8 and TIMP1, while previous studies have shown high levels of MMP9 secretion (Spiller et al., 2014; Lolmede et al., 2009), suggesting potential mechanisms by which M2c macrophages regulate fibrosis.

During the onset of injury, neutrophils are the first cells to infiltrate the wound site (Singer and Clark, 1999). Then, as wound healing progresses, neutrophils begin to apoptose as macrophages migrate into the site of injury (Singer and Clark, 1999). M2c macrophages have been previously shown to have a higher phagocytic capacity for apoptotic neutrophils, as well as nanoparticles, compared to the M0, M1, and M2a macrophage phenotypes (Zizzo et al., 2012; Capsoni et al., 1995). Here, we show that M2c macrophages upregulate phagocytic genes like *CD163*, *MARCO*, *FCRLA*, *FPR1* and *CD300E* compared to M0, M1, and M2a macrophages, highlighting potential roles for these genes in the highly phagocytic behavior of M2c macrophages.

This study had several limitations. First, a major limitation of any *in vitro* characterization of macrophages is that the relationship between *in vitro*-activated macrophages and those present *in vivo* is not clear. However, reference to gene expression signatures from *in vitro*-activated macrophages such as those generated in this study has been proposed as a more precise method for describing macrophages isolated from the *in vivo* environment (Xue et al., 2014), with recognition that they are not the same. Secondly, a limited number of human donors were used for profiling gene expression and protein secretion, and these biological characteristics were only analyzed at a single time point following polarization. In addition, functional assays were not conducted to validate the functional activity of the identified markers. However, this study was intended to serve as an initial analysis of M2c activation in macrophages, so that more in-depth follow-up studies could be conducted. Finally, a major limitation of our wound healing analysis is that we only used a single publicly available data set, and the data were obtained from heterogeneous tissues. However, very few studies have been conducted to characterize expression of the whole genome in healing tissue over time in humans, and none have first isolated macrophages before analysis. Despite these limitations, this study provides new insight into the role of IL-10-stimulated M2c macrophages during the wound healing response. Novel genetic markers differentially

upregulated in the M2c phenotype may provide new tools to study M2c macrophages *in vitro* and *in vivo*.

5. Conclusions

Next generation sequencing and subsequent validation of gene expression of macrophages polarized *in vitro* with IL-10 to the M2c phenotype identified 39 upregulated genes compared to control macrophages and 17 that were not shared by other phenotypes that are commonly studied and believed to be involved in wound healing (M1 and M2a). Many of the M2c-specific genes were involved in matrix remodeling, angiogenesis, and phagocytosis, and were upregulated at early times after injury in a human burn injury data set. Although preliminary, these results suggest that M2c macrophages may act at early stages of wound healing, in contrast to M2a macrophages, which act at later stages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imbio.2017.02.006>.

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

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Fig. 1.

RNA-seq analysis. (A) The number of differentially expressed genes between M1, M2a, and M2c macrophages compared to the M0 control, determined via the pseudo-alignment method of RNA-seq analysis (Method 3). The area of the circles is proportional to the total number of genes per cell type. (B) The number of differentially expressed genes between the M2c and M0 phenotypes that were identified in each method of RNA-seq analysis. (C) The top 25 most enriched gene ontology (GO) terms for the M2c phenotype compared to the M1 and M2a phenotypes. Macrophages were isolated from $n = 4$ human donors.

Fig. 2.

Alternative splicing analysis. The occurrence of five main types of alternative splicing events per phenotype as compared to M0 using paired-end analysis by donor (n = 4 human donors). Values in parentheses represent the actual number of detected events. Events were considered to be significant with a false discovery rate < 0.01 and an inclusion level difference of 0.05.

Fig. 3.

Nanostring analysis of 110 putative M2c-related genes identified by RNA-seq analysis. Euclidean hierarchical clustering of M0, M1, M2a, and M2c macrophage gene expression of log₂-transformed gene counts of the 110 genes determined from RNA-seq analysis to be upregulated in the M2c phenotype compared to M0 (gene counts averaged across n = 6 human donors).

Fig. 4.

Secretion of proteins involved in regulation of extracellular matrix. ELISA analysis of macrophage-conditioned media for select MMPs and TIMP1 (n = 4–7 human donors). Data are represented as mean ± SEM. One-way ANOVA with Tukey's post-hoc analysis, *p < 0.05, **p < 0.01.

Fig. 5.

Cluster analysis of publicly available data set using validated M2c genes. (A) Soft clustering of top 100 M1, 100 M2a and 17 M2c genes in human wound healing using publicly available data provided by Gabriel et al. (2014). Genes were assigned to each cluster based on a correlation threshold of 0.6. (B) Composition of the genes in each cluster, calculated by dividing the number of phenotype-associated genes in each cluster by the total number of genes for that phenotype in all four clusters.

Table 1

39 genes upregulated in M2c macrophages compared to M0. Genes are ranked from highest log fold change (LFC) of M2c/M0 expression to lowest with Benjamin–Hochberg adjusted p-value cutoff of < 0.05.

Gene ID	Description	LFC M2c/M0 (p-value)
<i>CD163</i> *	Cluster of differentiation 163	2.504 (5.6E-10)
<i>JAK3</i>	Janus Kinase 3	2.482 (2.3E-10)
<i>VCAN</i> *	Versican	2.319 (2.1E-07)
<i>NKG7</i>	Natural killer cell granulate protein 7	2.304 (9.5E-09)
<i>SOC3</i>	Suppressor of cytokine signaling 3	2.092 (2.5E-10)
<i>CD300E</i> *	Cluster of differentiation 300E	1.909 (3.0E-07)
<i>ENPP2</i>	Ectonucleotide purinophosphatase/phosphodiesterase 2	1.863 (7.5E-07)
<i>MMP7</i>	Matrix Metalloproteinase 7	1.588 (3.3E-05)
<i>IL2RA</i>	Interleukin 2 receptor subunit alpha	1.485 (3.7E-04)
<i>PCOLCE2</i> *	Procollagen C-endopeptidase enhancer 2	1.353 (2.5E-04)
<i>FPR1</i> *	Formyl peptide receptor 1	1.331 (2.1E-04)
<i>FAM20A</i>	Family with sequence similarity 20 member A	1.166 (1.2E-04)
<i>MMP8</i> *	Matrix Metalloproteinase 8	1.166 (9.2E-04)
<i>SLAMF1</i>	Signaling lymphocytic activation molecule family member 1	1.123 (1.9E-03)
<i>PDPN</i>	Podoplanin	1.068 (3.6E-03)
<i>FPR2</i>	Formyl peptide receptor 2	1.039 (5.8E-04)
<i>F5</i> *	Coagulation factor V	1.036 (5.8E-04)
<i>C1S</i>	Complement component 1, s subcomponent	0.983 (8.2E-04)
<i>SIGLEC10</i>	Sialic acid binding Ig like lectin 10	0.954 (1.2E-04)
<i>SH3PXD2B</i> *	SH3 and PX domains 2B	0.905 (8.8E-05)
<i>SECTM1</i>	Secreted and transmembrane 1	0.895 (2.9E-03)
<i>FCRLA</i> *	Fc receptor like A	0.887 (2.0E-03)
<i>MARCO</i> *	Macrophages receptor with collagenous structure	0.864 (6.7E-03)
<i>GAS6</i>	Growth arrest specific 6	0.862 (4.9E-04)
<i>CYP19A1</i>	Cytochrome P450 family 19 subfamily A member 1	0.855 (4.9E-03)
<i>GXYLT2</i> *	Glucoside xylosyltransferase	0.836 (1.6E-04)
<i>MCTP2</i> *	Multiple C2 and transmembrane domain containing 2	0.831 (6.3E-03)
<i>LIN7A</i> *	lin-7 homolog A, crumbs cell polarity complex component	0.829 (4.0E-04)
<i>ST6GAL1</i>	ST6 beta-galactoside alpha-2,6-sialyltransferase 1	0.807 (1.3E-04)
<i>TIMP1</i> *	Tissue Inhibitor of metalloproteinase 1	0.744 (1.2E-04)
<i>SRPX2</i> *	Sushi repeat containing protein, X-linked 2	0.741 (3.2E-03)
<i>KREMEN1</i>	Kringle containing transmembrane protein 1	0.741 (9.0E-03)
<i>CD226</i>	Cluster of differentiation 226	0.694 (2.0E-02)
<i>FCGR1B</i>	Fc fragment of IgG receptor 1B	0.679 (1.0E-03)
<i>PLOD2</i> *	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	0.676 (1.5E-02)

Gene ID	Description	LFC M2c/M0 (p-value)
<i>TMEM74B</i>	Transmembrane protein 74B	0.665 (5.4E-03)
<i>SERPINA1</i> *	Serpin family A member 1	0.642 (1.6E-02)
<i>COL22A1</i>	Collagen type XXII alpha 1 chain	0.632 (2.7E-02)
<i>FCGR1A</i>	Fc fragment of IgG receptor 1A	0.601 (7.0E-03)

* Genes that are differentially upregulated in the M2c phenotype compared to M0, M1 and M2a macrophages.

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