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Infant Alveolar Macrophages Are Unable to Effectively Contain Mycobacterium tuberculosis

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INTRODUCTION

Alveolar macrophages (AMφs) are long-lived, tissue-resident phagocytes, originating from fetal monocytes colonizing the airways in the first days of life (1, 2). Their occasional replenishment in the alveolar airways by peripheral monocytes in the steady state is accelerated following inflammatory necrosis (3). AMφs are trained by the lung microenvironment and their inflammatory responses are restrained to avoid inappropriate activation by harmless particulate matter and commensal microbes (2, 4). A number of pathogenic microorganisms exploit the AMφ niche (5) including Mycobacterium tuberculosis (Mtb) (6).

Infants are particularly vulnerable to severe tuberculosis (TB), such as TB meningitis and miliary TB (7). Studies performed prior to the availability of effective antibiotics against Mtb demonstrated that bacillary dissemination occurs in approximately one third of infants, compared with only one in 20 older children and adults (7). Histology from these studies showed that infants with untreated TB exhibit dysfunctional granuloma formation and failure to control mycobacterial replication in the lung (8, 9). However, the facets of infant immunity responsible for these phenomena have not yet been elucidated.
An incomplete understanding of mycobacterial immunity has also hampered delivery of an effective vaccine against tuberculosis. Current candidate vaccines enhance antigen-specific T-cell immunity and IFNγ production (10), however Mtb disseminates before this develops (11). Animal studies have shown that Mtb-infected AMφs are important in early bacillary containment, by initiating granuloma formation in the lung interstitium through the recruitment of other mononuclear cells (12–14). Human studies of primary AMφs have demonstrated that these cells exhibit a unique transcriptional and functional response to Mtb, distinct from model systems of human macrophage immunity (15–17). No studies to date have examined Mtb control in primary AMφs from human infants, predominantly due to difficulties in their sampling. We developed a method for obtaining AMφs from infants without active clinical lung inflammation or infection and used this cellular source to show that infant AMφs exhibit a multifaceted dysfunctional response to Mtb, including diminished ability to control Mtb replication. These data suggest that infant AMφs are less able to contain the replication and spread of Mtb. This may relate to the lack of exposure to microenvironmental signals required to drive development of innate mycobacterial immunity in the infant (4, 18), and provides a potential approach to therapeutically train AMφs in this vulnerable age group.

METHODS

Human Subjects and Samples

The research protocol was approved by the National Health Service Research Ethics Committee (Reference 14/SW/0100 and 15/NW/0409). Written informed consent was obtained from adult participants and the legal guardians of infant participants in accordance with the Declaration of Helsinki. Bronchoalveolar lavage (BAL) with upto 20 mL 0.9% saline was performed on infants undergoing rigid bronchoscopy for suspected airway abnormalities at Royal Manchester Children’s Hospital, using a ventilating Storz bronchoscope under sevoflurane inhalational anesthesia. In adults, BAL was performed with upto 50 mL 0.9% saline during flexible bronchoscopy for investigation of persistent abnormalities at Royal Manchester Children’s Hospital, using a saline during flexible bronchoscopy for investigation of persistent abnormalities at Royal Manchester Children’s Hospital, using a saline during flexible bronchoscopy for investigation of persistent abnormalities at Royal Manchester Children’s Hospital, using a saline during flexible bronchoscopy for investigation of persistent abnormalities at Royal Manchester Children’s Hospital, using a saline during flexible bronchoscopy for investigation of persistent abnormalities at Royal Manchester Children’s Hospital, using a saline during flexible bronchoscopy for investigation of persistent abnormalities at Royal Manchester Children’s Hospital, using a saline during flexible bronchoscopy for investigation of persistent abnormalities at Royal Manchester Children’s Hospital, using a saline during flexible 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Flow Cytometry
Cells were incubated at 4°C for 20 min with Live/Dead stain (Zombie UV Fixable Viability Kit, BioLegend) and mouse serum (Sigma-Aldrich). After washing with PBS, cells were incubated at 4°C for 20 min with the appropriate antibody cocktail (Panel 1: CD40 BV785, TLR2 AF700, CD200R AF647, CD119 APC-Vio770, CD3 FITC, CD19 FITC, CD56 FITC, CD66b FITC, CD235a FITC, CD64 PE-Cy7; or Panel 2: Sirpa APC, HLA-DR AF700, CD206 APC-Cy7, CD3 FITC, CD19 FITC, CD56 FITC, CD66b FITC, CD235a FITC, CD64 PE-Cy7), before washing and fixation with 3.7% paraformaldehyde at room temperature. Data were acquired on a BD Fortessa cytometer (BD Biosciences). In all experiments, single stain controls were prepared using compensation beads (OneComp eBeads, Fisher Scientific) and were used to standardize voltage settings. At least 50,000 cells were acquired from macrophage samples. Samples were analyzed after compensation was set using FlowJo (Version 10.3, Tree Star), and gating to determine percentage positive expression was determined using the fluorescence-minus-one principle.

Cytokine Bead Array
Culture supernatant was sterilized by 0.22 µm cellulose acetate membrane centrifuge tube filtration (Corning Inc.) before removal from Biosafety Level 3 conditions and storage at −80°C. After thawing, soluble inflammatory mediator production was quantified by multiplex cytokine bead array as per the manufacturer's instructions (Soluble Protein Human Flex Set, BD Biosciences). Briefly, supernatants were incubated with cytokine detection beads alongside a phycoerythrin-conjugated detection protein. Recombinant cytokine was produced to analyze a standard curve to fit the measurements of supernatant samples, with a lower limit of detection was 20 pg/mL for all cytokines. To allow sample measurement to fall within upper limit of detection for TNFα, IL6, IL8, and CCL4, it was necessary to pre-dilute supernatants 50-fold with assay buffer. Samples were acquired on a BD FACSVersus system, and data analyzed using FCAP Array (Version 3.0, Soft Flow Inc.).

RNA Isolation
Lysate from AMφs was stored at −80°C following cell disruption with buffer RLT (QIAGEN) containing 1% β-mercaptoethanol (Sigma-Aldrich). RNA was isolated from lysates using RNeasy Micro Kit (QIAGEN) according to the manufacturer’s instructions. RNA was quantified using a Qubit 2.0 Fluorimeter (Thermo Fisher Scientific). RNA samples were assessed using a 2200 TapeStation (Agilent Technologies) and deemed of acceptable quality if they had an RNA Integrity Number (RIN) of greater than 8.0.

RNA-Seq
The TruSeq® Stranded mRNA assay (Illumina Inc.) was used to generate libraries according to the manufacturer’s protocol. The loaded flow-cell was then paired-end sequenced (76 + 76 cycles, plus indices) on an Illumina HiSeq4000 instrument. Finally, the output data was demultiplexed allowing one mismatch and converted to fastq format by bcl2fastq software (Version 2.17.1.14, Illumina Inc.). The quality of the unmapped paired-end sequences was assessed by FastQC (Version 0.11.7, Babraham Institute). Trimmomatic (Version 0.36) was then used to trim sequence adaptors and low-quality reads. Reads were mapped against the reference human genome (hg38) and counts per gene were calculated using annotation from GENCODE 27 using STAR (Version 2.5.3). The minimum proportion of reads that were uniquely mapped and counted into annotated genes was 80%.

Normalization of uniquely mapped reads was calculated with DESeq2 (Version 1.16.1) using the median of ratios method that accounts for RNA composition and sequencing depth, after which the software performed principal component analysis (PCA) and calculated differential expression using the multiple comparison correction of Benjamini-Hochberg on differentially expressed (DE) genes in which a False Discovery Rate (FDR) <0.05 was considered significant. Gene ontology (GO) enrichment analysis (AmiGO2, Version 2.5) and identification of upstream regulators of gene expression (Ingenuity Pathway Analysis, QIAGEN, Version 44691306) were performed on DE genes with a FDR <0.05 (Fisher's Exact with FDR multiple test correction) and a real fold change >2. Gene set enrichment analysis (GSEA) was performed as described previously (21).

Statistical Analysis
Statistical analysis of in vitro functional data was undertaken using Prism (Version 7.0, GraphPad Software). Parametric distribution of the data was confirmed by the Shapiro-Wilk normality test. The significance levels were set at $p \leq 0.05$ and FDR $\leq 0.05$.

RESULTS
Recovery of Infant Alveolar Macrophages From Non-infamed Infant Lungs
To obtain AMφs from infants without active clinical inflammation we performed bronchoalveolar lavage (BAL) during rigid bronchoscopy for the investigation of suspected airway abnormality. Since BAL is usually performed by flexible
brachyoscopy for the investigation of infection/inflammation (22), this required the development of a method of instilling/recovering saline from the lower respiratory tract (Supplementary Video 1). Using this technique, we obtained BAL from 20 infants. We also performed BAL on 20 adults by flexible bronchoscopy (23). None of the infants had received BCG vaccination, while all adults had received BCG vaccination during childhood/adolescence. None of the participants had a contact history with an individual with Mtb or a family history of Mtb. The QuantiFERON-TB Gold Plus assay that analyses IFNγ release to Mtb-specific antigens was negative (IFNγ < 0.35 IU/L) in all participants. None of the infants had a history of chronic illness, and chronic conditions in adults included hypertension in 8/20 (40%), hypercholesterolemia in 6/20 (30%), ischemic heart disease in 4/20 (20%), type 2 diabetes mellitus in 4/20 (20%) and breast cancer in remission in 2/20 (10%). In adults, 8/20 (40%) had never smoked, 6/20 (30%) identified as an ex-smoker and 6/20 (30%) were current smokers. Procedures were well tolerated by all participants. Macrophage viability was >98% in all samples as assessed by trypan blue exclusion. Due to limited cell numbers, each sample was only able to contribute to one experiment (Supplementary Table 1).

**Infant and Adult AMφs Express Similar Levels of M1/M2 Activation Markers**

AMφs are known to express a unique combination of phenotypic markers due to the influence of the airway microenvironment (2), but few reports have described the immunophenotype of human infant AMφs (23). We measured surface marker expression of AMφs from seven infants and seven adults (Figure 1). There was no significant difference in the expression of markers reflecting classical M1-activation relevant to antimycobacterial function: TLR2 (mycobacterial recognition), HLA-DR (antigen presentation) (24), CD40 (co-stimulation) (25) and IFNGR1 (activation) (26). Similarly, there was no significant difference in markers indicating alternative M2-activation: CD200R, Sirpα and CD206 (2).

**Impaired Mtb Control and Altered Chemokine Production by Infant AMφs**

To compare the ability of infant vs. adult AMφs to restrict bacillary replication, we optimized a reporter assay that uses the autoluminescent strain Mtb-LuxG13 for use with primary human AMφs (Supplementary Figure 1). Mtb-LuxG13 is produced by transformation of *M. tuberculosis* H37Rv with bacterial luciferase, which confers only live bacilli with the ability to generate light (20). Bacterial autoluminescence correlated with CFU in liquid broth culture and following infection of AMφs (Supplementary Figures 1A–D). Infection of AMφs with a MOI 10:1 was associated with a higher autoluminescence (reflective of bacillary load) compared with MOI 5:1, despite a similar proportion of phagocytosed bacilli and replication rate, which was estimated from the fold-change in autoluminescence (Supplementary Figures 1E,F). Consistent with its putative biological function in mouse macrophages (5), treatment with exogenous IFNγ was associated with reduced bacillary replication in AMφs infected with Mtb-LuxG13, compared with untreated cells (Supplementary Figure 1G). Therefore, our assay yielded accurate and reliable measurements of mycobacterial phagocytosis and capacity to restrict mycobacterial replication by human AMφs.

Using this assay, we found no significant differences in the phagocytosis of Mtb-LuxG13 by AMφs from seven infant vs. seven adult participants (Figure 2A). However, the fold-change in mycobacterial autoluminescence (reflective of mycobacterial replication) was significantly higher in infant AMφs at 24 h (p < 0.0001) and 48 h (p < 0.0001) post-infection, compared with adult equivalents (Figure 2B). This difference was associated with an extended lag prior to mycobacterial replication in adult AMφs in the first 24 h post-infection, with an estimated mean doubling time of 58 h in adult AMφs, compared with 18 h in infant AMφs. In addition, infant AMφs produced significantly more CXCL8 (q = 0.007) in culture supernatants at 24 h post-infection, but significantly less CXCL9 (q = 0.007) compared with adult AMφs (Figure 2C).

**Transcriptional Disparity Between Mtb-Stimulated Infant vs. Adult AMφs**

To define why infant AMφs were less capable of controlling Mtb replication, we performed two RNA-Seq experiments to compare gene expression of: (i) AMφs from four infants and four...
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FIGURE 1 | Infant and adult AMs have a similar immunophenotype. (A) Representative flow cytometry plots showing gating strategy to differentiate AMs based on their high autofluorescence (in the 488 530/30 FITC channel) and high CD64 expression, from lineage positive cells (CD3+ T cells, CD19+ B cells, CD56+ NK cells, CD66b+ granulocytes, and CD235a+ erythrocytes) and monocytes (low autofluorescence in 488 530 /30 FITC channel, lineage negative and CD64+). (B) Flow cytometry of AMs from seven infant BAL samples (blue representative histogram) compared with seven adults (red representative histogram), with fluorescence minus one (gray representative histogram). Line represents median. MFI, mean fluorescence intensity.

adults following in vitro infection with M. tuberculosis H37Rv for 24 h; and (ii) freshly isolated AMs following 1 h adherence four infants and four adults.

There were 768 significantly differentially expressed (SDE) genes between infants and adults in the Mtb-stimulated samples (Figure 3A), compared with 591 SDE genes in the freshly isolated samples (Figure 3B). Consistent with this, the second principal component (25% variance) separated Mtb-stimulated samples from infant and adult participants (Figure 3A), but there was no infant vs. adult separation in a separate PCA of the freshly isolated samples (Figure 3B). Evaluation of SDE genes in Mtb-stimulated infant vs. adult AMs using gene ontology (GO) enrichment analysis revealed over-representation of genes involved in cellular processes and components relevant to mycobacterial immunity (Figure 3C).

Polarized Gene Expression Affecting Specific Functional Pathways in Mtb-Infected AMs

Mtb-stimulated infant AMs exhibited lower expression of genes that promote lysosomal maturation and mycobactericidal
activity in comparison with adult equivalents (Figure 4A). Consistent with the established role of IFNγ in initiating a transcriptional program that results in mycobacterial killing, we found that Mtb-stimulated infant AMφs also exhibited lower expression of genes involved the cellular response to IFNγ, including JAK2 and STAT1 (Figure 4A). Congruent with this, IPA Upstream Regulator analysis predicted that inhibition of IFNγ in infant AMφs was the most statistically significant (overlap p-value = 1.03E-51, activation z-score = -2.8) upstream factor responsible for the pattern of Mtb-stimulated SDE genes that we had observed.

We also examined all SDE genes encoding chemokines and found that Mtb-stimulated infant AMφs exhibited a lower expression of all genes encoding mononuclear chemoattractants compared with adult equivalents (Figure 4B). In contrast, Mtb-stimulated infant AMφs also displayed higher expression of all genes encoding neutrophil chemoattractants compared with adult equivalents (Figure 4B). Of the chemokines measured in culture supernatants, a similar trend was observed for CXCL8 which was higher in infants, and CXCL9 which was lower in infants (Figure 2C and Supplementary Figure 2).

Overall the infant vs. adult gene expression differences in Mtb-stimulated AMφs (lysosome function, mycobactericidal activity, response to IFNγ and chemokine expression) appeared relatively specific, because there was no clear infant vs. adult pattern of gene expression among enriched GO terms for other important antimycobacterial functions such as the regulation of innate cytokine production (IFNγ, IL12, TNF, IL6, and IL1β) and cell death (Supplementary Figure 3A). There was also no clear infant vs. adult pattern of
expression of genes encoding Mtb phagocytosis receptors (Supplementary Figure 3B) (29–31).

We also tested whether the gene expression exhibited by Mtb-infected infant or adult AMs may be concordant with previously published transcriptomic studies of Mtb-stimulated macrophages (27, 28), using Gene Set Enrichment Analysis (GSEA). We found significant overrepresentation of genes that have previously been associated with infection of AMs with Mtb H37Rv vs. the avirulent strain H37Ra \( (p = 0.03) \) (Figure 4C, Supplementary Table 2) as well as genes associated with severe clinical TB \( (p = 0.048) \) (Figure 4D, Supplementary Table 3) in our transcriptomic data of Mtb-infected infant AMs, relative to adult equivalents.

We then examined RNA-Seq data from the freshly isolated samples to ascertain whether differential infant vs. adult gene expression in the steady state condition might predict the differences that we had observed in the Mtb-stimulated samples. Of the 13 SDE genes encoding chemokines in the Mtb-stimulated samples, two neutrophil chemoattractants \( (CXCL1 \) and \( CXCL2) \) were significantly more highly expressed in freshly isolated infant AMs compared with adult equivalents. Of the 26 Mtb-stimulated SDE genes involved in IFNγ signaling, lysosome
function and mycobactericidal activity, none were differentially expressed in freshly isolated infant vs. adult AMφs. Therefore, the infant vs. adult differences in gene expression observed in Mtb-stimulated AMφs were not observed in the steady state condition.

We also undertook GO enrichment analysis of the SDE genes from freshly isolated samples to identify other areas of cellular functioning that may differ between infant and adult AMφs in steady state conditions. This unexpectedly revealed significant enrichment (FDR 4.78E-07) of genes involved in regulation of cell cycle, in which freshly isolated infant AMφs exhibited higher expression of genes involved in both the positive and negative regulation of cell cycle, compared with adult equivalents (Supplementary Figure 4).

**DISCUSSION**

We provide the first clinically relevant comparison of infant vs. adult human AMφs. Following Mtb infection, infant AMφs were less able to restrict mycobacterial replication, despite similar degrees of bacterial phagocytosis. They also exhibited reduced expression of genes involved in lysosome function, mycobactericidal activity and response to IFNγ. Furthermore, Mtb-stimulated infant AMφs also exhibited lower expression of
chemokines that recruit mononuclear cells and exhibited higher expression of chemokines that recruit neutrophils. Our results are consistent with historical autopsy studies of untreated infants with TB, in whom granulomas are characterized by bacillary outgrowth, fewer mononuclear cells and increased neutrophils (8, 9). The clinical relevance of our data is further illustrated by enrichment of a previously described set of genes associated with disseminated TB in the transcriptome of our Mtb-stimulated infant AMφs (28).

The key role of IFNγ in mycobacterial immunity has been defined in part by monogenic defects in the “IFNγ/IL12 circuit” that cause susceptibility to avirulent mycobacterial infection, collectively termed Mendelian Susceptibility to Mycobacterial Disease (32). Functionally, ligation of the IFNγ receptor of Mtb-infected murine macrophages results in antimycobacterial effector action such as ROS production, phagolysosome maturation, autophagy and cytokine/chemokine production (5). However, few previous studies have tested the effect of exogenous IFNγ on Mtb replication in human primary AMφs (15, 33). Using an autoluminescent reporter assay, we demonstrate a reduced rate of Mtb replication in IFNγ-treated AMφs from adult participants. Importantly, our data also show that infant AMφs may be intrinsically less responsive to IFNγ compared with adult equivalents, and that IFNγ is a master regulator of the transcriptional differences observed. The unresponsiveness of infant AMφs to IFNγ might be caused by their lack of exposure to viral infections, which have been shown to drive training and “innate macrophage memory” through CD8+ T cell-mediated priming of AMφs by IFNγ (4). Infant AMφs also exhibited lower expression of the IFNγ-stimulated gene (ISG) CYBB, which encodes a membrane-bound subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system responsible for ROS production, mutations of which are associated with susceptibility to mycobacterial infection (34). In addition, infant AMφs exhibited lower expression of genes involved in antimycobacterial phagosome maturation (DRAM2 and UVRAG) that are not known to be ISGs (35). Alongside this, infant AMφs exhibited lower expression of genes involved in lysosome functioning (ACP5 and FUCAL), including cholesterol breakdown (LIPA) and efflux (ABCA2) (36–38). Mtb preferentially utilizes host cholesterol as a fuel source and reduced cholesterol breakdown may lead to intracellular persistence of bacteria (39). Furthermore, lysosomal dysfunction is associated with impaired macrophage migration (40), which may lead to poorer containment of Mtb (14).

As the first cells to be infected by Mtb, AMφs are key producers of chemokines (5). Recruitment of mononuclear cells is non-redundant in mycobacterial immunity and granuloma formation (41). For example, Ccr2-/- and Cxcr3-/- mice exhibit decreased monocyte and lymphocyte recruitment and dysfunctional granuloma formation (42, 43). In particular, chemokines that signal through CXCR3 (CXCL9, CXCL10, CXCL11) (41). We also show that infant AMφs display higher expression of genes encoding chemokines that attract neutrophils through CXCR1 and CXCR2 (CXCL1, CXCL2, CXCL5, CXCL6, PPBP, CXCL8). Neutrophil accumulation may be detrimental, as demonstrated by observations of Mtb-infected necrotic neutrophils promoting mycobacterial outgrowth, and improved survival of Mtb-infected mice if neutrophil infiltration is inhibited (44, 45). Vulnerability to severe TB and dysfunctional granuloma production in infants may therefore partly occur through disordered chemokine production by their AMφs.

We found that unstimulated infant AMφs exhibited higher expression of genes involved in both the positive and negative regulation of cell cycle compared with adult equivalents. Early childhood is a period of rapid structural change in the lung, including exponential increase in the number of alveoli (46). Investigators estimate that each alveolus contains up to five AMφs (47), and so the dominance of cell cycle associated genes may reflect macrophage expansion to fill the developing niche.

The main limitation of this study is the small number of participants. A greater number of participants may help with understanding the variability within the infant and adult participant groups. Adult participants should ideally be relatively young, as macrophage function may be impaired in older adults. Furthermore, we cannot rule out if macrophage function was confounded in some participants by exposure to cigarette smoke, inhalational anesthesia, or gastro-esophageal reflux which commonly co-exists with laryngomalacia (48–50). Another potential source of confounding in our study was that adults, but not infants, had received BCG vaccination, which has been shown to educate hematopoietic stem cells to produce trained monocytes and macrophages (51). Despite these limitations we were able to elicit statistically significant functional and transcriptomic differences between infant and adult AMφs. Now that feasibility of sampling is demonstrated, our data should prompt future studies that comprehensively compare infant and adult lung microenvironments, in particular factors known to affect early control of Mtb infection such as interstitial macrophages, dendritic cells, alveolar epithelial cells, respiratory microbiota and soluble factors/opsonins (14, 52, 53). Ideally, these studies would also define further the extent of infant AMφ dysfunction in mycobacterial immunity, including areas that we did not specifically assess functionally (e.g., cell death, response to IFNγ, phagolysosomal maturation, autophagy, eicosanoid production) as well as mechanistic studies to better understand the relevance of infant AMφ dysfunction described in our data (e.g., chemokine production) (5). This work should also assess the expression of bacterial virulence factors in Mtb-infected infant AMφ compared with adult equivalents, given our finding that a previously determined transcriptomic signature of mycobacterial virulence was significantly enriched in our Mtb-stimulated infant AMφ transcriptomic data (27). Non-human primate models could explore whether the observed pattern of AMφ dysfunction in the human infant results in delayed recruitment of mononuclear cells, impaired TH1 immunity and granuloma formation, and increased haematogenous spread of bacilli (54).
Novel and fundamental insights into mycobacterial immunity are required to overcome the current impasse in TB vaccination and therapeutics. Taken together, our results provide the first evidence that age-dependent differences in AM function may contribute to clinical vulnerability to TB. Improved understanding of the age-dependent microenvironmental factors that may drive trained immunity may inform the design of novel therapeutics with broad clinical applications against infectious and allergic disease.

DATA AVAILABILITY STATEMENT

The RNA-Seq data for this study can be found in the ArrayExpress database (EMBL-EBI) under accession number E-MTAB-7679.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Health Service Research Ethics Committee (Reference 14/SW/0100 and 15/NW/0409). Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AG, PA, and TH conceived the idea for the study and designed the experiments. JN assisted setting up the infant bronchoalveolar lavage methodology and obtained clinical samples from infant participants. PF-S and JC assisted with initiating the macrophage infection assays. AG collected the samples, performed the experiments, and received assistance from DM and EC. AG analyzed the RNA-Seq experiment with assistance from IP. AG wrote the first draft of the manuscript. All authors contributed to critical review of the manuscript and approved the manuscript for submission.

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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.2020.00486/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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