

**CONTROLLING DENDRITIC CELL FUNCTION BY TARGETING  
IRON-MODULATING PROTEINS IDENTIFIED THROUGH  
TRANSCRIPTIONAL ANALYSIS**

by

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# CONTROLLING DENDRITIC CELL FUNCTION BY TARGETING IRON-MODULATING PROTEINS IDENTIFIED THROUGH TRANSCRIPTIONAL ANALYSIS

Anna Selene Roessing, M.S.

University of Pittsburgh, 2018

Dendritic cells (DC) are key orchestrators of the body's immune responses. DC control T cell proliferation and differentiation while displaying cognate antigen (Ag) to Ag-specific T cells. DC cytokine secretion is central to the differentiation of T cells into distinct, functional subsets. Stimulation with bacteria-derived molecules such as lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR4) leads to interleukin-12 (IL-12) production by DC and supports T helper type 1 (Th1) generation in interacting cluster of differentiation 4 (CD4+) T cells. Fungal interactions with the Dectin-1 receptor yields IL-6, IL-23, and IL-1 $\beta$  production by DC to mediate Th17 cell polarization. DC, especially those exposed to IL-33, support the generation of IL-5- and IL-13-secreting Th2 cells. However, there are no identified DC-produced cytokines which have been shown to directly underlie Th2 polarization.

The aim of this project was to identify novel genes and signaling pathways by which CD4+ T cell polarization is directed following DC TLR4/ST2 (IL1RL1) ligation. Our preliminary data demonstrated that IL-33 and LPS stimulate the expression of several iron-modulating proteins in DC, such as lipocalin 2 (Lcn2), hemoglobin- $\alpha$ , and cytochrome b5 reductase 3 (Cyb5R3), which act as a previously unappreciated DC nitric oxide (NO) sequestration system. **Given these findings, we hypothesized that TLR4/ST2 ligation on DC causes expression of iron-modulating proteins which sequester NO.** We found that while TLR4/ST2 receptor ligation on DC induces a NO sequestration system in DC, it involves the

expression of hemoglobin- $\alpha$  and potentially other redundant reductase proteins, but not Cyb5R3. Instead, we determined that Cyb5R3 acts as a regulator of DC stimulatory capacity toward CD4<sup>+</sup> T cells. Specifically, Cyb5R3 functions as a negative regulator of glycolysis. In Cyb5R3-expressing cells, unstimulated and IL-33-stimulated cells favor oxidative phosphorylation (OXPHOS) while LPS stimulation leads a shift towards glycolysis. Finally, Cyb5R3-deficient DC are both more inflammatory and more stimulatory, producing greater amounts of IL-12 and expressing more stimulatory surface markers, ultimately leading to Th1 cell polarization of naïve T cells, especially upon DC stimulation with LPS.

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## **1.0 INTRODUCTION**

### **1.1 DENDRITIC CELL MATURATION AND ACTIVATION**

First identified by Ralph Steinman in 1973 [1], DC are hematopoietic cells that are specialized for antigen presentation and have roles in both innate and adaptive immune responses. They express various pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), and C-type lectins [2]. PRRs bind molecular motifs that are characteristic of particular pathogens or that are associated with cellular damage. Ligation of PRRs induces the maturation of DC, initiating signaling pathways that lead to cellular activation and marked changes in gene expression and metabolism that direct the expression of cytokines, co-receptors, chemokines, and other functional molecules [3]. The change from resting DC to activated DC involves a transition in which the cells become more dendritic in appearance and more secretory, allowing them to become better at interaction with other cells [4]. DC activated by PRRs drive the activation of naïve T cells toward antigen-specific T cells.

Metabolic studies in DC following PRR ligation have shown how their biochemical activity also shapes their maturation and functionality following a shift from resting state to activation. Changes in DC appearance are accompanied by changes in cellular metabolism that are necessary and essential to activation [2]. As an example, DC activation in response to TLR ligation leads to an increase in glucose consumption and lactic acid production due to a rapid

increase in glycolytic flux that occurs within minutes of stimulation by TLR agonists [5]. This glycolytic flux fulfills the need for citrate in activated DC. In DC, the export of citrate from mitochondria into the cytoplasm is especially important for fatty acid synthesis. Fatty acid synthesis is linked to the requirement of activated DC to increase the size of their endoplasmic reticulum (ER) and Golgi apparatus. These organelles are integral in protein synthesis and secretion, producing proteins that are essential to DC activation and cellular interaction [5, 6]. In this way, DC activation and maturation regulate antigen capturing, processing and presentation, and the expression of costimulatory molecules [7].

## **1.2 CD4+ T CELL POLARIZATION AND DIFFERENTIATION**

Once DC are matured and activated, they are able to display the peptide antigens they have generated from processed extracellular proteins on their surface through their major histocompatibility complex (MHC) receptors for potentially responsive T cells. These peptides are presented in major histocompatibility complex class II (MHCII) receptors and allow for peptide inspection through the T cell receptor (TCR) expressed by interacting T cells. At the immune synapse, adhesion molecules allow DC and T cells to come into contact, sequentially triggering TCR stimulation by the MHC-peptide complexes if the TCR is cognate to the complexes [8, 9]. In order for a naïve T cell to polarize towards a CD4+ T cell, the TCR must engage with peptide presented on MHCII as well as direct binding of the CD4 surface marker to the MHC [10]. This CD4-MHCII interaction strengthens the signal between the TCR and the peptide [9]. Co-stimulation occurs through engagement of T cell-expressed CD28 by B7 molecules on DC, amplifying the signaling initiated by the TCR up to 100-fold. Depending on the level of co-stimulation and the amount of peptide presented, between 6 and 30 hours of TCR

stimulation is required for a naïve T cell to become committed to a T helper cell subset [11]. After a cell has committed to a subset and begins division, T cells proliferate rapidly in response to IL-2, which is produced in either a paracrine or autocrine manner by activated T cells. These CD4<sup>+</sup> T cells are now fully functional immune cells of a specific Th subset.

CD4<sup>+</sup> T cells play a critical role in the immune response. They have the capacity to help B cells produce antibodies, to induce macrophages to develop microbicidal activity, to recruit other cells to sites of infection (such as neutrophils, eosinophils, and basophils) and to produce cytokines important for adaptive immune responses [12]. To fulfill these functions, CD4<sup>+</sup> T cells differentiate to become Th1, Th2, Th9, Th17, T regulatory (Treg), and T follicular helper (Tfh) cells, each with a unique cytokine profile that mediates their effector functions. In order for differentiation to occur, activation of a set of cytokine signaling pathways and lineage-specific transcription factors is required [13]. For the purposes of relevance to this discussion, the scope will be limited to Th1 and Th2 differentiation.

IL-12 and interferon- $\gamma$  (IFN- $\gamma$ ) are the critical cytokines to initiate the downstream signaling cascade for Th1 cell differentiation [14]. IL-12 is secreted in large amounts by DC after their activation through their PRRs [15]. The master regulator for Th1 differentiation, T-box transcription factor (T-bet), is crucial, not only for its ability to activate the set of genes to promote Th1, but also for its suppression of the development of opposing cell lineages [16, 17]. T-bet significantly enhances the production of IFN- $\gamma$  and plays an important role in suppressing Th2 differentiation [17]. T-bet suppresses the development of Th2 cells through the inhibition of the IL-4 gene and the Th2 master regulator GATA-binding protein 3 (GATA3) [18, 19].

IL-4 and IL-2 are the critical cytokines for initiating Th2 differentiation. The major transcription factor involved in Th2 differentiation is the IL-4-induced signal transducer and

activator of transcription 6 (STAT6), which is responsible for upregulating the expression of the master regulator GATA3 [20, 21]. While much progress has been made in the field of T cell biology, the exact pathway underlying DC polarization of Th2 cells has yet to be clarified. Therefore, three mechanisms of GATA3 involvement in Th2 differentiation have been theorized. These include 1.) enhanced Th2 cytokine production, 2.) selective proliferation through recruitment of growth factor independent-1 (Gfi-1), and 3.) inhibition of Th1 differentiation [22]. Further research has also suggested the involvement of several other transcriptional factors activated downstream, such as STAT3, STAT5, and suppressor of cytokine signaling-1 (SOCS-1) as well as several cytokines, including IL-2 and IL-6 [23- 26].

### **1.3 EXOGENOUS AND ENDOGENOUS MOLECULES THAT SHAPE DC CAPACITY FOR TH CELL POLARIZATION**

As previously addressed in section 1.1, DC express various PRRs that interact with molecular motifs of cellular and microorganismal origin. The molecules these receptors interact with greatly affect how CD4+ T cells will become polarized. Pathogen-associated molecular patterns (PAMPs) are exogenous molecules derived from microorganisms that are recognized by the PRRs of DC as well as many epithelial cells. In contrast, damage-associated molecular patterns (DAMPs) are endogenous, cell-derived molecules that initiate and perpetuate immunity in response to trauma and tissue damage [27].

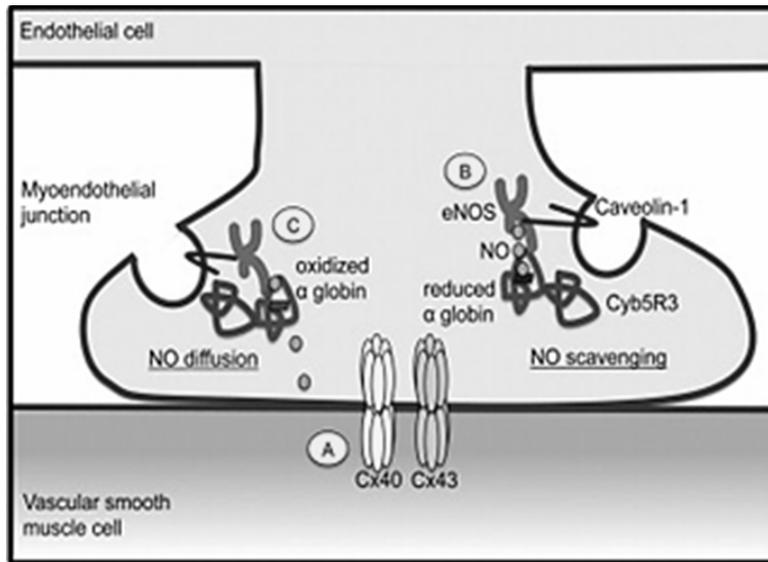
Bacterial and viral motifs are the most common drivers of Th1 responses [28]. Surface membrane-expressed TLRs such as TLR4 and TLR5 recognize PAMPs such as LPS and bacterial flagellin. However, TLR3, TLR7, and TLR9, expressed on endosomal compartments within DC, interact with viral and bacterial nucleic acid PAMPs, such as double-stranded RNA,

single-stranded RNA, and unmethylated double-stranded DNA, respectively [28]. In contrast, Th2 responses have been linked to helminth molecules as well as cell damage motifs. For instance, receptors such as the mannose receptor on DC bind to the PAMP *Schistosoma mansoni* soluble egg antigen (SEA) [28]. Similarly, ST2 binds to the DAMP IL-33, following its release from injured cells. IL-33, and similar motifs released from cells that are normally localized to a cellular compartment under homeostatic conditions, are sometimes referred to as alarmins [29].

IL-33, a member of the IL-1 superfamily of cytokines, is expressed by stromal cells, such as epithelial and endothelial cells, and upregulated following pro-inflammatory stimulation. IL-33 can function as both a traditional cytokine and as a regulatory factor of gene transcription. It mediates its biological effects through receptor interaction with ST2 and IL-1 receptor accessory protein (IL-1RAcP), both of which are widely expressed, particularly by innate immune cells. IL-33 has been shown to strongly induce Th2 polarization and can promote the pathogenesis of Th2-related diseases such as asthma and anaphylaxis. However, IL-33 has also shown various protective effects in cardiovascular diseases such as atherosclerosis, obesity, and type 2 diabetes [30]. Interestingly, both the LPS receptor TLR4 and IL-33 share a common signaling pathway. Under normal physiological conditions, upon activation of a TLR or IL-1R superfamily member, the transmembrane receptor's Toll/Interleukin-1 receptor (TIR) domain dimerizes with the TIR domain of the cytosolic adaptor molecules. This triggers the adaptor proteins myeloid differentiation primary response 88 (MyD88) and IL-1R-associated kinase (IRAK) to activate downstream mitogen-activated protein kinase (MAPK)-kinases through tumor necrosis factor receptor-associated factor 6 (TRAF6) signaling. In turn, this leads to the activation of activator protein 1 (AP-1) through c-Jun N-terminal kinases (JNKs). TRAF6 also activates the inhibitor of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) kinase complex, allowing access to the active form of NF- $\kappa$ B

[31]. Thus, the effects of IL-33 are either pro- or anti-inflammatory, leading to Th1 or Th2 polarization, depending on the PAMPs and DAMPs present in the environment and the interacting PRRs on DC.

#### 1.4 NITRIC OXIDE SEQUESTRATION PATHWAY



**Figure 1. Endothelial Cell Expression of Hemoglobin- $\alpha$  and Cyb5R3 Facilitates NO Scavenging.** In endothelial cells, NO diffusion out of the cell into the myoendothelial junction is controlled by the iron redox state of hemoglobin- $\alpha$ , which is modulated by Cyb5R3. When the iron group bound to hemoglobin- $\alpha$  globin is reduced to  $Fe^{2+}$ , NO is sequestered. However, when the iron group bound to hemoglobin- $\alpha$  is oxidized to  $Fe^{3+}$ , NO diffusion is permitted [32]. This figure illustrates a similarly functioning pathway with fewer proteins than our research has identified in DC.

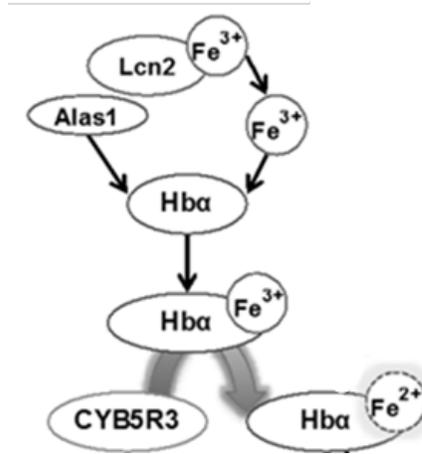
As the NO scavenging pathway to be discussed in this thesis has yet to be described in immune cells, this explanation will pertain to the relevant similarities in protein expression and function published in endothelial cells (EC).

The hemoglobin- $\alpha$  pathway of NO sequestration was first identified in 2012 as a regulator of NO on vascular activity in arterial EC (Figure 1) [33]. While the sequestration

mechanism itself is due to the redox state of the iron group bound to hemoglobin- $\alpha$ , there are upstream proteins involved which must first be activated in order for this downstream NO sequestration to be achieved [34].

The protein acting as the initiator of this pathway in EC, and potentially in immune cells, is Lcn2 (Figure 2). Lcn2 is a secreted protein that sequesters iron from bacterial siderophores and has been shown to act as a regulator of human hematopoiesis [35, 36]. In this pathway, Lcn2 introduces ferric ( $\text{Fe}^{3+}$ ) iron to the system, which signals to aminolaevulinic acid synthase 1 (Alas1). Alas1 is the ubiquitous, rate-limiting enzyme, formed by the condensation of glycine and succinyl-CoA. It has the important housekeeping function of providing heme to non-erythroid tissues [37]. Once it is signaled by Lcn2, Alas1 initiates the production of hemoglobin- $\alpha$ . Hemoglobin- $\alpha$  allows the  $\text{Fe}^{3+}$  introduced by Lcn2 to become a bound iron group (Figure 2). Hemoglobin- $\alpha$  is comprised of two  $\alpha$  and two  $\beta$  globin subunits in a heterocyclic ring of four pyrrole molecules which are known as a porphyrin [38]. The hemoglobin- $\alpha$  tetramer is able to interact with carbon dioxide as well as carbon monoxide [39]. In this pathway, hemoglobin- $\alpha$  is able to sequester NO following the reduction of its  $\text{Fe}^{3+}$  group to ferrous ( $\text{Fe}^{2+}$ ) iron. This sequestration only occurs following the redox activity of Cyb5R3 (Figure 2). Cyb5R3 is a flavoprotein enzyme involved in the transfer of electrons from the physiological electron donor nicotinamide adenine dinucleotide (NADH) through its flavin adenine dinucleotide (FAD) domain. It is also critically important in many oxidation and reduction reactions [40]. For the NO sequestration pathway in EC, Cyb5R3 reduces ferric iron once it is bound by hemoglobin- $\alpha$  [32-34, 41]. This creates a free electron on the ferrous iron group bound to hemoglobin- $\alpha$ , which is more energetically favorable than ferric iron (which lacks a free electron). The free electron allows for iron nitrosylation to occur as the ferrous iron coupled to hemoglobin- $\alpha$  sequesters NO,

preventing its diffusion from the cell [33]. Alternatively, the absence of either hemoglobin- $\alpha$ , for the iron group to be bound, or Cyb5R3, for the ferric iron group to be reduced to ferrous iron, permits free diffusion of NO from the EC.



**Figure 2. Schematic of NO Sequestration Proteins and Activity.** As the pathway has been described in vascular endothelial cells, Lcn2 sequesters iron from bacterial siderophores to bring ferric ( $Fe^{3+}$ ) iron to the hemoglobin pathway. Upon the introduction of ferric iron, the enzyme Alas1 initiates production of hemoglobin- $\alpha$ , the protein which accepts the iron. Once hemoglobin- $\alpha$  accepts ferric iron, it is able to be reduced by Cyb5R3 to ferrous ( $Fe^{2+}$ ) iron. It is ferrous iron that has a free electron, allowing iron nitrosylation which functions as a NO sink.

## 2.0 MATERIALS AND METHODS

### 2.1 MICROARRAY AND DATA ANALYSIS

C57BL/6 mice were used to generate bone-marrow derived DC (BMDC) through a seven-day culture with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), plated at  $4 \times 10^6$  cells per plate. Fresh complete RPMI 1640 media and cytokines were added on days two, four, and six of BMDC culture. On day seven, fifteen C57BL/6 samples with five replicates per treatment were harvested six hours following exposure to media alone, *E. coli*-derived LPS (100 ng/ml), or rIL-33 (20 ng/ml) and used to complete an Illumina BeadArray microarray (MouseWG-6\_Version2), which probed for 45,200 transcripts. The data were then analyzed with GeneSpring (version 14.8). Raw data were baseline transformed to the mean of all samples and averaged over replicates. Gene expression was filtered by percentile (30<sup>th</sup>-100<sup>th</sup>). A One-way ANOVA with Tukey HSD post hoc, asymptotic p-value, and Benjamini-Hochberg FDR correction was used for treatment comparison between gene lists.

All mice were bred and maintained in accordance with the University of Pittsburgh's Department of Laboratory Animal Resources guidelines and all animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

## 2.2 WESTERN BLOTTING

C57BL/6 BMDC (generated as in section 2.1) or Rosa26ER<sup>T2</sup> x Cyb5R3<sup>Fl/Fl</sup> whole splenic protein (from mice which received five days of intraperitoneal tamoxifen injections) was left untreated or stimulated approximately eighteen hours with media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). Samples were then collected by centrifugation at 1500rpm for five minutes, lysed using RIPA buffer, pelleted by centrifugation at 1300rpm for twenty minutes at 4°C, and protein quantified by BCA assay.

Proteins were run on 4-12% Tris-Glycine Mini Gels in an XCell SureLock™ Mini-Cell box at 125V for ninety minutes. The blots were carefully transferred to a PVDF membrane using the XCell II™ Blot Module at 25V for two hours. Membranes were incubated at room temperature in 5% fetal bovine serum (FBS) for one hour to block background and rocked overnight in a cold room with anti-conjugated protein prepared in 5% FBS. The following day, secondary antibodies were added following washes with tris-buffered saline/Tween 20 (TBST) and incubated for one hour. Detection reagents were added following washes with TBST and incubated for five minutes. Proteins were then detected using film.

## 2.3 MIXED LYMPHOCYTE REACTIONS AND ELISAS

C57BL/6 or Rosa26ER<sup>T2</sup> x Cyb5R3<sup>Fl/Fl</sup> mice were used to generate BMDC through a seven-day culture as outlined in section 2.1. When Rosa26ER<sup>T2</sup> x Cyb5R3<sup>Fl/Fl</sup> BMDC were used, 4-hydroxytamoxifen was added to half of the plates, along with fresh media and cytokines, on days two, four, and six of BMDC culture. On day seven, cells were harvested and CD11c bead

purified to isolate DC. DC were either left untreated or stimulated for eighteen hours with LPS (100 ng/ml), or rIL-33 (20 ng/ml) in 24-well plates. The following day, DC were plated in a five-day mixed lymphocyte reaction (MLR) with untouched CD4 bead-purified T cells isolated from Balb/c splenocytes.

CD4<sup>+</sup> T cell cytokine production was measured by Mouse IL-5 ELISA MAX™ and Mouse IFN- $\gamma$  ELISA MAX™ enzyme-linked immunosorbent assays (ELISAs) from the supernatants following protein quantification.

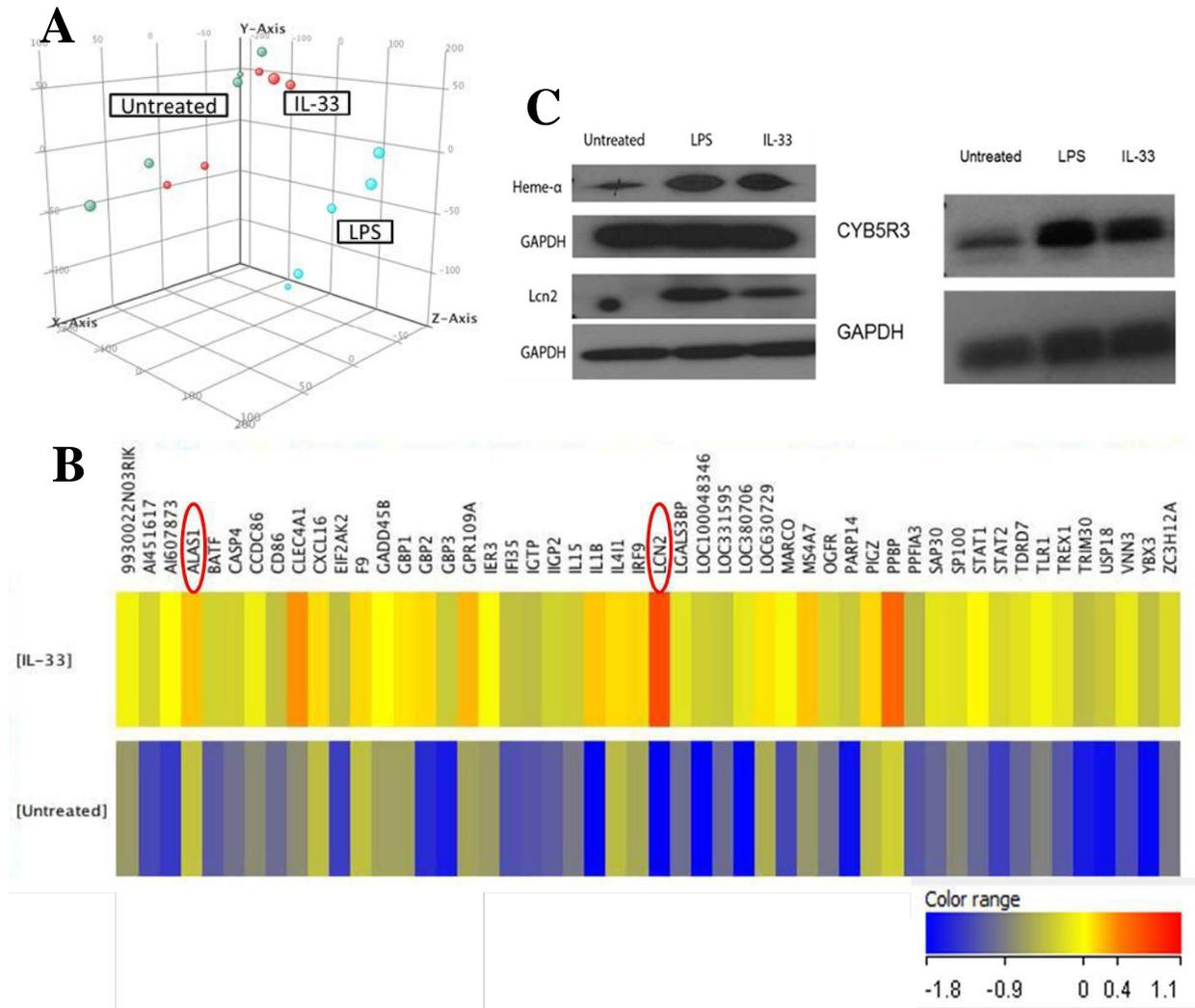
## **2.4 DENDRITIC CELL PHENOTYPING**

Rosa26ER<sup>T2</sup> x Cyb5R3<sup>Fl/Fl</sup> mice were used to generate BMDC through a seven-day culture as outlined in section 2.1. 4- hydroxytamoxifen was added to half of the plates, along with fresh media and cytokines, on days two, four, and six of BMDC culture. On day seven, cells were harvested and CD11c bead purified to isolate DC. DC were either left untreated or stimulated for eighteen hours with LPS (100 ng/ml), or rIL-33 (20 ng/ml) in 24-well plates.

The following day, GolgiPlug™ Protein Transport Inhibitor (containing Brefeldin A) was added to the cells for five hours. DC were collected and stained for the cell surface markers CD11b (V450 (1:300)), CD11c (PE/Cy7 (1:200)), CD80 (APC, (1:300)). CD86 (PerCPCy5.5 (1:300)), MHCII FITC (1:100)), and the cytokine IL-12p40 (PE (1:50) intracellular). Samples were run on a BD LSR II™ Flow Cytometer and analyzed using FlowJo® version 10.

### 3.0 RESULTS AND ANALYSIS

#### 3.1 MICROARRAY ANALYSIS OF STIMULATED DENDRITIC CELLS FOR DIFFERENTIAL GENE EXPRESSION



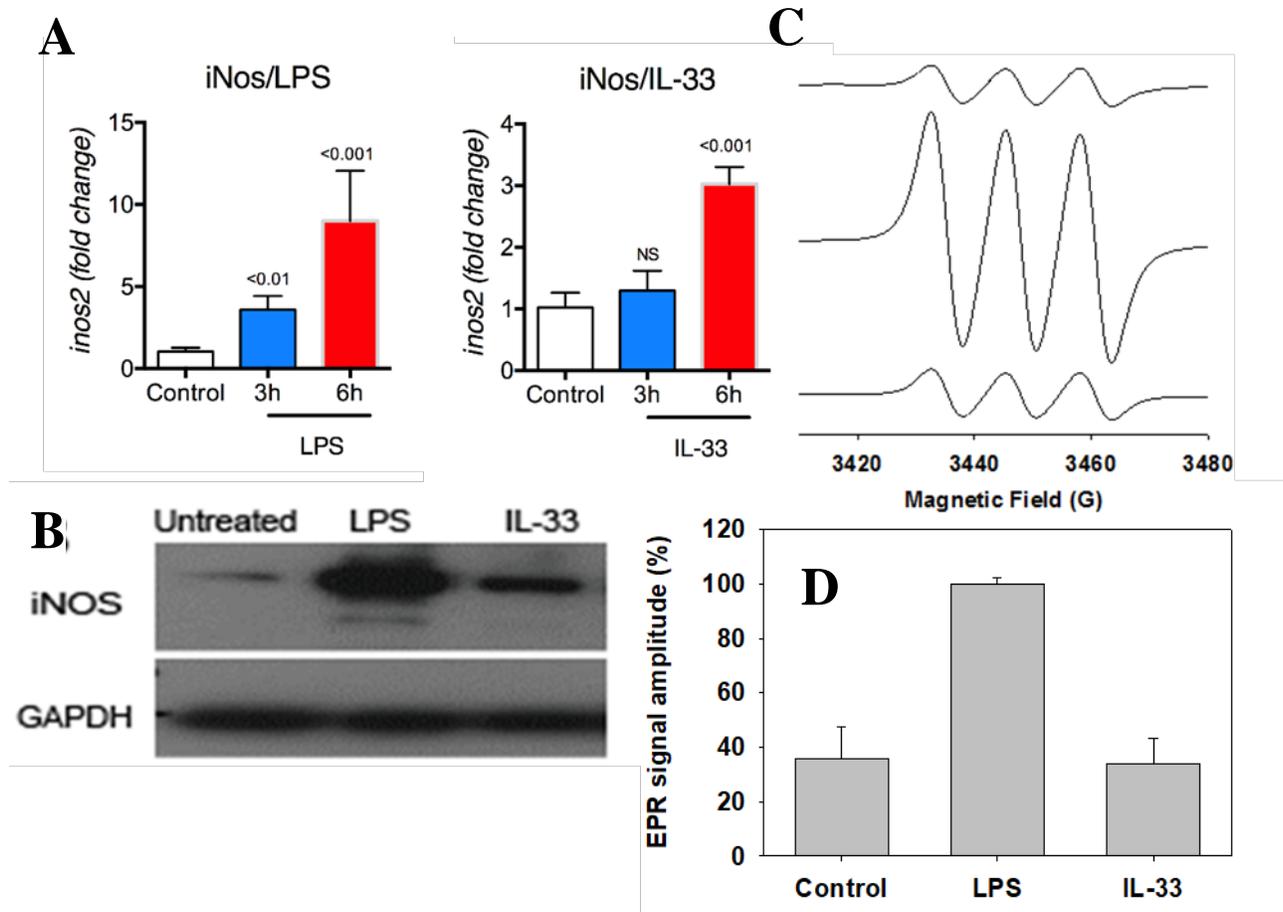
**Figure 3. Microarray Analysis and Western Blotting of Stimulated Dendritic Cells.** **A.** PCA analysis of C57BL/6 BMDC samples following six hours of stimulation in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). n=5 samples per treatment group; 15 total. Data is representative of one experiment. Green= Untreated, Red= rIL-33, Aqua= LPS **B.** Top fifty genes upregulated in fold-change expression analysis of rIL-33 versus Untreated. Genes are listed in alphabetical order from left to right. **C.** Western blot images of C57BL/6 BMDC samples following eighteen hours of stimulation in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). Anti-mouse antibodies were used to probe for hemoglobin-a, Lcn2, and the GAPDH loading control. Anti-goat antibody was used to probe for Cyb5R3.

As previously mentioned in section 1.2, there are no DC-produced cytokines identified which have been shown to directly underlie Th2 polarization of CD4<sup>+</sup> T cells. In our attempts to elucidate this, we have shown that overnight stimulation of C57BL/6 BMDC in media alone, in LPS, or in rIL-33, with subsequent co-culture in an allogeneic MLR yields Th2 polarization by IL-33-stimulated DC and Th1 polarization by LPS-stimulated DC [42]. This led to the observation that, while IL-33- and LPS-stimulated DC are polarizing CD4<sup>+</sup> T cells toward different subsets, they are utilizing the same MyD88 signaling pathway [43]. Thus, we began investigating why TLR4 and ST2 ligation both activate the same MyD88-dependent pathway but generate different functional outcomes through the identification of novel genes.

We began to address this question by looking at C57BL/6 BMDC samples that were harvested six hours following exposure to media alone, LPS, or rIL-33 and completing an Illumina microarray which was then analyzed with GeneSpring. After the normalization and averaging of the data, a principle component analysis (PCA) was used to determine the similarity of the replicate sets. We established that the three treatment groups appeared to represent distinct gene subsets (Figure 3A). Following the PCA, a fold-change expression analysis was completed between the IL-33-stimulated DC and the DC in media alone, specifically looking at those genes upregulated in the IL-33-stimulated DC (Figure 3B). Using Ingenuity<sup>®</sup> Pathway Analysis, the genes with the highest expression above baseline in the IL-33-stimulated DC were analyzed for function. From this, we came to identify Alas1 and Lcn2 as iron-modulating proteins, both of which were found in the NO sequestration pathway involving hemoglobin- $\alpha$  and upregulated by IL-33 stimulation of DC. Since this NO sequestration pathway had only been described in vascular EC, we verified our gene expression data and searched for other proteins of the hemoglobin- $\alpha$  pathway through Western blots of BMDC following eighteen hours of treatment

in media alone, LPS, or rIL-33 (Figure 3C). Notably, the expression of Lcn2, hemoglobin- $\alpha$ , and Cyb5R3 increase with BMDC stimulation by IL-33 and LPS. This suggested that these stimuli are utilizing the same MyD88 signaling pathway in DC to activate these proteins following their receptor ligation and may trigger a potential NO sequestration pathway.

### 3.2 IL-33 INDUCTION OF INOS YIELDS LOWER NITRIC OXIDE SECRETION FROM DENDRITIC CELLS COMPARED TO LPS-STIMULATED DENDRITIC CELLS

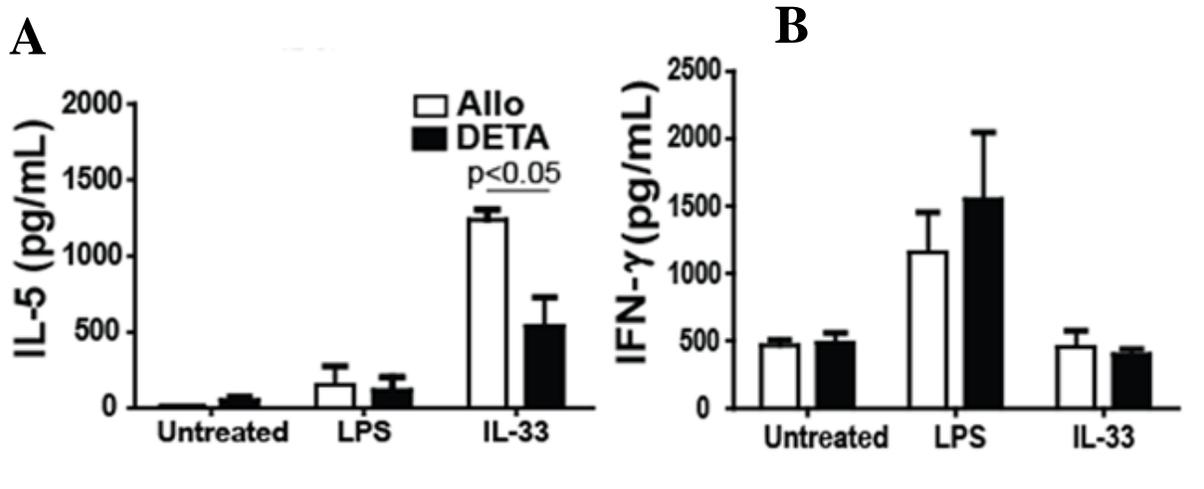


**Figure 4. IL-33 Induces Lower NO Secretion from iNOS Compared to LPS.** **A.** mRNA analysis of iNOS expression C57BL/6 BMDC samples following three or six hours of stimulation in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml) as designated. **B.** Western blot image of C57BL/6 BMDC samples following eighteen hours of stimulation in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). Anti-mouse antibodies were used to probe for iNOS and the GAPDH loading control. This blot is representative of three completed blots. **C.** EPR spectra of NO secretion from whole C57BL/6 BMDC, Top: Control DC, Middle: LPS-stimulated DC, Bottom: IL-33-stimulated DC. **D.** EPR spectra amplitude quantified. Statistical analysis by Student's paired t-test,  $p = 0.029$  (control vs LPS),  $p < 0.001$  (LPS vs IL-33), Control and LPS,  $n = 4-6$ .

Following the identification of these upregulated proteins responsible for the hemoglobin- $\alpha$  NO sequestration system in vascular EC in our DC, we wanted to evaluate the response of the NO-producing, DC protein inducible nitric oxide synthase (iNOS). iNOS is an enzyme stimulated by cytokines and bacterial products in a number of cells, including monocytes and macrophages. It

is one of three isoforms of NOS that are responsible for producing NO in the body [2]. Both the messenger RNA (mRNA) and protein levels from a C57BL/6 mouse showed induction of iNOS at levels roughly three-fold over the control after incubation with rIL-33. This level of expression is lower than that caused by LPS, which was roughly ten-fold over the control (Figure 4A and B). Using electron paramagnetic resonance spectroscopy (EPR), we measured the NO secreted by the DC following overnight stimulation. Interestingly, IL-33 stimulates a comparable amount of NO secretion as DC in media alone when compared to LPS-treated DC (Figure 4C and D). Together, this demonstrated that IL-33 induces iNOS mRNA and protein but does not stimulate increased NO secretion. This also suggested that stimulation of DC with IL-33 does not lead to NO secretion in the canonical iNOS pathway and provided further evidence of an active NO sequestration system activated by ST2 ligation.

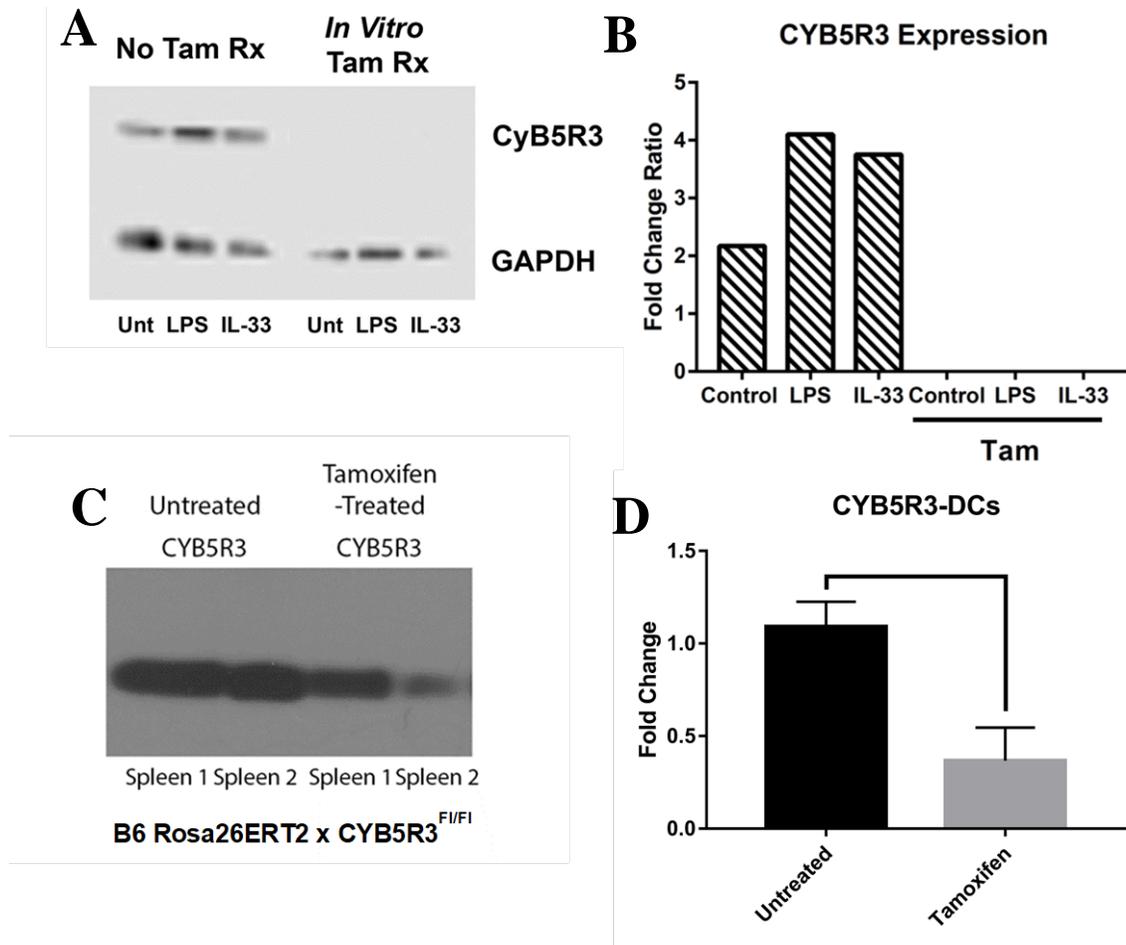
### 3.3 INCREASED NITRIC OXIDE LEVELS MAY LIMIT THE CAPACITY OF DENDRITIC CELLS FOR TH2 POLARIZATION



**Figure 5. Increased NO Levels may Limit DC Th2 Polarization Capacity.** C57BL/6 BMDC were stimulated overnight with media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml) for use in an MLR with bead purified CD4+ Balb/c T cells with or without the exogenous NO donor DETA. Five days later, cytokine production was measured by ELISA. For **A**, the IL-5 response from CD4+ T cells and **B**, the IFN- $\gamma$  response from CD4+ T cells. Statistical analysis by Student's paired t-test.

After understanding the effect of IL-33-stimulation on iNOS and DC NO production, we chose to address how the addition of NO impacted IL-33-mediated DC Th2 polarization. Using C57BL/6 BMDC, stimulating them for eighteen hours in media alone, with LPS, or with rIL-33, and subsequently co-culturing them in an allogeneic MLR with or without the NO donor diethylenetriamine (DETA), we measured cytokine production by ELISA five days later for IL-5 (as a Th2 marker) and IFN- $\gamma$  (as a Th1 marker). The results indicated that the addition of NO dampens the normally robust IL-5 response seen from IL-33-DC-stimulated T cells (Figure 5A). Interestingly, the addition of NO had no effect on IFN- $\gamma$  from LPS-stimulated T cells (Figure 5B). This demonstrated that increased NO levels may limit the ability of IL-33-stimulated DC to mediate Th2 polarization specifically.

### 3.4 MOUSE MODEL FOR THE EFFECTIVE KNOCKDOWN OF CYB5R3 EXPRESSION IN VITRO AND IN VIVO



**Figure 6. Mouse Model is Effective for Knockdown of Cyb5R3 Expression.** **A.** Western blot image of 4-hydroxytamoxifen treated or untreated Rosa26ERT<sup>2</sup> x Cyb5R3<sup>F1/F1</sup> BMDC samples following eighteen hours of stimulation in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). Anti-goat antibody was used to probe for Cyb5R3 and anti-mouse was used for the GAPDH loading control. **B.** Densitometry analysis for Cyb5R3 expression as a fold change ratio normalized to GAPDH. **C.** Western blot image of five-day tamoxifen treated or untreated Rosa26ERT<sup>2</sup> x Cyb5R3<sup>F1/F1</sup> splenocytes following eighteen hours of stimulation in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). Anti-goat antibody was used to probe for Cyb5R3. **D.** qRT-PCR analysis of CD11c bead purified Rosa26ERT<sup>2</sup> x Cyb5R3<sup>F1/F1</sup> BMDC for Cyb5R3 expression. Error expressed as STDEV.

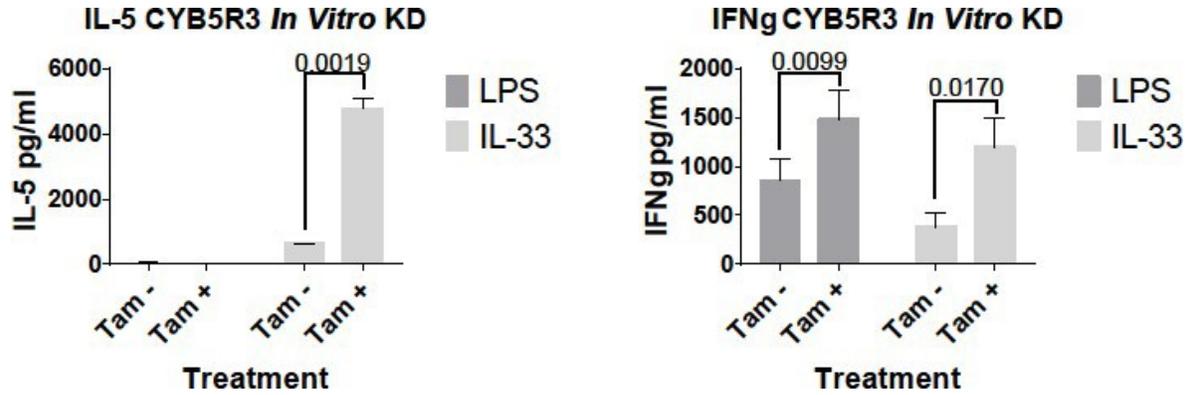
Once it was established that the proteins responsible for the hemoglobin- $\alpha$  NO sequestration pathway were expressed in our DC and the differential effects of IL-33 and LPS stimulation on DC NO secretion and DC function were understood, we realized that in order to best study the pathway, the proteins must be studied individually. Cyb5R3 was chosen as the first protein in the

NO sequestration pathway to evaluate due to its reductase activity, which is vitally important in the pathway (see section 1.3). To target this protein, our collaborator, Dr. Adam Straub of the University of Pittsburgh Vascular Medicine Institute developed a B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J x Cyb5R3<sup>F1/F1</sup> (Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup>) mouse colony. This model was designed as a Tamoxifen-inducible global knockout of Cyb5R3 in all cells.

The *in vitro* efficacy of the model was evaluated by harvesting Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> BMDC, stimulating them overnight in media alone, LPS, or rIL-33, and culturing them in the presence or absence of 4-hydroxytamoxifen to evaluate expression knockdown. Regardless of the presence or absence of LPS or IL-33 DC stimulation, there was no induction of Cyb5R3 expression in the DC treated with 4-hydroxytamoxifen (Figure 6A). This was also evident when quantified as a fold change ratio over glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Figure 6B).

Similarly, to evaluate the *in vivo* efficacy, whole spleens were harvested from untreated Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> mice and Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> mice that were treated for five days with tamoxifen for a whole splenocyte western blot. This illustrated that there is knockdown of Cyb5R3 protein when compared to tamoxifen-untreated spleens (Figure 6C). Splenocytes were also harvested for CD11c purification for quantitative real-time polymerase chain reaction (qRT-PCR) analysis to evaluate expression-level knockdown of Cyb5R3 (Figure 6D). These results demonstrated that there was a significant decrease in the expression of Cyb5R3 following tamoxifen treatment *in vivo* when compared to the control. Together, these results provided evidence that these mice were useful tools for further *in vitro* and *in vivo* studies involving Cyb5R3.

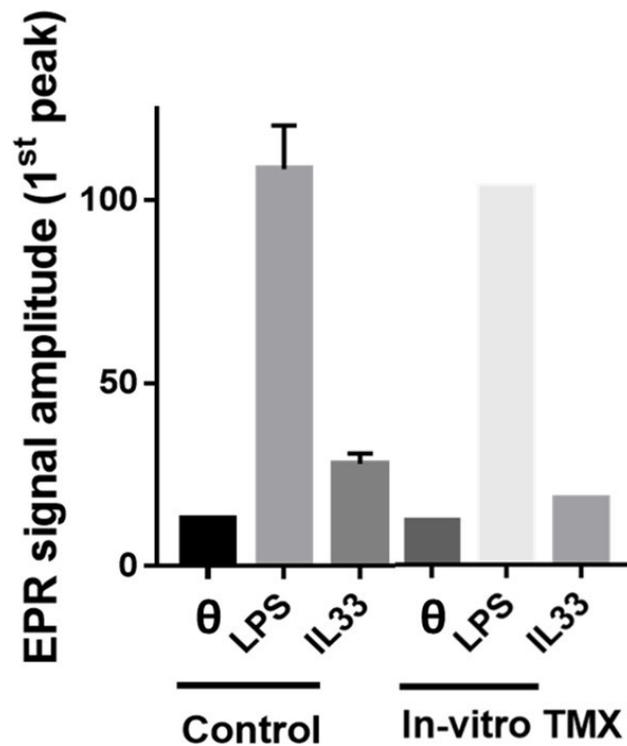
### 3.5 CYB5R3 FUNCTIONS AS A REGULATOR OF DENDRITIC CELL STIMULATORY CAPACITY



**Figure 7. Cyb5R3 regulates DC Stimulatory Capacity.** 4-hydroxytamoxifen treated or untreated Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> BMDC were stimulated overnight with media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml) for use in an MLR with bead purified CD4<sup>+</sup> Balb/c T cells. Five days later, cytokine production was measured by ELISA for the IL-5 and the IFN- $\gamma$  response from CD4<sup>+</sup> T cells. Statistical analysis by Student's paired t-test.

Once it was established that the Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> mice were reliable for studies, BMDC from these mice were treated with 4-hydroxytamoxifen, harvested, stimulated for eighteen hours with LPS or rIL-33, used in an allogeneic MLR, and cytokine production was measured. In the presence of Cyb5R3 in DC, production of IL-5 and IFN- $\gamma$  by T cells following IL-33-DC- or LPS-DC-stimulation is dampened, respectively (Figure 7). However, in the absence of Cyb5R3 in DC, production of IL-5 by T cells following IL-33-DC-stimulation and IFN- $\gamma$  production by T cells following IL-33-DC- and LPS-DC-stimulation is robust (Figure 7). This suggested a previously unappreciated role for Cyb5R3 as a regulator of DC stimulatory capacity.

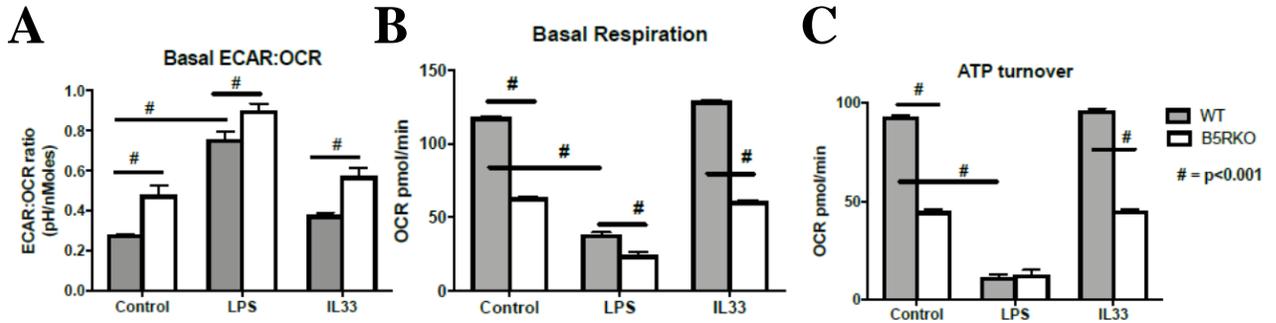
### 3.6 CYB5R3 DOES NOT HAVE A ROLE IN THE NITRIC OXIDE SEQUESTRATION PATHWAY OF DENDRITIC CELLS



**Figure 8. Cyb5R3 does not have a role in the NO Sequestration Pathway.** EPR spectroscopy was used to capture NO secretion from whole 4-hydroxytamoxifen treated or untreated Rosa26ER<sup>T2</sup> x Cyb5R3<sup>Fl/Fl</sup> BMDC samples following eighteen hours of stimulation in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). Data is representative of three experiments.

When BMDC from Rosa26ER<sup>T2</sup> x Cyb5R3<sup>Fl/Fl</sup> mice were treated with 4-hydroxytamoxifen, harvested, and stimulated for eighteen hours with media alone, LPS, or rIL-33 for NO measurement by EPR, the results were unexpected. The knockout of Cyb5R3 resulted in no change in NO secretion by DC as compared to the Cyb5R3-expressing DC, regardless of LPS or IL-33 stimulation. This demonstrated that Cyb5R3 is not involved in the NO sequestration pathway in DC as previously hypothesized.

### 3.7 DELETION OF CYB5R3 FROM CD11c+ BMDC SHIFTS METABOLISM TOWARDS GLYCOLYSIS



**Figure 9. Deletion of Cyb5R3 from CD11c+ BMDC Causes a Metabolic Shift.** Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> BMDC were treated with 4-hydroxytamoxifen or left untreated, CD11c bead purified, and stimulated overnight in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). Metabolism analysis was performed using a Seahorse XFe96 Bioanalyzer to obtain **A.** basal ECAR:OCR, **B.** basal respiration, and **C.** ATP turnover of Cyb5R3-expressing and -deficient cells. Statistical analysis by Student's paired t-test.

After our results indicated that Cyb5R3 was not functioning as a member of the NO sequestration pathway as had been identified in vascular EC [33, 34], we chose to evaluate the metabolism of these DC. Cyb5R3 has been suggested to be important to cellular metabolic control [44]. Given the importance of the precise modulation of DC metabolism to their functionality [2], we evaluated how the deletion of Cyb5R3 impacted DC metabolism at baseline and following stimulation with LPS or rIL-33. BMDC were harvested from Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> mice, left untreated or treated with treated with 4-hydroxytamoxifen, CD11c bead purified, and stimulated overnight in a 96-well plate with media alone, LPS, or rIL-33 for use the following day in a Seahorse XFe96 Bioanalyzer. This was used to measure the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of the DC. From these readings, the basal ECAR:OCR ratio, basal respiration, and ATP turnover rates could be determined,

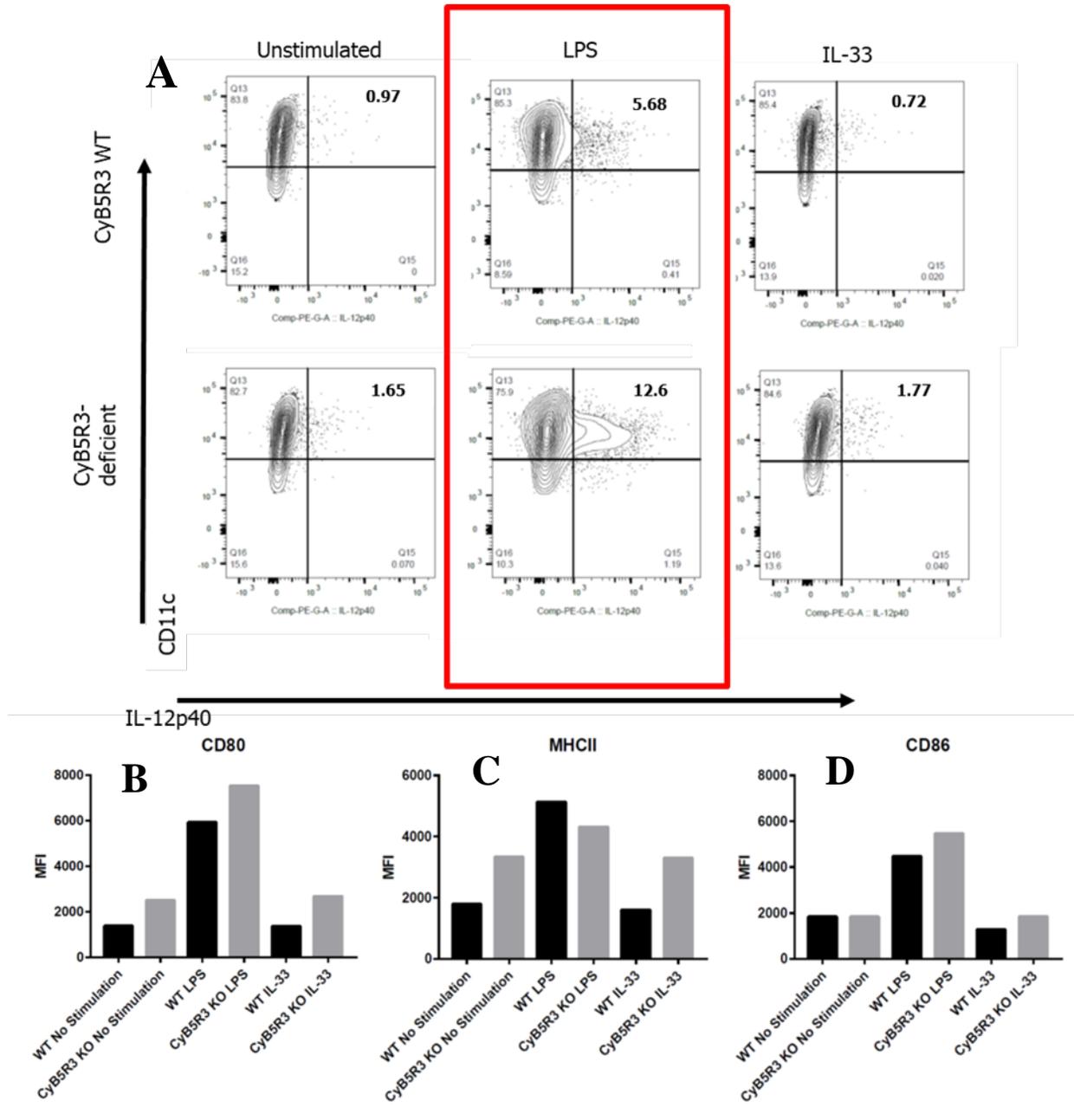
which gave insight to the oxidative phosphorylation, glycolysis and fatty acid oxidation occurring in the cells.

Analysis of the ECAR:OCR ratio showed an increase in the excretion of lactic acid (following its conversion from pyruvate) over the amount of oxygen consumed across all stimulations when Cyb5R3 was absent. This suggested a shift from OXPHOS to glycolysis when Cyb5R3 was absent (Figure 9A).

Analyzing the basal respiration, it was noted there was high oxygen consumption in the untreated and IL-33-stimulated Cyb5R3-expressing DC. This was followed by a significant decrease in the amount of oxygen consumed after Cyb5R3 knock out. This again suggested a shift from OXPHOS to glycolysis when Cyb5R3 was absent and corroborated the conclusions of the ECAR:OCR ratio (Figure 9B).

Analyzing the ATP turnover, high oxygen consumption was again noted in the untreated and IL-33-stimulated Cyb5R3-expressing DC, which significantly decreased following Cyb5R3 knock out. Due to ATP production being the terminal reaction in the electron transport chain in OXPHOS, we could link the oxygen consumption rate with the amount of ATP produced as a means of determining the DC's metabolism. Taken together, these three measurements illustrate that the deletion of Cyb5R3 from CD11c+ DC causes a metabolic shift from OXPHOS to glycolysis.

### 3.8 CD11c+ CYB5R3-DEFICIENT DENDRITIC CELLS SHOW AN INCREASE IN STIMULATORY MARKERS AND INFLAMMATORY CYTOKINES



**Figure 10. CD11c+ Cyb5R3-Deficient DC show an Increase in Stimulatory Markers and Inflammatory Cytokines.** *Rosa26ER<sup>T2</sup> × Cyb5R3<sup>F1/F1</sup>* BMDC were treated with 4-hydroxytamoxifen or left untreated and stimulated overnight in media alone, LPS (100 ng/ml), or rIL-33 (20 ng/ml). The following day, GolgiPlug was added for 5 hours to prevent cytokine leakage. These cells were then stained for CD11b, 11c, CD80, CD86, MHC11, and IL-12p40 **A**. y-axis: CD11c, x-axis: IL-12, numbers indicate IL-12hi, CD11c+ population counts **B-D**. Mean fluorescence intensity of CD80, MHCII, and CD86, respectively. Data is representative of two experiments.

From the results of the metabolic analysis suggesting that the Cyb5R3-deficient DC utilize glycolysis, we next decided it was pertinent to look at the cytokines and stimulatory markers produced by these DC.

Using BMDC harvested from Rosa26ER<sup>T2</sup> x Cyb5R3<sup>F1/F1</sup> mice, DC were left untreated or treated with 4-hydroxytamoxifen, CD11c bead purified, and stimulated overnight with media alone, LPS, or rIL-33. The DC were then treated with GolgiPlug for five hours to prevent cytokine secretion and stained for CD11b, CD11c, CD80, CD86, MHCII, and IL-12p40. Looking at the Cyb5R3-expressing flow plots and gating the cells on CD11c+ DC and IL-12hi, it was noted that there was low production of IL-12 in these DC when unstimulated, which increased upon LPS stimulation and decreased with IL-33 stimulation. Strikingly, with Cyb5R3 deficiency, there was an increase in LPS-induced IL-12 production, with a slight increase in IL-12 production in unstimulated and IL-33-stimulated DC. Together, this shows that in absence of Cyb5R3, DC become more inflammatory.

Next, looking at the mean fluorescence intensity of the stimulatory surface markers CD80, MHCII, and CD86, it was noted that there was a universal increase in DC surface expression of each of these markers when Cyb5R3 was knocked out, regardless of stimulation. The only exceptions to this were MHCII following LPS-stimulation of Cyb5R3-deficient DC and CD86 with no stimulation in Cyb5R3-deficient DC. Taken together, these data suggest that in the absence of Cyb5R3, DC become more inflammatory and stimulatory.

## 4.0 DISCUSSION

### 4.1 TLR4/ST2 RECEPTOR LIGATION ON DC INDUCES A NITRICOXIDE SEQUESTRATION SYSTEM THAT DOES NOT RELY ON CYB5R3

Straub et al. have previously demonstrated that a NO sequestration pathway exists in vascular EC and is capable of regulating smooth muscle tone through NO diffusion control [33]. To our knowledge, the expression of hemoglobin- $\alpha$  and Cyb5R3 as well as a NO sequestration pathway in DC is a novel discovery (Figure 3). Previous literature has shown that DC-produced NO can modulate T cell polarization *in vitro*, with NO synergizing with IL-12 to expand Th1 cells while blocking IL-4-mediated Th2 responses [45, 46]. Our data support this idea, following IL-33 stimulation, and demonstrate that other Th2 cytokines, namely IL-5, are also dampened in the presence of NO, with no effect on Th1 polarization following LPS stimulation (Figure 5). Though IL-33 is known to be a pleiotropic cytokine [42], its polarization of CD4+ T cells towards Th2 cells following DC stimulation with IL-33 and its inability to increase DC NO production by iNOS highlight important differences between ST2/TLR4 signaling in the NO sequestration system and iNOS signaling pathway (Figures 4 and 5).

It is interesting that Cyb5R3 does not function in the sequestration pathway as it does in EC (Figure 8). It is possible that an as yet undetermined hemoprotein reductase is present and is reducing the hemoglobin- $\alpha$ -bound iron group. This may also be due to a redundant reductase protein functioning in the place of Cyb5R3. Due to there being four isoforms of Cyb5R in humans [47, 48], it is feasible that another could be reducing the Fe<sup>3+</sup> group on hemoglobin- $\alpha$  to

Fe<sup>2+</sup>, permitting NO sequestration.

#### **4.2 CYB5R3 ACTS AS A REGULATOR OF DENDRITIC CELL STIMULATORY CAPACITY TOWARDS CD4+ T CELLS**

Our results indicate that Cyb5R3, which does not function in the NO sequestration pathway in DC (Figure 8), acts as a regulator of DC stimulatory capacity. When Cyb5R3 is present in DC, cytokine production levels of IL-5 and IFN- $\gamma$  by T cells are significantly dampened regardless of LPS-DC- or IL-33-DC-stimulation. However, when Cyb5R3-deficient DC are stimulated with IL-33 and LPS, robust production of IL-5 and IFN- $\gamma$  by T cells occurs (Figure 7).

The idea that Cyb5R3 is important as a signaling regulator for DC is not an unheard-of concept. Although it functions as a positive regulatory factor in DC, dendritic cell-specific transmembrane protein (DC-STAMP), a multi-membrane protein in the Golgi apparatus of mature DC, has been shown to cause BMDC to produce less IL-6, IL-12, tumor necrosis factor alpha (TNF- $\alpha$ ), and IL-10 and have impaired T cell activation [49]. Therefore, if a positive stimulatory regulator such as DC-STAMP exists, it is likely that a negative stimulatory regulator such as Cyb5R3 exists, preventing too much T cell activation and an inappropriate immune response.

### **4.3 CYB5R3 IS A NEGATIVE REGULATOR OF GLYCOLYSIS AND DENDRITIC CELLS SHOW A SHIFTED METABOLIC PROFILE IN ITS ABSENCE**

As discussed in the last section, Cyb5R3 is important as a negative regulator for DC activation (Figure 7), with its absence leading to unchecked T cell stimulation. Our data illustrated a similar result following our metabolic and flow cytometry analyses (Figures 9 and 10). Results of the basal ECAR:OCR, basal respiration, and ATP turnover between the Cyb5R3-expressing and the Cyb5R3-deficient cells suggest that the metabolism of the DC shifts from OXPHOS to glycolysis following a loss of Cyb5R3 expression (Figure 9). This is further corroborated by the cytokine and surface marker expression analyses.

Analysis of the Cyb5R3-expressing cells showed that there is low production of IL-12 in unstimulated DCs, which increases following LPS stimulation, and decreases with IL-33 stimulation. However, in the Cyb5R3-deficient DC, an increase in IL-12 production is noticeable in unstimulated DC, with a robust increase following LPS stimulation and slight IL-12 production following IL-33 stimulation. Analysis of the DC stimulatory surface markers CD80, MHCII, and CD86, provides further evidence of the importance of Cyb5R3 in the negative regulation of glycolysis. In Cyb5R3-expressing cells, the expression of CD80, MHCII, and CD86 is minimal in unstimulated and IL-33-stimulated-DC, with an increase in LPS-stimulated DC. However, in Cyb5R3-deficient DC, there is an increase in DC surface marker expression, regardless of stimulation. These data together demonstrate that in the absence of Cyb5R3, DC become more inflammatory. It is known that the activation of DC by pro-inflammatory stimuli causes them to undergo a metabolic switch from OXPHOS to glycolysis, leading to the upregulation of pro-inflammatory cytokines and stimulatory surface markers [50]. By knocking

out Cyb5R3, DC are left without an important internal metabolic regulator, producing the same effects seen when Cyb5R3 is present and DC are stimulated with LPS.

Taken together, this study has elucidated specific proteins integral to DC ability to determine CD4<sup>+</sup> T cell lineage and improved our understanding of the metabolic shifts that occur during TLR4/ST2 ligation.

## APPENDIX

**Table 1: Abbreviations**

<b>Abbreviation</b>	<b>Definition</b>
<b>Ag</b>	antigen
<b>Alas1</b>	aminolaevulinic acid synthase 1
<b>AP-1</b>	activator protein 1
<b>BMDC</b>	bone marrow-derived dendritic cells
<b>CD</b>	cluster of differentiation
<b>Cyb5R3</b>	cytochrome b5 reductase 3
<b>DAMPs</b>	damage-associated molecular patterns
<b>DC</b>	dendritic cells
<b>DC-STAMP</b>	dendritic cell-specific transmembrane protein
<b>DETA</b>	diethylenetriamine
<b>EC</b>	endothelial cells
<b>ECAR</b>	extracellular acidification rate
<b>ELISAs</b>	enzyme-linked immunosorbent assays
<b>EPR</b>	electron paramagnetic resonance spectroscopy
<b>ER</b>	endoplasmic reticulum
<b>FAD</b>	flavin adenine dinucleotide
<b>FBS</b>	fetal bovine serum
<b>Fe<sup>2+</sup></b>	ferrous iron
<b>Fe<sup>3+</sup></b>	ferric iron
<b>GAPDH</b>	glyceraldehyde 3-phosphate dehydrogenase
<b>GATA3</b>	GATA-binding protein 3
<b>Gfi-1</b>	growth factor independent-1
<b>GM-CSF</b>	granulocyte-macrophage colony-stimulating factor
<b>IFN-<math>\gamma</math></b>	interferon gamma
<b>IL</b>	interleukin
<b>IL-1RAcP</b>	IL-1 receptor accessory protein
<b>iNOS</b>	inducible nitric oxide synthase
<b>IRAK</b>	IL-1R-associated kinase
<b>JNK</b>	c-Jun N-terminal kinases
<b>Lcn2</b>	lipocalin 2
<b>LPS</b>	lipopolysaccharide
<b>MAPK</b>	mitogen-activated protein kinase
<b>MHC</b>	major histocompatibility complex
<b>MLR</b>	mixed lymphocyte reaction
<b>mRNA</b>	messenger RNA

<b>MyD88</b>	myeloid differentiation primary response 88
<b>NADH</b>	nicotinamide adenine dinucleotide
<b>NF-<math>\kappa</math>B</b>	nuclear factor kappa B
<b>NLRs</b>	nucleotide-binding oligomerization domain-like receptors
<b>NO</b>	nitric oxide
<b>NOD</b>	nucleotide-binding oligomerization domain
<b>OCR</b>	oxygen consumption rate
<b>OXPPOS</b>	oxidative phosphorylation
<b>PAMPs</b>	pathogen-associated molecular patterns
<b>PCA</b>	principle component analysis
<b>PRRs</b>	pattern recognition receptors
<b>qRT-PCR</b>	quantitative real-time polymerase chain reaction
<b>RIG-1</b>	retinoic acid-inducible gene 1
<b>RLRs</b>	retinoic acid-inducible gene 1-like receptors
<b>SEA</b>	soluble egg antigen
<b>SOCS-1</b>	suppressor of cytokine signaling-1
<b>ST2</b>	IL1RL1/IL-1 receptor like 1
<b>STAT</b>	signal transducer and activator of transcription
<b>T-bet</b>	T-box transcription factor
<b>TBST</b>	tris-buffered saline/Tween 20
<b>TCR</b>	T cell receptor
<b>Tfh</b>	T-follicular helper cell
<b>Th</b>	T helper cell type
<b>TIR</b>	Toll/Interleukin-1 receptor
<b>TLRs</b>	Toll-like receptors
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor alpha
<b>TRAF6</b>	tumor necrosis factor receptor-associated factor 6
<b>Treg</b>	T-regulatory cell

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