Transcriptional and Histochemical Signatures of Bone Marrow Mononuclear Cell-Mediated Resolution of Synovitis

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Osteoarthritis (OA) may result from impaired ability of synovial macrophages to resolve joint inflammation. Increasing macrophage counts in inflamed joints through injection with bone marrow mononuclear cells (BMNC) induces lasting resolution of synovial inflammation. To uncover mechanisms by which BMNC may affect resolution, in this study, differential transcriptional signatures of BMNC in response to normal (SF) and inflamed synovial fluid (ISF) were analyzed. We demonstrate the temporal behavior of co-expressed gene networks associated with traits from related in vivo and in vitro studies. We also identified activated and inhibited signaling pathways and upstream regulators, further determining their protein expression in the synovium of inflamed joints treated with BMNC or DPBS controls. BMNC responded to ISF with an early pro-inflammatory response characterized by a short spike in the expression of a NF-ƙB- and mitogen-related gene network. This response was associated with sustained increased expression of two gene networks comprising known drivers of resolution (IL-10, IGF-1, PPARG, isoprenoid biosynthesis). These networks were common to SF and ISF, but more highly expressed in ISF. Most highly activated pathways in ISF included the mevalonate pathway and PPAR-γ signaling, with pro-resolving functional annotations that improve mitochondrial metabolism and deactivate NF-ƙB signaling. Lower expression of mevalonate kinase and phospho-PPARγ in synovium from inflamed joints treated with BMNC, and equivalent IL-1β staining between BMNC- and DPBS-treated joints, associates with accomplished resolution in BMNC-treated joints and emphasize the intricate balance of pro- and anti-inflammatory mechanisms required for resolution. Combined, our data suggest that BMNC-mediated resolution is characterized by constitutively expressed homeostatic mechanisms, whose expression are enhanced following inflammatory stimulus. These mechanisms translate into macrophage proliferation optimizing their capacity to counteract inflammatory damage and improving their general and mitochondrial metabolism to endure oxidative stress while driving tissue...
repair. Such effect is largely achieved through the synthesis of several lipids that mediate recovery of homeostasis. Our study reveals candidate mechanisms by which BMNC provide lasting improvement in patients with OA and suggests further investigation on the effects of PPAR-γ signaling enhancement for the treatment of arthritic conditions.

Keywords: macrophage, arthritis, mevalonate pathway, oxidative stress, joint therapy

INTRODUCTION

Osteoarthritis (OA) is a common and debilitating condition that similarly affects horses and people (1, 2). Because chronic synovial inflammation is a hallmark of OA and often the single driver of related degenerative changes (3–7), the use of anti-inflammatory drugs (steroidal and non-steroidal) has been a logical and long-accepted approach for the treatment of many arthritic conditions (8, 9). However, acute inflammation is not simply a clinical sign to alleviate. Acute inflammation is a critical event in promoting tissue repair and setting the stage for endogenous resolution of the inflammatory process and recovery of homeostasis (10). Importantly, anti-inflammatory and pro-resolving effects are not the same, and resolution is not merely the passive termination of the inflammatory process. Anti-inflammation is based on inhibiting key pro-inflammatory mediators, such as chemokine and cytokine production and leukocyte extravasation to the site of injury. Resolution is an active process driven primarily by macrophages and their derived cytokines and lipid mediators, which shift the phlogistic phase of inflammation into a non-phlogistic process that culminates with tissue repair and recovery of homeostasis (11, 12). Most importantly, the recruitment of macrophages and the production of pro-resolving mediators is triggered by enzymes synthesized during the acute inflammatory process (13). Macrophages play such a fundamental role in resolving inflammation and promoting tissue repair that impaired macrophage chemotaxis and/or macrophage depletion results in inefficient healing or chronic inflammation (14–16). Blocking acute inflammation with anti-inflammatory medications interferes, at least to some degree, with macrophage recruitment and the pro-resolving response, and often prevents effective resolution and recovery of homeostasis (12, 13). Targeted therapies for chronic joint inflammation should therefore have pro-resolving properties, which precisely combine pro- and anti-inflammatory mechanisms (12).

Synovial macrophages are the central drivers of the inflammatory response in osteoarthritic synovium (17, 18). In fact, synovial macrophage activation is directly related to disease activity, severity, and pain in OA-affected patients (19). However, this relationship is not causative. Synovial macrophages are also essential keepers of synovial homeostasis through phagocytic clearance and secretion of anti-inflammatory and pro-resolving cytokines, chemokines, enzymes, and growth factors (20–23). Following injury, synovial macrophages proliferate to form a protective immunological barrier in the synovial lining for intra-articular structures (24, 25). When regulatory functions are overwhelmed by the amount of damage, synovial macrophages upregulate inflammation, signaling to monocytes and other leukocytes (e.g., neutrophils and lymphocytes) to help counteract the increased demands for tissue repair and restore homeostasis (17, 26). During the progression of OA, the recruitment of myeloid monocytes into joints seems to be impaired (27), which combined with continuous joint damage, overwhelms the pro-resolving mechanisms of synovial macrophages, leading to degeneration (17, 27, 28).

The mononuclear cell fraction of bone marrow aspirates (bone marrow mononuclear cells -BMNC) is a rich source of pro-resolving macrophages that have been used therapeutically to improve tissue repair and inflammation resolution (29–39). Macrophages within BMNC are the main drivers of such effect, which’s documented pro-resolving functions include increased production of IL-10 (30, 33), diverse types of prostaglandins and specialized lipid mediators (13, 40, 41). The production of these molecules induce decreased production of IL-6 (33), increased phagocytic clearance of debris and apoptotic cells (efferocytosis) (30, 39) and enhanced PPAR-gamma signaling (42–47). Increasing the numbers of myeloid macrophages present in osteoarthritic knees, by injection of BMNC, restored joint homeostasis with long-lasting effects (48). Similarly, BMNC therapy increased counts of pro-resolving macrophages and induced marked resolution of joint inflammation in in vivo and in vitro models of equine synovitis (49, 50). In these models, there was a coordinated spectrum of pro-inflammatory, pro-resolving and anti-inflammatory events, including increased IL-10, IGF-1, and PGE2 production, and self-limiting IL-1 signaling (α and β). These events are all innately required for efficient synovial homeostasis and tissue repair and are commonly antagonized by therapeutic corticosteroids (30, 51–53). While these findings partially explain the durable effects of BMNC in the treatment of OA, little is known about BMNC-related mechanisms of resolution. Therefore, our purpose was to identify cellular mechanisms from BMNC driving joint homeostasis that could

Abbreviations: OA, osteoarthritis; BMNC, bone marrow mononuclear cells; IL, interleukin; IGF-1, insulin-like growth factor 1; PGE2, prostaglandin E2; NF-κB, nuclear factor – kappa B; SF, normal synovial fluid; ISF, inflamed synovial fluid; DPBS, Dulbecco’s phosphate buffered saline; LPS, lipopolysaccharide; CSF1, colony-stimulating factor 1; SDF-1, stromal derived factor-1; IL-1ra, interleukin-1 receptor antagonist; WGCNA, weighted gene co-relation analysis; FPKM, fragments per kilobase of transcript per million mapped reads; DEGs, differentially expressed genes; GO, gene ontology; IPA, ingenuity pathway analysis; PPAR-γ peroxisome proliferator-activated receptor-gamma; PPARGCA1, PPAR-γ co-activator 1-α; TLR3, toll-like receptor 3; MAFB, transcription factor MAFB; SIRT2, sirtuin 2; FN1, fibronectin 1; PCA, principal component analysis; BP, biological process; STAT, signal transducer and activator of transcription; DAMP, damage-associated molecular pattern.
be used for developing targeted pro-resolving joint therapies and uncovering biomarkers of arthritis resolution. The aim of this study was to identify transcriptional signatures of BMNC leading to inflammation resolution using RNA-sequencing and relate these to the expression of key gene products in the synovial membrane. We hypothesized that gene networks linked to macrophage proliferation, negative regulation of inflammatory response, and to a lesser extent, NF-κB signaling, would be temporally upregulated in response to inflammation.

MATERIALS AND METHODS

Study Design

Samples used in the current report were obtained from two previous studies using the same horses. These in vitro (50) and in vivo (49) studies were counterparts of a larger project assessing the effects of BMNC on joint inflammation resolution. Briefly, eight skeletally mature Thoroughbred horses (3-9 years old, median 5 years; 2 females and 6 castrated males) free of OA or systemic inflammation were used under IACUC approval and oversight. General and musculoskeletal health were confirmed by clinical, hematological and orthopedic evaluations. Following sternal bone marrow aspiration for BMNC isolation, synovitis was induced in both radiocarpal joints, as a way of producing more homogeneous inflammation and inflamed synovial fluid (ISF) than could be acquired from naturally occurring OA. Normal synovial fluid (SF) was collected from healthy middle carpal joints. BMNC from each horse were cultured independently (not pooled) in neat (100%) autologous SF or ISF and harvested at 0 (uncultured), 48 and 96 hours, and 6 and 10 days for RNA isolation. RNA-seq was used to identify transcriptional signatures of BMNC in response to acute joint inflammation. The transcriptome of BMNC was assessed over time within the same group (SF or ISF), as well as comparatively between groups at each time point (Figure 1). The expression of 9 potential upstream regulator genes identified following bioinformatical analysis was assessed by immunohistochemistry in the synovium of inflamed joints from the same horses, 6 days after treatment with BMNC or Dulbecco’s phosphate buffered saline (DPBS). Four protein coded by genes identified in key activated pathways were also assessed by IHC.
**BMNC Isolation, Induction of Synovitis, and Synovial Fluid Harvest**

Bone marrow harvest and processing of BMNC, and induction of the synovitis model were performed as previously described in our related study (49). Briefly, BMNC were isolated by density gradient centrifugation. Synovitis was induced by intra-articular injection of 0.5 ng lipopolysaccharide (LPS) into each radiocarpal joint (49, 54). At peak inflammation (8 hours following induction of the model), SF and ISF were collected using aspecific technique. Synovial fluid cytology (SF and ISF) was performed to confirm the health of normal joints and ensure LPS effectively induced synovitis. Synovial fluid was then centrifuged (5,000g; 20 min; 4°C) for cell depletion and the cell-free supernatant used as autologous growth medium. Parameters used to differentiate SF from ISF included quantification of cytokine as reported in our related study (50), total protein (<2.5 g/dL in SF and >4g/dL in ISF) and synovial fluid cytology (total nucleated cells/µL < 1,500 in SF and ~130,000 in ISF; neutrophil count <10% in SF and >80% in ISF).

**BMNC Culture in SF and ISF**

BMNC were plated in 24 well culture plates (2x10⁶ viable cells/50 µL DPBS/well) and covered with 500 µL SF or ISF. Cell viability was assessed at baseline using trypan blue and ranged from 74-96% across horses. Well contents were carefully mixed and plates incubated at 37°C in 5% CO₂ and 90% humidity. Remaining SF and ISF was preserved at 4°C for later addition of medium to replenish cell nutrients (200 µL added every 48 hours). All conditions and time points were performed in duplicate wells with cells from one well used for RNA-sequencing and the other for flow cytometry (macrophage activation markers CD14, CD86, CD206 and IL-10). Conditioned medium was aspirated at the same time points of cell harvest (48 and 96 hours and 6 and 10 days) centrifuged, and the cell-free supernatant used for cytokine and growth factor quantification (FGF-2, GM-CSF, IL-1β, IL-6, MCP-1, IL-10, TNF-α, SDF-1, IFN-γ, IL-1ra, and PGE₂) using a PGE₂ ELISA kit (KGE004B; R&D Systems) and the Milliplex Map Equine chemokine/cytokine bead based array (Eqctlmmag-93K, MilliporeSigma). Details and findings from flow cytometry and cytokine and growth factor quantification are reported elsewhere (50) and were used in this study as a trait for weighted gene co-relation network analysis (WGCNA).

**Transcriptome Analysis of BMNC**

**Cultured in SF and ISF**

RNA Isolation and Sequencing

Cultured cells were recovered in 10 mM EDTA, centrifuged (12,000g; 10 min; 4°C) and the cell pellet placed in guanidinium chloride-phenol (Trizol® Life Technologies, 15596018, Carlsbad, CA). RNA was purified with on-column DNase digest (DirectZol™ RNA microprep kit, R2061, Zymo Research, Irvine, CA), quantified (Qubit® 3.0 Fluorometer, 33216, ThermoFisher Scientific, Carlsbad, CA), and stored at -80°C. RNA quality was assessed (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA) and cDNA libraries prepared using TruSeq DNA Library Preparation kits (Illumina, Inc., San Diego, CA), followed by sequencing (NovaSeq 6000 S4, Illumina) to generate an average of 34.5 (range, 24-54) million stranded paired-end reads (2 x 150 nt) per sample.

**Bioinformatics Pipeline**

Reads were trimmed for quality and adapters with TrimGalore 0.4.3 and mapped to the equine reference genome (EquCab 3.0) (55) using STAR (56) algorithm (version 2.7.2a) and GeneCounts, and expression values determined as gene length corrected trimmed mean of M-values (GeTMM) (57) with the Ensembl v104 annotation. Differentially expressed genes (DEGs) were determined using DESeq2 based upon a false discovery rate (FDR) adjusted P-value (q-value) <0.05 after Benjamini-Hochberg correction for multiple testing, by comparing datasets from consecutive time points within SF or ISF, and by comparing ISF to SF datasets at any given time point. The cutoff set for considering a transcript expressed prior to analysis was 10 fragment alignments. DEGs were represented by principal component analysis using JMP Pro 13 and by volcano plots using Origin software (version 2019, OriginLab, Northampton, MA, USA).

**Functional Genomics**

We adopted a multidisciplinary approach to functional genomics by employing several bioinformatics tools to tease out the biological significance of our data. We used WGCNA and DAVID in a semi-supervised analysis to identify biological processes of interest, and IPA to identify upstream regulators and activated and inhibited signaling pathways. By using this approach, we took advantage of both the superior annotation of biological processes from DAVID and the better pathway annotation of IPA. Together, these tools enabled us to make associations to our previous clinical studies to start to draw clinical translations to our findings.

Weighted gene co-relation analysis was performed using WGCNA version 1.66 package in R to construct gene co-expression networks as described elsewhere (58, 59). Gene co-expression clusters were generated from the whole transcriptome in SF and ISF datasets separately over time. Only genes expressed in at least 50% of samples in each dataset were included in the analysis (16,318 genes in SF and 18,038 genes in ISF). In order to normalize the data, the GeTMM values for each gene were log2 transformed. Next, a pairwise correlation matrix was constructed between all pairs of genes across the samples, and a matrix of weighted adjacency was generated by raising co-expression to a power β = 9, as determined for our sample set (58, 60). A topological overlap matrix (TOM) was then assembled and used as input for hierarchical clustering analysis. Then, a dynamic tree cutting algorithm was used to identify gene clusters or modules (i.e., genes with high topological overlap) in an unsupervised fashion. Gene modules were visualized by heatmap plot (TOMplot) of the gene network topological overlap. Module relationships were summarized by a hierarchical clustering dendrogram and TOMplot of module eigengenes (MEs). Associations between gene modules and traits of interest were tested by correlating MEs to trait score. Module-trait
correlations were visualized using a heatmap plot and only modules with trait relationship significance (R²) higher than 0.7 and a p-value ≤0.05 were considered for further analysis. Traits of interest used for WGCNA included: timeline from our previous study, previously reported CD14, CD86, CD206 and IL-10 expression measured by flow cytometry, and IL-10, IGF-1, MCP-1, IL-1β, TNF-α, PGE₂, and SDF-1 concentrations quantified in conditioned SF and ISF (50). Module memberships (MM; correlation between each gene expression profile (GeTMM) and the ME of a given module as an indicator of the intramodular connectivity) and gene significance (GS; correlation between the gene expression profile (GeTMM) and the trait score (e.g. cytokine concentration in conditioned SF/ISF) as a measure of biological relevance) were calculated (58). Genes (network nodes) having MM ≥ 0.90, P-value < 0.05, and GS ≥ 0.5 were identified as intramodular hub genes (61). Gene ontology (GO) analysis was performed on the entire gene list derived from each module as described above using DAVID Bioinformatics Resources version 6.8 (62) to functionally annotate their biological processes (BP). Of note, no single time point was chosen to determine the module-trait correlations. The entire timeline of the study was itself a trait. Therefore, genes within each module were co-expressed at all time points and thus dominant (overrepresented) BPs for a given module were the same at all time points.

To predict upstream regulators relevant for each set of DEGs, analysis was performed using the Ingenuity Pathway Analysis software (IPA, 2018) (63). The analysis output provided a P-value of overlap, activation Z-scores, and the downstream targets for each predicted upstream regulator. Z-scores were used to predict activation state (activation or inhibition) of each upstream regulator. Predicted upstream regulators were considered significant if they had P < 0.05 and activation Z-score >2 (activated) or <−2 (inhibited). Subsequently, we investigated overlap between the predicted upstream regulators for each set and the DEGs from the same set to identify potential regulators among those DEGs. Genes in common between the two analyses with Z-scores (generated by IPA) matching the direction of fold change (generated by DESeq2) were defined as potential regulators. To investigate the interaction and relationships between potential upstream regulators, all known protein-protein interactions were referenced and matched using STRING version 10.5 (64). Potential upstream regulators of high interaction were selected to have their protein expression assessed. STRING version 10.5 (64). Potential upstream regulators of high interaction were selected to have their protein expression assessed.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded synovial membrane samples from inflamed joints of 6 horses treated with either BMNC or DPBS were sectioned at 5-7 μm and baked at 38°C for 48 hours. Sectioned tissues were processed with the BOND-MAX system (Leica Microsystems, Buffalo Groove, IL) using antibodies for the following gene-products, identified as key upstream regulators or key genes from most activated pathways: peroxisome proliferator-activated receptor γ (PPARγ; rabbit anti-human, clone 16643-1-AP, ThermoFisher Scientific), phospho- PPARγ (rabbit anti-human, clone PA536763, ThermoFisher Scientific), PPARγ co-activator 1 alpha (PPARGCA1; rabbit anti-human, clone PA5-38021; ThermoFisher Scientific), mevalonate kinase (MVK; rabbit anti-human, clone PA528650, ThermoFisher Scientific), 3-Hydroxy-3-Methylglutaryl-CoA Synthase 1 (HMGS1; rabbit anti-human, clone PA529488, ThermoFisher Scientific), colony-stimulating factor 1 (CSF1; rabbit anti-mouse, clone PA5-95279; ThermoFisher Scientific), interleukin-1β (IL-1β; rabbit anti-human, clone P420B; Invitrogen), transcription factor MAFB (MAFB; rabbit anti-human, clone PA5-40756; ThermoFisher Scientific) and sirtuin 2 (SIRT2; rabbit anti-human, clone PA3-200; ThermoFisher Scientific). Positive controls included equine liver, heart, and kidney. Negative controls were prepared with mouse (for PPARγ, phospho-PPARγ, MVK, HMGS1, PPARGCA1, IL-1β, MAFB, SIRT2 antibodies) or goat (CSF1) IgG (Santa Cruz Biotechnology, Inc.). Photographs of representative areas were scored by 3 experienced investigators for staining intensity (0-absent, 1-mild, 2-moderate, 3-intense) and distribution (0-absent, 1-scattered, 2-focal, 3-across the entire villi lining) as previously described (49). Composite scores for immunohistochemical data were presented as median and 95% confidence interval and analyzed by paired t-tests with significance set as p ≤ 0.05 using Prism GraphPad 7.

**RESULTS**

**Temporal Transcriptional Changes in BMNC Following Culture in SF and ISF**

**Differential Gene Expression**

Principal component analysis (PCA) of DEGs between BMNC cultured in SF and ISF showed clear differences in clustering patterns as early as 48 hours, and progressively diverged over time, representative of differences in BMNC response to normal and inflammatory environments (Figure 2A). Volcano plots depict up and downregulated DEGs when comparing ISF to SF cultures at each time point (Figure 2B). Vertical and horizontal comparisons were made, with the number of DEGs between any two conditions reported (Figure 2C). In vertical comparisons, ISF cultures were compared to their SF counterparts at each time point. Counts of upregulated genes were most remarkable at 96 hours and 6 days. In horizontal comparisons, each subset of BMNC was compared with its nearest time point to analyze gene expression variation from BMNC response along the timeline.
From baseline to 48 hours, when myeloid progenitors in BMNC commit to the monocyte/macrophage lineage, the number of DEGs was highest among all time points, for both SF and ISF. The expression patterns of DEGs identified in all vertical and horizontal comparisons were also visualized by heat map (Figure 3A). Further, Venn diagrams were used to illustrate the intersection between DEGs identified by horizontal comparisons and revealed that SF and ISF cultures shared 64.9% of DEGs over the 10 days, while those expressed exclusively in the SF or ISF dataset represented 15.0% and 20.1%, respectively (Figure 3B). Upset plots elucidating the intersection between DEGs identified by vertical comparisons revealed that most DEGs were exclusively expressed in ISF cultures at 6 days (Figure 3C). An entire list of DEGs is available at Supplementary Table 1.

Co-Expression Network Analysis From BMNC in Response to Inflammation

WGCNA provided further insights into the patterns of gene co-expression and the identification of genes with the highest
interaction or connectivity (hub genes) among SF and ISF datasets separately (hub genes are denoted by bold cells in Supplementary Table 2). Co-expression analysis of 18,038 genes in ISF identified 11 module eigengenes (i.e., clusters) (Figure 4A). Among these, modules turquoise, green, blue, brown, black and pink were positively associated with three of the assigned traits. The turquoise and green modules were positively associated with IL-1β concentrations in conditioned ISF, and thus interpreted as having an overall pro-inflammatory nature. Modules blue, brown, black and pink were positively associated with the timeline. The blue module was also associated with CD86 expression assessed by flow cytometry, which denotes macrophage activation (50). In the SF dataset, analysis of the 16,318 genes identified the same 11 gene modules; however, the timeline was the only trait with a positive relationship to MEs, which like ISF included the blue, brown, black and pink modules (Figure 4B). Thus, the IL-1β-related green and turquoise modules are the standout, inflammation-associated differences between ISF and SF cultures, while the blue module also differed in the number of positively associated traits. Since events associated with inflammation resolution would only be present in an inflammatory environment, further dissection of WGCNA findings were centered on data from ISF cultures, while data from SF cultures were used as a point of comparison.

To assess the temporal behavior of each module, the mean expression profile (mean GeTMM values for all genes) for each module was plotted over time for both SF and ISF separately (Figure 5A). This comparison revealed that the blue and brown modules exhibited increasing mean expression profiles that similarly dominated over time in both SF and ISF, which however, were higher in ISF. The pink and black modules had lesser expression among modules identified which completely

FIGURE 3 | Differentially expressed genes (DEGs) in BMNC cultured in normal (SF) and inflamed autologous synovial fluid (ISF). (A) Heatmap of DEGs (FDR<0.05) identified among all possible comparisons (n=8456) between SF and ISF cultures over 10 days. The heatmap was created using Log10 transformed GeTMM values expressed on a color scale denoting high (red) and low (blue) expression. Each dataset (SF and ISF) included all DEGs displayed in a fixed position for comparison of the effect of culture medium over the same genes. (B) Venn diagram illustrating the intersection between DEGs identified by horizontal comparisons in either SF or ISF cultures. (C) Upset plots elucidating the intersection between DEGs identified by vertical comparisons. The nature of each intersection is indicated by the dots under the vertical bars, which denote the number of DEGs in each intersection, while horizontal bars represent the number of DEGs in each comparison.
overlapped between SF and ISF, and therefore are not graphically represented. Additional comparisons for a given module between ISF and its SF counterpart also included the functional annotation of the genes within such modules (Figures 5B–E). The blue, brown, black and pink modules completely overlapped between ISF and SF regarding their gene list, HUB genes list (Supplementary Table 2) and functional annotations (Supplementary Tables 3, 4). Exclusive to ISF, the green module peaked at 48 hours, the same time at which the blue and brown modules started to exhibit increased expression.

![Diagram](image-url)

**FIGURE 4** | Weighted gene co-expression network analysis (WGCNA): module-trait relationships. (A) WGCNA of 18,038 genes in ISF identified 11 modules, eigengene (ME), of which 6 were positively associated ($R^2 \geq 0.7$, $p \leq 0.05$) with the assigned traits. The turquoise and green modules were positively associated with IL-1β quantitation in ISF conditioned by BMNC. MEs blue, brown, black and pink were positively associated with the timeline. The blue module was also associated with CD86 expression assessed by flow cytometry (29). (B) In the SF dataset, analysis of the 16,318 genes identified the same 11 gene modules; however, timeline was the only trait with a positive association to MEs blue, brown, black and pink modules, as in ISF.
FIGURE 5 | Expression profile, gene ontology enrichment and overrepresented biological processes within dominant modules. The green and turquoise modules were only significant in ISF and positively associated with IL-1β quantification in conditioned ISF, and overall associated with pro-inflammatory mechanisms. The blue and brown modules were significantly associated to the timeline for both SF and ISF, while the blue module was positively associated to CD86 expression in ISF.

(A) Mean expression profiles of significant modules in SF and ISF derived from all transcripts in each cluster. In ISF, the presence of the pro-inflammatory green and turquoise modules are associated to increased mean expression of the homeostatic/pro-resolving blue and brown modules. Overrepresented Biological Processes (BPs) in the blue (B), brown (C), green (D), turquoise (E) modules and their corresponding fold enrichment. The complete list of significant modules, BPs and related genes for each module is presented in Supplementary Tables 2, 3.
comparison to its SF counterpart. The mean expression profile in
the ISF’s turquoise module progressively decreased from
baseline. Functional annotation of genes within each module
was then inspected (Figures 5B–E).

Modular Gene Ontology Enrichment and
Overrepresented Biological Processes (BPs)
Overrepresented BPs were ranked based on fold enrichment
and having an FDR <0.05 (Supplementary Table 3). For cases in
which a large list of BPs met this criterion, BPs with FDR <0.01
were given priority attention. Fold enrichment was determined
by comparing the background frequency of total genes annotated
to a certain BP in the specified species to the sample frequency of
genes under such BP. Overrepresentation was defined by a
positive fold enrichment value (65). Since the gene list for
modules blue, brown, pink and black completely overlapped
between SF and ISF (Supplementary Table 2), overrepresented
BPs in any of these modules were the same for both groups
(Figures 5B–E). For the blue module, isoprenoid biosynthesis
was the most overrepresented of the 79 BPs identified by GO.
Given the high number of overall (n=4148) and hub genes
(n=699) in this module, a diversity of BPs was identified
within it, and is collectively discussed below. Of note, genes
identified with pro-resolving functions in the related previous
studies (IL10 and IGF1) (49, 50) also allocated to the blue
module. In the brown module, while the most overrepresented
BP was “antigen presentation via MHC class II”, most BPs in this
module related to mitochondrial response to oxidative stress and
energy metabolism homeostasis. Overrepresented BPs in the pink
and black modules constituted a minor list and were associated
with a variety of cell homeostasis and housekeeping functions.

Exclusive to ISF cultures, BPs in the IL-1β-associated green
module were primarily associated with macrophage response to
damage, including mitosis, adjustment of lipid and glucose
metabolism following circadian distress, activation of the
amphiregulating-STAT3 axis (GO:0032355—response to
estradiol) and noncanonical NF-κB signaling, thus, likely a
module with a pro-inflammatory signature. In the turquoise
module, also associated with IL-1β production, overrepresented
BPs reflected the response of myeloid progenitors to stress and
IL-4 signaling, a key event in the response of pro-resolving
macrophages, amplying chromatin opening for enhanced
mRNA transcription (44–46). In summary, ISF triggered an
early pro-inflammatory response in BMNC progenitors (green
module) leading to macrophage commitment and priming
(turquoise module). These events enhanced the constitutive
expression of homeostatic mechanisms from macrophages
(blue and brown modules) required to counteract damage and
recover homeostasis (Figures 5A–E).

Pathway Analysis, Upstream Regulators and Their
Network Interactions
Ingenuity Pathway Analysis revealed activated and inactivated
pathways in SF and ISF cultures (Table 1, Supplementary
Table 4). Our pathway analysis results from 0–48 hours
(performing with IPA) agrees with findings from GO analysis
and points repeatedly to activation of the mevalonate pathway
and isoprenoid biosynthesis (superpathways of cholesterol
biosynthesis, geranylgeranyl diposphate biosynthesis, cholesterol
biosynthesis I, II and III, and mevalonate pathway 1). The patterns of expression of genes involved in these
pathways, comparing BMNC cultured in SF and ISF
(Figure 6), highlight the increased expression of genes such as
ACAA2, HADHA ACAT2 and FDPS in ISF, essential for the
synthesis of isoprenoids and mitochondria beta-oxidation of fatty
acids. Additional pathways activated at 0–48 hours included
unfolded protein response in agreement with overrepresented BPs
in the blue module, and estrogen biosynthesis, in agreement with
the BP “response to estradiol” from the green module, peaking at 48
hours and progressively decreasing.

From 48–96 hours, the PPAR- signaling pathways were
repeatedly identified, which agrees with the identification of
PPARG as highly connected upstream regulator, depicting the
activation of the PPAR-γ and PPAR-α signaling pathways.
Comparisons for BMNC cultured in SF and ISF for the
expression of genes involved in these pathways (Figure 6),
highlight the higher expression of PPAR genes in ISF. It also
highlights the higher expression of NFκB2 and RELB genes,
encoding for drivers of non-canonical NF-κB signaling, which
has essential pro-resolving functions. After 96 hours of culture in
ISF, a mix of pathways involved in cartilage metabolism
(Heparan Sulfate Biosynthesis), cellular homeostasis/
inflammation resolution (Cell Cycle: G2/M DNA Damage
Checkpoint Regulation, Unfolded Protein Response) and
leukocyte migration during inflammation (Netrin Signaling)
were observed to be activated. This mixed profile may have
resulted from continuously challenging BMNC with ISF every 48
hours as performed in our model (50). Inhibited pathways
included leukocyte extravasation signaling, IL-6 across the time
course, IL-15 production and acute phase response signaling, key
players in the development and maintenance of synovitis and
degenerative processes observed in osteoarthritis. While the
“Osteoarthritis Pathway” was identified as the third most
activated pathway in SF at 6-10 days, neither the model used
in our study, nor the list of genes involved in such functional
annotation support such a finding.

Twenty-three potential upstream regulators were identified as
activated (p <0.05 and a Z-score ≥2) in ISF and 35 in SF
(Figure 7A, Supplementary Table 5, Supplementary
Figure 1). Within these, 5 transcription factors (EIF4E, LARP1,
MAFB, NF2E2L2 and SIRT2 genes), the transmembrane receptor
TREM2, the enzymes LPL and PIK3R1, and the multifunctional
receptor GABARAP were conserved between ISF and SF.
Inactivated upstream regulators (p <0.05 and a Z-score ≤2)
were also identified in both ISF (n=17) and SF (n=32). Within
these, IL1B and its downstream signaling transcription factor
RELA, the mitochondria fission receptor MFN2, and the
transcription factor GATA1 were conserved between ISF and SF.

Interaction networks generated by STRING among activated
and inhibited upstream regulators in ISF cultures revealed
predicted interactions between upstream regulators in and
outside their same module (Figure 7B). Among activated
upstream regulators, the blue module was overrepresented in ISF (50%; including PPARG, SCAP, MAFB and SIRT2) followed by the brown module (CSF1, LARP1, LPL, NFE2L1) and turquoise module (including CDKN1A, CDKN2A, EIF4E, TG) equally representing 16.6% of upstream regulators, while the grey module (including CSF1, LARP1, LPL, NFE2L1) equally followed by the green module (8.3%; STAT3 and GABARAP) were minimally represented. Amongst inhibited upstream regulators in ISF (Figure 7B), the turquoise module was overrepresented (70.5%) and largely related to the inflammation-derived oxidative stress and an antioxidant response relying on lipid biosynthesis and activation of the PPAR signaling pathway. While the IL-6 pathway inhibition was common to both SF and ISF, it was a more frequent in ISF cultures. A detailed list of activated and inactivated pathways is presented on Supplementary Table 4.

**DISCUSSION**

In this study, we identified differential transcriptional signatures of BMNC in response to ISF and SF. These same BMNC were previously shown to resolve synovitis following exposure to an inflamed synovial environment *in vivo* and *in vitro* (49, 50). We demonstrate a temporal behavior of co-expressed gene networks and their association with traits from our previous studies (49, 50).
as well as with the expression of key proteins in the synovium by immunohistochemistry. Our findings illustrate the elaborate balance of pro- and anti-inflammatory mechanisms shifting dominance through the recovery of joint homeostasis. BMNC responded to ISF with an early pro-inflammatory response (green module), characterized by a short spike in the expression of NF-κB-related genes, coincident with the peak of IL-1β secretion in conditioned ISF.

FIGURE 6 | Heatmaps depicting the patterns of expression of genes involved in the most highly activated signaling pathways associated with resolution of synovitis, comparing BMNC cultured in SF and ISF. Further details are available at Supplementary Table 4.
This response was associated with increased expression of the blue and brown modules, 2 gene networks with homeostatic functions comprising known drivers of resolution, which were more highly expressed in ISF and were dominant among activated upstream regulators. Significant differences in the expression of phosphorylated PPARγ and mevalonate kinase in synovial membranes from inflamed joints treated with BMNC, and equivalent IL-1β signaling between BMNC- and DPBS-treated joints, emphasize the fine tuning of so-called pro-inflammatory pathways that must remain active at physiological levels during the resolution process. These observations highlight the differences between the
pro-resolving effects associated with BMNC therapy compared to the anti-inflammatory effects observed following clinical treatment with corticosteroids (30, 49–51, 69, 70).

The short spike of expression of the pro-inflammatory green module observed in ISF, is essential to trigger a cascade of events that culminates in a pro-resolving response. NF-κB-related and mitogen genes co-expressed in the green module (Supplementary Table 3) play a key role in promoting the proliferation of macrophages necessary to counteract damage (24, 25). NF-κB-related genes also induce increased expression of
genes with anabolic and anti-inflammatory functions within the blue and brown modules, which have critical roles in driving resolution of joint inflammation. The top four overrepresented BPs in the green module relate to a group of genes sharing important mitogenic activity. Genes such as MAD1L1 and CHAMP1 encode proteins that interact and regulate cell structure organization preceding mitosis (71, 72). NR4A3 and NR1D2 encode transcriptional activators involved in proliferation, survival and differentiation of myeloid progenitor cells, and in adjusting myeloid progenitor cell metabolism to oxidative stress (73, 74). NR1D2 does so by activation of IL-6 transcription, which is also required to induce expression of the IL-4 receptor and related downstream regulatory functions of macrophages, including their self-renewal (75–77). The BP “response to estradiol” was characterized by the expression of the amphiregulin (AREG) and STAT3 genes. Macrophages are an important source of amphiregulin produced during acute inflammation. The AREG/ERK/STAT3 signaling axis is required for the differentiation of progenitor cells during tissue repair and establishing a pro-resolving response (78–81). AREG was more highly expressed in ISF and exhibited progressively decreasing expression over time, as the resolving response progressed (Supplementary Table 6). Additionally, estrogen accelerates the resolution of inflammation through the regulation of IL-10/STAT3-mediated deactivation of pro-inflammatory responses, such that post-menopausal women are prone to developing chronic inflammation (82). STAT3 was the activated upstream regulator in ISF cultures with the highest connectivity. The NIK/NF-ƙB signaling in the green module was highlighted by the expression of RELB and NFKB2. Both RELB and NFKB2 are subunits of the non-canonical NF-ƙB signaling, which in macrophages, can exert both pro- and anti-inflammatory effects (83, 84). Non-canonical NF-ƙB signaling is critical to produce SDF-1ƙ and recruit monocytes to the site of damage immediately following injury (83). Further, during monocyte-macrophage differentiation, non-canonical NF-ƙB signaling prevents hyperactivation of new macrophages by accelerating the removal of RelA and c-Rel (canonical NF-ƙB subunits) from pro-inflammatory gene promoters preventing overt inflammation. As such, blocking non-canonical NF-ƙB by inactivation of its IKKƙ subunit results in increased inflammation (84). In both SF and ISF cultures, RELB expression was positively regulated, with decreasing expression over time, while RELA was downregulated (Figure 6, Supplementary Table 6). Combined, these signatures illustrate a fraction of molecular drivers of the acute response of BMNC to inflammation, which also sets the stage for establishing a pro-resolving response. Increased expression of the blue module in response to inflammation, in parallel with the surge of the green module, and over a timeline associated with resolution in our previous studies (49, 50), suggests a pro-resolving identity. Genes encoding established drivers of joint inflammation resolution (IL-10, IGF-1) allocated to the blue module (Supplementary Table 2). A major functional signature of this module was the activation of the mevalonate pathway and isoprenoids biosynthesis, comprised by the expression of genes encoding central drivers of the mevalonate/isoprenoid pathway (COQ2, HMGCR, FDP5, HMGCS1, GPPS1, MVK, PDSS1, PDSS2, GGDP5, FDP5, ACA2, MADH1A, ACAT2. In agreement, the super pathway of cholesterol biosynthesis, geranylgeranyl biosynthesis (an isoprenoid) and mevalonate pathway had the highest activation scores in ISF cultures between 0 and 48 hours, as detected by IPA. The roles of the mevalonate pathway in stereodigenesis, counteracting oxidative stress and inflammation resolution, are well documented in the macrophage response to damage and inflammation resolution (85–88). Deficiency of mevalonate kinase (MVK), a key enzyme in the mevalonate pathway, causes reduced synthesis of isoprenoids, leading to mitochondrial damage, subsequent oxidative stress and severe inflammation (89, 90). Importantly, exogenous isoprenoid treatment in models of inflammation induces decreased oxidative stress and production of inflammatory markers, by increasing expression of the NF-ƙB inhibitor IƙBƙ and antioxidant selenoproteins (86, 91–93). Gene expression for MVK and HMGCS1, key enzymes in the mevalonate pathway, exhibited a similar expression profile between themselves (Figure 6, Supplementary Table 6). These enzyme genes were more highly expressed in SF, suggesting that inflammation negatively affects this pathway. A similar pattern of gene expression for IL10 and IGF1 was observed in our previous study, in which both genes were more highly expressed in SF than ISF (50). However, concentrations of IL-10 and IGF-1 in ISF were higher than in SF, because the decreased relative production in ISF was compensated for by higher macrophage counts (50). Our ongoing lipidomic study on BMNC-conditioned SF and ISF from the same samples used in this study may help elucidate if a similar context applies for isoprenoid production. Interestingly, it was recently evidenced that transcriptional dysregulation of the mevalonate pathway is a key signature of the overt inflammation caused by SARS-CoV-2 infection, highlighting its importance for inflammation resolution (88). Differences in MVK expression detected in synovial membranes suggests that the in situ activity of MVK in synovitis resolution happens earlier in time, as suggested by our pathway analysis and in vivo study (49).

The second overrepresented BP in the blue module “proteasome ubiquitin-dependent protein catabolism” was comprised of genes that characterize the formation of the 26S proteasome. Inflammation-derived oxidative stress damages nascent proteins that become misfolded and targeted for degradation (94). The 26S proteasome is essential for the degradation of these proteins, preventing aggregate formation, which is part of the pathogenesis of several conditions (94). Such observation from GO analysis agrees with the IPA findings where the “Unfolded Protein Response” was identified among the most activated pathways following isoprenoid biosynthesis through the mevalonate pathway. The next overrepresented BP was “protein localization to the Cajal body”. These are coiled bodies found in the nucleus of proliferating or metabolically active cells and are implicated in telomere homeostasis (95, 96). This BP was represented primarily by genes encoding chaperonin containing...
talless (CCT) proteins that are critical regulators of telomerase folding and trafficking (97). Depletion of CCT proteins cause Cajal body and telomerase mislocalization and failure of telomere elongation (97). CCTs are also required for folding of cytoskeletal proteins during cell proliferation (98). The fourth dominant BP, “response to thyroid hormone stimulus”, reflects the effects of triiodothyronine on regulation of macropage maturation and responses. Such responses include controlling cell migration and conferring protection against endotoxemia and LPS exposure, in great part through proliferation of tissue resident macrophages (99, 100). In addition, half of the genes characterizing this BP encode for cathepsins that mediate endolysosomal protein degradation. In summary, the dissection of a minute part of the blue module, illustrates the effect of isoprenoids and thyroid hormones in improving metabolism and performance of BMNC-derived macrophages to counteract the effects of inflammatory oxidative stress (85, 89, 90, 99, 100). This response is, at least partially, achieved by adjusting proteostasis through the 26S proteasome and Cajal bodies, preventing degenerative protein aggregate formation during increased cell transcription, proliferation and metabolism in response to inflammation (94, 97, 98).

The signature of the brown module were BPs that comprised of a series of gene groups encoding proteins that regulate the mitochondrial respiratory chain and mitochondria-mediated regulation of energy metabolism (101, 102). There is growing evidence of the pivotal role of mitochondria in energy metabolism adjustments required for inflammation resolution as shown in the brown module (103–105). In the face of inflammatory challenges, enhanced cell respiration induces oxidative stress, activating alternative sources of energy driving gluconeogenesis and enhancing mitochondrial fatty acid oxidation (101, 102) as denoted by the increased expression of genes such ACAAl2 and HADHA in ISF cultures (Figure 6). These genes were, however, associated with the isoprenoid biosynthetic pathway, highlighting the recently reported role of mitochondrial isoprenoid biosynthetic process (106). Enhancing the mitochondrial respiratory chain is a homeostatic mechanism that prevents mitochondrial DNA damage and its subsequent cytosolic and extracellular release signaling through damage-associated molecular pattern (DAMP) receptors (107). The peroxisome proliferator-activated receptor-gamma (PPAR-γ) co-activator 1-α (PPRC1A; PPRGC1A gene), a master regulator of mitochondria biogenesis and liver gluconeogenesis was among the outstanding genes involved in mitochondrial regulatory functions listed in the brown module (101) and exhibited a trend for higher expression in BMNC treated joints (Figure 8). Following interaction with PPRGC1A, PPAR-γ exhibits increased activity, interacting with a multitude of transcription factors and PPAR-γ responsive elements (43). PPAR-signaling was an important activated pathway identified by IPA in both SF and ISF cultures between 48 and 96 hours (Table 1, Figure 6), denoting its homeostatic functions. Among different PPARs, PPAR-γ was one of the most highly connected upstream regulators. In macrophages and other cells, PPAR-γ signaling is a cornerstone of tissue repair and inflammation resolution, exhibiting myriad functions that are either PPAR-γ-mediated or -dependent (43). Examples include shifting the production of pro-inflammatory cytokines towards anti-inflammatory and pro-resolving mediators, driving apoptosis and clearance of neutrophils, enhancing macrophage traffic, recruitment, phagocytosis and effector cell activation, improving mitochondrial respiratory performance, and the overall transcriptome of a regulatory response driving recovery of homeostasis (43, 47, 101, 108). Of note, some isoprenoids, the signature of the blue module, as well as some specialized pro-resolving molecules, signal through PPAR-γ, conferring increased production of IL-10, resistance to inflammatory stimuli and attenuated NF-κB activation following LPS stimulation (43, 81, 109–111). Gene expression for PPARG was higher in ISF compared to SF. Significantly lower staining for phospho-PPAR-γ expression in synovial membranes detected by IHC, combined with the timing at which PPARG was identified as an Upstream Regulator, suggest that its activity modeling synovitis resolution almost overlap with the acute phase of inflammation. Importantly, PPAR-signaling findings from IPA at 48–96 coincide with the time at which pro-resolving effects were observed in our previous in vivo and in vitro studies (49, 50). Histochemical findings for phospho-PPAR-γ and PPRGC1A in BMNC compared to DPBS-treated controls suggests PPAR-γ signaling was not only a BMNC response to the inflamed synoval environment, but also part of the beneficial effects of BMNC on treated joints. Moreover, there is recent evidence that PPRGC1A expression is required for chondrocyte metabolism and cartilage homeostasis, with PPRGC1A knockouts exhibiting delayed endochondral ossification, disruption of physeal morphology and severe premature osteoarthritis (112).

Also in the brown module, CSFI was identified as an upstream regulator gene and was more highly expressed in ISF than SF cultures. The marked proliferation of BMNC in ISF observed in vitro (50) may also be a response from CSF-1 signaling (50). Following a spike of proliferation, stimulation of macrophage progenitors with interferon-gamma (IFN-γ) or LPS induce maturation and cell cycle arrest, increasing MHC-II expression and developing the capability to quickly respond to inflammatory stimuli and antigen presentation (113). Given that synovitis in our model was induced by injection of LPS, it is not surprising that “antigen processing and presentation of polysaccharide antigen via MHC class II” was a dominant BP in the brown module, particularly considering that IFN-γ was not detected by immunoassay in conditioned SF or ISF in a preliminary screening performed in our study (50). Combined, these observations from the brown module highlight the importance of macrophage proliferation and maturation, and PPRGC1A/PPAR-γ signaling during macrophage-mediated joint homeostasis, identifying the need for further investigation of the therapeutic roles of PPAR-γ-agonists in the recovery of joint health.

The combined overrepresented BPs in the turquoise module, exclusive to ISF, reflect the dynamic of serine/threonine phosphorylation, autophosphorylation and dephosphorylation during cytokine signaling and subsequent mRNA transduction
In vivo, our findings suggest that IL-1β expression was significantly higher in ISF (50), in agreement with the overall agreement between different analytical tools used in this study. A study of the transcriptome analysis from synovial samples from naturally inflamed joints treated with BMNC and DPBS would complement our current observations and further illustrate the molecular drivers of the synovial response to BMNC injection.

CONCLUSION

Our current data suggest that BMNC-derived mechanisms of resolution are primarily represented by constitutively expressed homeostatic mechanisms, whose expression is enhanced to counteract tissue damage. These homeostatic mechanisms translate into macrophage proliferation, enlarging the "macrophage army" to fight aggressors, also improving their general and mitochondrial metabolism to better resist the challenges of inflammatory oxidative stress. Such effect is partially achieved through the synthesis and signaling of lipid mediators that promote recovery of homeostasis. Further exploration of BPs and pathways not dissected in this study may identify additional targets for future investigations. The combined findings of our equine studies (27, 49, 50) and human clinical trials (48, 119) highlight the long-lasting and superior pro-resolving effects of BMNC in the treatment of arthritic conditions. This study reveals important transcriptional signatures of BMNC-induced resolution of synovitis and reinforce that pro-resolving macrophages do not fit within commonly described pro- or anti-inflammatory phenotypes established in artificial in-vitro systems (120, 121). Current knowledge, including our study, suggests that in vivo, macrophages are by default homeostatic cells that, following injury, drive inflammation with the purpose of counteracting tissue aggressors, further guiding inflammation resolution and recovery of homeostasis (24, 25, 39, 122–125). Our study also highlights candidate mechanisms by which BMNC provide lasting improvement in patients with OA. Therapeutic enhancement of PPAR-γ signaling in joints with chronic inflammation may represent a novel strategy for resolving joint inflammation. Defining multiple mechanisms of macrophage-mediated synovitis resolution may provide means to develop pharmacological pro-resolving therapies, bypassing the need for more invasive bone marrow aspiration and further advancing the treatment of many inflammatory arthropathies, not just OA.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author. The current RNAseq data was deposited in the Gene Expression Omnibus (GEO; GSE18552) repository.
ETHICS STATEMENT

This study was conducted in compliance with the Animal Welfare Act and the approval of the Virginia Tech Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BCM, INM, and LAD designed studies. BCM obtained and processed the samples. TSK and SCL mapped and quantified the sequenced libraries. HE-SA and SCL performed DEGs analysis. BCM and HE-SA performed functional genomics, figures preparation, data interpretation and conceptualization. KES performed immunohistochemical assays, which were scored by BCM, HE-SA, and KES. BCM prepared the manuscript. All authors edited, reviewed, and approved the manuscript.

REFERENCES

1. McIlwraith, CW, Frisbie, DD, Kawcak, CE. The Horse as a Model of Naturally Occurring Osteoarthritis. *Bone Joint Res* (2012) 1:297–309. doi: 10.1302/2046-3758.11.2000132
2. Murphy, LB, Cisternas, MG, Pasta, DJ, Helmick, CG, Yelin, EH. Medical Expenditures and Earnings Losses Among US Adults With Arthritis in 2013. *Arthritis Care Res* (2018) 70:869–76. doi: 10.1002/acr.23425
3. Sellam, J, Benrahem, F. The Role of Synoviocytes in Pathophysiology and Clinical Symptoms of Osteoarthritis. *Nat Rev Rheumatol* (2010) 6:625. doi: 10.1038/nrrheum.2010.159
4. Goldring, MB, Otero, M. Inflammation in Osteoarthritis. *Curr Opin Rheumatol* (2011) 23:471–8. doi: 10.1097/BOR.0b013e328343bc2b1
5. Mathiessen, A, Conaghan, PG. Synovitis in Osteoarthritis: Current Understanding With Therapeutic Implications. *Arthritis Res Ther* (2017) 19:18. doi: 10.1186/s13075-017-1229-9
6. Smith, MD. The Normal Synovium. *Open Rheumatol J* (2011) 5:100–6. doi: 10.2174/1874312901105010100
7. Lopes, ERPa, Filiberti, A, Husain, SA, Humphrey, MB. Immune Contributions to Osteoarthritis. *Curr Osteoporos Rep* (2017) 15:593–600. doi: 10.1007/s11914-017-0411-y
8. McIlwraith, C. Principles and Practices of Joint Disease Treatment. In: D S, and MW Ross, editors. *Diagnosis and Management of Lameness in the Horse*. St Louis, MO: Elsevier Saunders (2011). p. 840–52.
9. Hunter, D. Treatment of Osteoarthritis. In: N Arden, editor. *Atlas of Osteoarthritis*, vol. pp . London: Springer Healthcare (2018). p. 80–99.
10. Nathan, C, Ding, A. Nonresolving Inflammation. *Cell* (2010) 140:871–82. doi: 10.1016/j.cell.2010.02.029
11. Serhan, CN, Chiang, N, Van Dyke, TE. Resolving Inflammation: Dual Anti-Inflammatory and Pro-Resolution Lipid Mediators. *Nat Rev Immunol* (2008) 8:343–9. doi: 10.1038/nri2294
12. Sugimoto, MA, Sousa, LP, Pinho, V, Perretti, M, Teixeira, MM. Resolution of Inflammation: What Controls Its Onset? *Front Immunol* (2016) 7. doi: 10.3389/fimmu.2016.00160
13. Buckley, CD, Gilroy, DW, Serhan, CN. Resolving Lipid Mediators and Mechanisms in the Resolution of Acute Inflammation. *Immunity* (2014) 40:315–27. doi: 10.1016/j.immuni.2014.02.009
14. Degenouts, CKE, Goumans, M-J, Bakker, W. Mononuclear Cells and Vascular Repair in HHT. *Front Genet* (2015) 6:114. doi: 10.3389/fgene.2015.00114
15. Godwin, JW, Pinto, AR, Rosenthal, NA. Macrophages are Required for Adult Salamander Limb Regeneration. *Proc Natl Acad Sci USA* (2013) 110:9415–20. doi: 10.1073/pnas.1300291110
16. Wynn, TA, Barron, L. Macrophages: Master Regulators of Inflammation and Fibrosis. *Semin Liver Dis* (2010) 30:245–57. doi: 10.1055/s-0030-1255354
17. Bondeson, J, Wainwright, SD, Lauder, S, Amos, N, Hughes, CE. The Role of Synovial Macrophages and Macrophage-Produced Cytokines in Driving Aggrecanases, Matrix Metalloproteinases, and Other Destructive and Inflammatory Responses in Osteoarthris. *Arthritis Res Ther* (2006) 8: R187. doi: 10.1186/ar2099
18. Manferdini, C, Paolella, F, Gabusi, E, Silvestri, Y, Gambari, L, Cattini, L, et al. From Osteoarthritic Synovium to Synovial-Derived Cells Characterization: Synovial Macrophages are Key Effector Cells. *Arthritis Res Ther* (2016) 18:83–3. doi: 10.1186/s13075-016-0983-4
19. Kraus, VB, McDaniel, G, Huebner, JL, Stabler, TV, Pieper, CF, Shipes, SW, et al. Direct In Vivo Evidence of Activated Macrophages in Human Osteoarthritis. *Arthritis Care and Research* (2016) 24:1613–21. doi: 10.1002/acr.2016.04.010
20. Van Weeren, PR. General Anatomy and Physiology of Joints. In: F. D, CW McIlwraith, CE Kawcak and PR van Weeren, editors. *Joint Disease in the Horse*, St Louis, MO: Elsevier (2016). p. 23–52.
21. Fichadiya, A, Bertram, KL, Ren, G, Yates, RM, Krawetz, RJ. Characterizing Heterogeneity in the Response of Synovial Mesenchymal Progenitor Cells to Synovial Macrophages in Normal Individuals and Patients With Osteoarthritis. *J Inflamm (London England)* (2016) 13:12–2. doi: 10.1186/s12950-016-0120-9
22. Bellac, CL, Dufour, A, Krisinger, MJ, Loonchanta, A, Starr, AE, Dow, P, et al. Macrophage Matrix Metalloproteinase-12 Dampens Inflammation and Neutrophil Influx in Arthritis. *Cell Rep* (2014) 9:618–32. doi: 10.1016/j.celrep.2014.09.006
23. Alivernini, S, MacDonald, L, Elmesmari, A, Finlay, S, Tolusso, B, Gigante, MR, et al. Distinct Synovial Tissue Macrophage Subsets Regulate Inflammation and Remission in Rheumatoid Arthritis. *Nat Med* (2020) 26:1285–306. doi: 10.1038/s41591-020-0939-8
24. Culemann, S, Grueneboom, A, Nicolas-Avila, JA, Weidner, D, Lammermann, T, Germain, RN. Resident Macrophages in the Normal Synovium: A Unique Subpopulation of Macrophages. *Arthritis Res Ther* 2011.00052
25. Uderhardt, S, Martins, AJ, Tsang, JS, Lammermann, T, Germain, RN, Resident Macrophages. Closely Tissue Microenvironments to Prevent Neutrophil-Driven Inflammatory Damage. *Cell* (2019) 177:541–55.e17. doi: 10.1016/j.cell.2019.02.028
26. Kennedy, A, Fearon, U, Veale, DJ, Godson, C. Macrophages in Synovial Inflammation. *Front Immunol* (2011) 2:52–2. doi: 10.3389/fimmu.2011.00052
27. Menarim, BC, Gillis, KH, Oliver, A, Ngo, Y, Werre, SR, Barnett, SH, et al. Macrophage Activation in the Synovium of Healthy and Osteoarthritic Equine Joints. *Front Vet Sci* (2020) 7:568756–6. doi: 10.3389/fvets.2020.568756
28. Scanzello, CR, Goldring, SR. The Role of Synovitis in Osteoarthritis Pathogenesis. *Bone* (2012) 51:249–57. doi: 10.1016/j.bone.2012.02.012

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.734322/full#supplementary-material
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39. Giraldi-Guimarães A, de Freitas HT, Coelho B, Macedo-Ramos H, Mendez-Lopez J, et al. Comparison of the Autologous Bone Marrow-Derived Mononuclear Cells as Treatment of Injured Tendons in a Goats Model. *Vet Med Int* (2010) 2010:250978. doi: 10.4061/2010/250978.

38. Song F, Tang J, Geng R, Hu H, Zhu C, Cui W, et al. Frequency of Mixed Th2/Th17 Allergic Airway Inflammation in Horses With Recurrent Airway Obstruction. *Vet Med Int* (2014) 2014:415672. doi: 10.1155/2014/415672.

37. Chu CR, Fortier LA, Williams A, Payne KA, McCarrel TM, Bowers ME, et al. Autologous Implant of Bone Marrow Mesenchymal Stem Cells and Bone Marrow Mononucleated Cells in Collagenase-Induced Tendinitis of Equine Superficial Digital Flexor Tendon. *Vet Med Int* (2010) 2010:250978. doi: 10.4061/2010/250978.

36. Tsubosaka Y, Maehara T, Imai D, Nakamura T, Kobayashi K, Nagata N, et al. Systemic Analysis of Ppar Signaling Axis Is Driving the Expansion of the RXR Heterodimer Cistrome, Providing Complex Ligand Responsiveness in Macrophages. *Nucleic Acids Res* (2018) 46:4423–39. doi: 10.1093/nar/gky157.

35. Menarim B, Fortini G, Alvarez P, Gómez J, Jarrín C, Ramírez A, et al. Autologous Implant of Bone Marrow Mononuclear Stem-Cells as Treatment for Equine Bicipital Tendinitis: Case Report. *Arch Med Vet* (2012) 44:291–5. doi: 10.4067/S0301-732X2012000300013.

34. Nguyen TL, Nguyen HP, Nguyen TK. The Effects of Bone Marrow Mononuclear Cell Transplantation on the Quality of Life of Children With Cerebral Palsy. *Health Qual Life Outcomes* (2018) 16:164. doi: 10.1186/s12955-018-0992-x.

33. Levy BD, Chish CB, Schmidt B, Gronert K, Serhan CN. Lipid Mediator Class Switching During Acute Inflammation: Signals in Resolution. *Nat Immunol* (2001) 2:612–6. doi: 10.1038/89759.

32. Crovace A, Lacitignola L, Rossi G, Francioso E. Histological and Immunohistochemical Evaluation of Autologous Cultured Bone Marrow Mesenchymal Stem Cells and Bone Marrow Mononucleated Cells in Collagenase-Induced Tendinitis of Equine Superficial Digital Flexor Tendon. *Vet Med Int* (2010) 2010:250978. doi: 10.4061/2010/250978.

31. Croasdell A, Duffney PF, Kim N, Lacy SH, Sime PJ, Phipps RP. Ppar Signaling of Macrophages Populations Reveals Marked Diversity in Expression With Critical Roles in Resolution of Inflammation and Airway Immunity. *J Immunol* (2012) 189:2614–24. doi: 10.4049/jimmunol.1200495.

30. Barussi FC, Bastos EZ, Leite LM, Fragoso FY, Senegal AC, Brofman PR, et al. Intratracheal Therapy With Autologous Bone Marrow-Derived Mononuclear Cells Reduces Airway Inflammation in Horses With Recurrent Airway Obstruction. *Respir Physiol Neurobiol* (2016) 232:35–42. doi: 10.1016/j.resp.2016.07.002.

29. Chu CR, Fortier LA, Williams A, Payne KA, McCarrel TM, Bowers ME, et al. Minimally Manipulated Bone Marrow Concentrate Compared With Microfracture Treatment of Full-Thickness Chondral Defects: A One-Year Study in an Equine Model. *J Bone Joint Surg* (2018) 100:138–46. doi: 10.2106/JBJS.17.00132.

28. Crovace A, Lacitignola L, Rossi G, Francioso E. Histological and Immunohistochemical Evaluation of Autologous Cultured Bone Marrow Mesenchymal Stem Cells and Bone Marrow Mononucleated Cells in Collagenase-Induced Tendinitis of Equine Superficial Digital Flexor Tendon. *Vet Med Int* (2010) 2010:250978. doi: 10.4061/2010/250978.
Mitochondrial Fatty Acid Oxidation in Human Atrial Myocardium via Pparα Activation? Antioxid Redox Signal (2014) 21:1156–63. doi: 10.1089/ars.2013.5868

103. Hernández-Agulera A, Rull A, Rodríguez-Gallo E, Riera-Borrull M, Luciano-Mateo F, Camps J, et al. Mitochondrial Dysfunction: A Basic Mechanism in Inflammation-Related Non-Communicable Diseases and Therapeutic Opportunities. Mediat Inflamm (2013) 2013:135698–8. doi: 10.1155/2013/135698

104. Raoof R, van der Vlist M, Willemen HLDM, Prado J, Versteeg S, Vos M, et al. Macrophages Transfer Mitochondria to Sensory Neurons to Resolve Inflammatory Pain. bioRxiv (2020) 2020.02.12.940445:1–32. doi: 10.1101/2020.02.12.940445

105. Ramond E, Janet A, Courreuil M, Charbit A. Pivotal Role of Mitochondria in Macrophage Response to Bacterial Pathogens. Front Immunol (2019) 10. doi: 10.3389/fimmu.2019.02461

106. Cao X, Yang S, Cao C, Zhou YJ. Harnessing Sub-Organelle Metabolism for Biosynthesis of Isoprenoids in Yeast. Synth Syst Biotechnol (2020) 5:179–86. doi: 10.1016/j.sysbio.2020.06.005

107. Xuan W, Song D, Yan Y, Yang M, Sun Y. A Potential Role for Mitochondrial DNA in the Activation of Oxidative Stress and Inflammation in Liver Disease. Oxid Med Cell Longev (2020) 2020:5835910. doi: 10.1155/2020/5835910

108. Heming M, Gran S, Jauch S-L, Fischer-Riepe L, Russo A, Klotz L, et al. Peroxisome Proliferator-Activated Receptor-γ Modulates the Response of Macrophages to Lipopolysaccharide and Glucocorticoids. Front Immunol (2018) 9:983–3. doi: 10.3389/fimmu.2018.00893

109. Lee YJ, Kim BM, Ahn YH, Choi JH, Choi YH, Kang JL. STAT6 Signaling Mediates Pparα Activation and Resolution of Acute Sterile Inflammation in Mice. Cells (2021) 10:501. doi: 10.3390/cells10030501

110. Asada K, Sasaki S, Tuda T, Chida K, Nakamura H. Antiinflammatory Roles of Peroxisome Proliferator-Activated Receptor Gamma in Human Alveolar Macrophages. Am J Respir Crit Care Med (2004) 169:195–200. doi: 10.1164/rccm.200207-740OC

111. Giannakis N, Sansbury BE, Patsalos A, Hays TT, Riley CO, Han X, et al. Dynamic Changes to Lipid Mediators Support Transitions Among Macrophage Subtypes During Muscle Regeneration. Nat Immunol (2019) 20:626–36. doi: 10.1038/s41590-019-0356-7

112. Prein C, Beier F. PGC1α is Required for Chondrocyte Metabolism and Cartilage Homeostasis. Osteoarthritis Cartilage (2020) 28:525–6. doi: 10.1016/j.joca.2020.02.040

113. Xaus J, Comalada M, Barrachina M, Herrero C, Gofalons E, Soler C, et al. The Expression of MHC Class II Genes in Macrophages Is Cell Cycle Dependent. J Immunol (2000) 165:6364–71. doi: 10.4049/jimmunol.165.11.6364

114. McCubrey JA, May WS, Duronio V, Mufson A. Serine/threonine Phosphorylation in Cytokine Signal Transduction. Leukemia (2000) 14:9–21. doi: 10.1038/sj.leu.2401657

115. Takekeda T, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, et al. Essential Role of Stat6 in IL-4 Signalling. Nature (1996) 380:627–30. doi: 10.1038/380627a0

116. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic Responses in Mouse Models Poorly Mimic Human Inflammatory Diseases. Proc Natl Acad Sci USA (2013) 110:3507–12. doi: 10.1073/pnas.1222878110

117. Karagiannis AE, Kapetanovic R, Summers KM, McGregor BC, Hume DA, Pirie RS. Comparative Transcriptome Analysis of Equine Alveolar Macrophages. Equine Vet J (2016) 49:375–82. doi: 10.1111/eij.12584

118. McIvor C, Beier F. PGC1α is Required for Chondrocyte Metabolism and Dynamic Changes to Lipid Mediators Support Transitions Among Macrophage Subtypes During Muscle Regeneration. Nat Immunol (2019) 20:626–36. doi: 10.1038/s41590-019-0356-7

119. Goncarov V, Jakobson B, Blums K, Briede I, Patetko L, Erglis K, et al. The Comparison of Knee Osteoarthritis Treatment With Single-Dose Bone Marrow-Derived Mononuclear Cells vs. Hyaluronic Acid Injections. Med (Kaunas Lithuania) (2017) 53:101–8. doi: 10.1016/j.med.2017.02.002

120. Stables MJ, Shah S, Camon EB, Lovering RC, Newson J, Bystrom J, et al. Transcriptomic Analyses of Murine Resolution-Phase Macrophages. Blood (2011) 118:e192–208. doi: 10.1182/blood-2011-04-345330

121. Pietras EM, Mirantes-Barbeito C, Fong S, Loeffler D, Kovtonyuk LV, Zhang S, et al. Chronic Interleukin-1 Exposure Drives Haematopoietic Stem Cells Towards Precocious Myeloid Differentiation at the Expense of Self-Renewal. Nat Cell Biol (2016) 18:607–18. doi: 10.1038/ncomms13346

122. Sergijenko A, Roelofs AJ, Riemen AHK, De Bari C. Bone Marrow Contribution to Synovial Hyperplasia Following Joint Surface Injury. Arthritis Res Ther (2016) 18:166–6. doi: 10.1186/s13075-016-1060-8

123. Rynaczar RE, Anasetti C. Expression of CD86 on Human Marrow CD34(+) Cells Identifies Immunocompetent Committed Precursors of Macrophages and Dendritic Cells. Blood (1998) 91:3892–900. doi: 10.1182/blood.V91.10.3892.3892_3900

124. St Clair EW. Interleukin 10 Treatment for Rheumatoid Arthritis. Ann Rheum Dis (1999) 58:1102. doi: 10.1136/ard.58.2008.1102

125. Olingy CE, San Emeterio CL, Ogle ME, Krieger JR, Bruce AC, Pfau DD, et al. Non- Classical Monocytes are Biased Progenitors of Wound Healing Macrophages During Soft Tissue Injury. Sci Rep (2017) 7:447. doi: 10.1038/s41598-017-00477-1

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