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Innate Immune Cell Phenotypes Are Dictated by Distinct Epigenetic Reprogramming

Kevin Douglas Adams
Brigham Young University

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Kevin Douglas Adams

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Master of Science

Steven M. Johnson, Chair
Richard A. Robison
Kim L. O'Neill

Department of Microbiology and Molecular Biology

Brigham Young University

ABSTRACT

Innate Immune Cell Phenotypes Are Dictated by Distinct
Epigenetic Reprogramming

Kevin Douglas Adams
Department of Microbiology and Molecular Biology, BYU
Master of Science

The innate immune system is the first line of host defense against external exposures. During these initial encounters, antigen presenting cells - specifically monocytes and macrophages - modulate further inflammatory responses. Macrophages exist along a spectrum of phenotypic programs; on the inflammatory M1 end they enhance immune activity while on the anti-inflammatory M2 end they suppress further immune activation. Furthermore, within M2 macrophages there exist many subpopulations, namely M2a and M2d, each with specific roles during infection or exposure. We sought to compare the epigenetic profiles of these subpopulations of macrophages to determine key regulatory gene networks and factors that could be exploited for therapeutic benefit.

While traditionally viewed as primitive and nonspecific, a growing body of clinical and experimental evidence argues the innate immune system develops memory as a result of previous exposures, allowing the innate system to respond with enhanced and broad immunological protection upon exposure to a secondary stimulus. This biological process of innate immunity has been termed trained immunity. Trained immunity shares many phenotypic and epigenetic characteristics with adaptive immune memory; however, one of the starkest distinctions is the propensity of trained immunity to develop against heterologous stimuli. Innate memory is not antigen specific, frequently protecting the host against unrelated organisms.

Keywords: monocytes, macrophages, epigenetics, trained immunity

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CHAPTER 1: Introduction

1.1 Background

Macrophages

White blood cells, or leukocytes, include the aggregate of diverse cell types that make up the human immune system. These cells are distinguished by their physical and functional characteristics which they develop in response to various internal and external cues. Cells of the mononuclear phagocyte system are one such example of a leukocyte subpopulation which display many different possible phenotypes based on the external stimulation received[1]. Monocytes are found circulating in peripheral blood, differentiating into macrophages upon entering tissue. These macrophages are capable of regulating many other aspects of the immune system; they can boost an immune response by activating helper T cells via the major histocompatibility complex (MHC) and inflammatory cytokines or they can suppress an immune response by activating regulatory T cells via anti-inflammatory cytokines [1-3]. Consequently, these cells undergo many epigenetic changes in order to carry out their stimulus- or tissue-specific functions [2, 3].

Peripheral blood monocytes may be stimulated into macrophages using the stimulatory cytokine macrophage colony-stimulating factor (M-CSF) to produce different cytokines and other peptides according to the needs of the immune system [4, 5]. During bacterial infection macrophages typically adopt an M1 or classically activated phenotype which allows them to produce antimicrobial peptides as well as immune-stimulatory cytokines which promote inflammation [2, 4]. To mimic this bacterial stimulation, researchers use Interferon Gamma (IFN-γ) [5, 6]. During wound healing the macrophages adopt an M2 or alternatively activated phenotype which allows them to suppress the immune system and boost cell proliferation [4, 7].

To mimic this alternative activation researchers use Interleukin 4 (IL-4) which is sufficient for M2 activation [4, 6]. The use of IL-4 produces an M2a activation, so to drive further polarization researchers use IL-6 which produces an M2d activation state which closely resembles tumor associated macrophages (TAMs) found in cancer patients (Figure 1- 1).

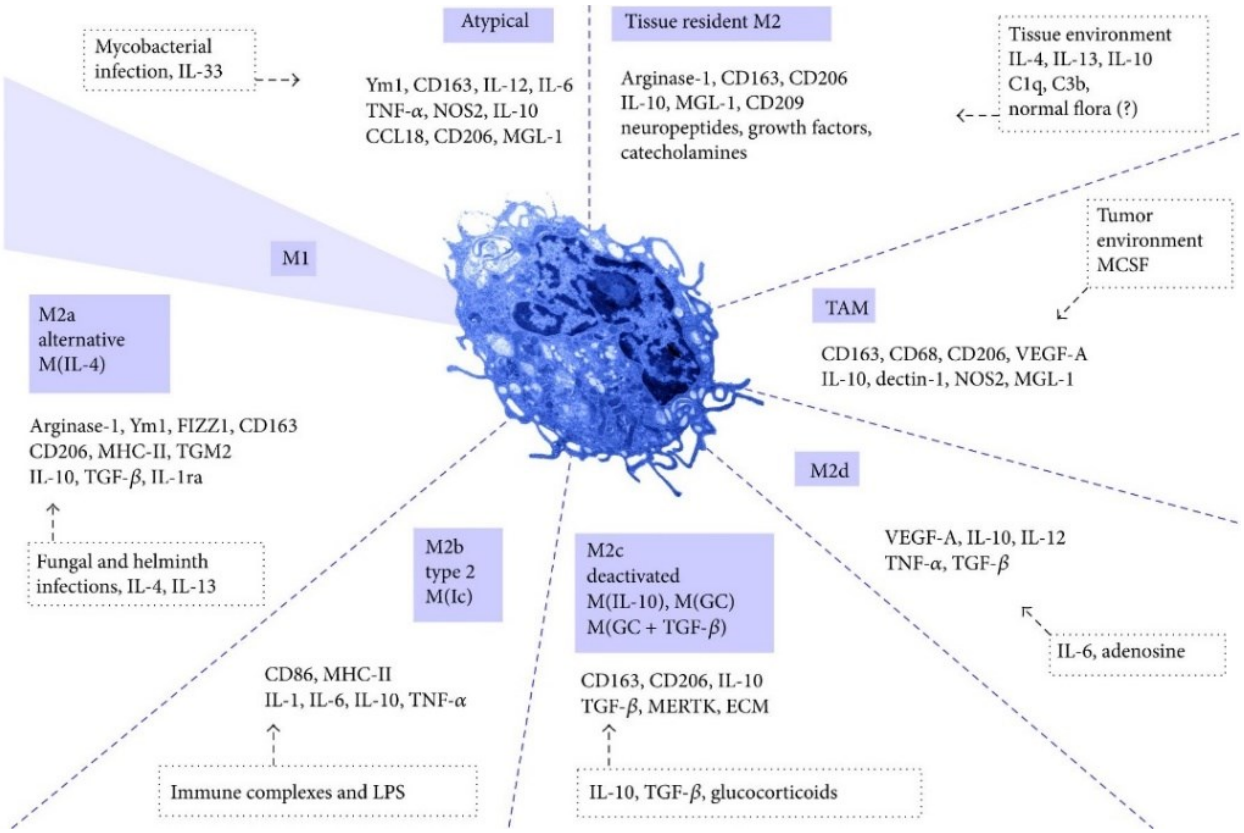

Figure 1- 1: Overview of macrophage polarization via external stimuli. Most research does not distinguish M2d and TAM phenotypes [4].

Since macrophages exist within a spectrum of phenotypes they must be tightly regulated to prevent disease [1]. Inflammatory cytokines produced during healing may result in chronic disease while immunosuppressive cytokines produced during disease or infection may prevent the body from clearing that infection or disease. This is seen in a range of diseases from tuberculosis [8] and cryptococcus [2] infections to atherosclerosis [9, 10] and cancer [11, 12],

where appropriate macrophage activation typically results in the maintenance of health, while errant activation often results in a disease state [13].

The Role of Macrophages in Cancer

Macrophage-related diseases often result from inappropriate macrophage polarization. Because macrophages are central regulators in the body's immune response, they will sometimes infiltrate tumors while in an M1 phenotypic state because the tumor secretes a gradient of monocyte chemotactic factors [14]. Once the monocytes or macrophages have been drawn into the tumor mass, they are forced to switch to an M2 phenotypic state by the tumor microenvironment [3, 13]. As the TAMs are converted to the M2d phenotype these macrophages begin producing cytokines and growth factors which suppress the immune system [1] and induce angiogenesis [15, 16], respectively [11]. Due to their presence within and contribution to the growth of tumors, a high concentration of TAMs within a tumor correlates with poor prognosis for the patient. In some tumors as much as 50% or more of the tumor mass is composed of TAMs [12] and studies suggest that as TAM concentration increases the chance of survival decreases [17, 18] leading physicians and researchers to use the presence or absence of a large TAM population as a prognostic indicator [19].

U-937 Cell Line

The U-937 monocyte cell line was derived from a histiocytic monocyte cancer in a 37-year-old man and was propagated for the specific purpose of researching monocyte cancers [20]. It was later discovered this monocyte cell line could be induced to adopt characteristics of activated macrophages. Using phorbol 12-myristate 13-acetate (PMA) researchers could drive the stimulation from monocyte to macrophage [21] in a similar manner to peripheral blood monocytes being induced to macrophages using M-CSF [5, 22]. It is understood that these

stimulated U-937s exhibit similar functional characteristics as stimulated macrophages derived from peripheral blood draws [21-23]; however, the similarity between epigenetic landscapes is not well defined. Analysis of chromatin architecture comparing U-937s to human peripheral monocytes is necessary for future macrophage studies.

Chromatin

Chromatin is the combination of DNA and DNA-associated proteins, and functions under various states of compaction, thereby influencing the accessibility of genes for transcription. Chromatin is primarily composed of DNA wrapped around histone octamers to form what are known as nucleosomes. The DNA between nucleosomes is known as linker DNA and is an important target for chromatin studies. Nucleosomes may condense to form heterochromatin or remain open and accessible for transcription factor binding [5] (Figure 1- 2). Heterochromatic regions block transcription factor binding, effectively silencing genes bound up in condensed chromatin. Chromatin compaction acts as an epigenetic regulator of cellular identity, driving the production or silencing of lineage-determining proteins [3, 5].

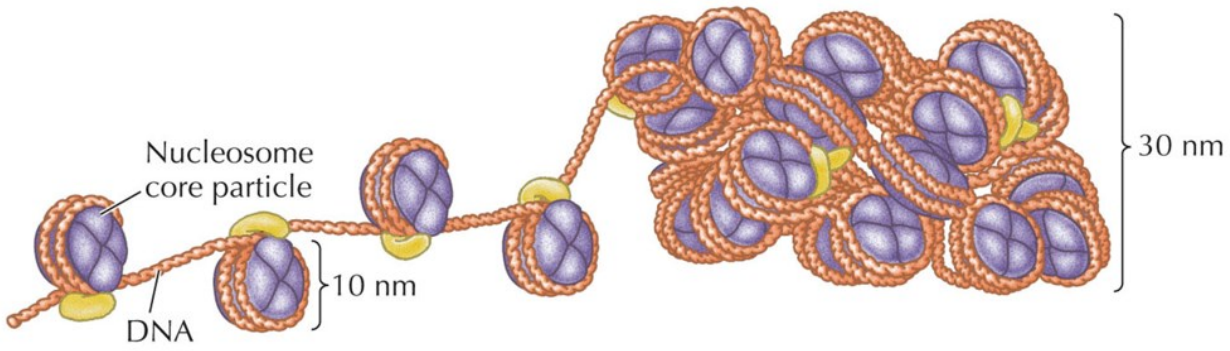

Figure 1- 2: Chromatin compaction. Depiction of open chromatin or “beads on a string” juxtaposed with condensed or heterochromatin [24].

Macrophages may adopt a wide spectrum of polarization states which are currently not well understood at an epigenetic level. Upon receiving external stimuli these macrophages are able to remodel their chromatin, opening up new potential drug targets [25]. Known targets include Activated Protein C [26] and Tie-2 [16] which were discovered using targeted PCR and microarray analysis, respectively. Tie-2 expressing macrophages exhibit a distinct RNA expression pattern associated with angiogenesis, indicating that the RNA or chromatin footprint of macrophages may have strong association with patient prognosis [19]. Open chromatin has been mapped in other cell types using a method known as DNase-seq, which uses DNase I to degrade the linker DNA between nucleosomes within open chromatic regions. The remaining DNA, which is bound up in nucleosomes or transcription factors, is liberated from those proteins and sequenced [27, 28]. This technique gives a high-resolution map of the open chromatin across an entire genome and may be used in conjunction with transcriptomic research [6, 7, 27].

Through transcriptome and chromatin research, targets have been discovered which can either kill TAMs or induce macrophages to alter polarization for tumor rejection [13, 18]. Clodronate is a drug which induces apoptosis in macrophages when it accumulates during phagocytosis [29]. Clodronate efficiently and specifically kills any macrophages, thereby decreasing both the detrimental and beneficial macrophages at the same time [30]. Antibodies targeting the Interleukin 10 receptor (IL-10R) are able to effectively block immune suppression in macrophages and dendritic cells; however, anti-IL-10R antibodies also affect B cells, natural killer cells, cytotoxic and helper T cells, mast cells, granulocytes, keratinocytes, and endothelial cells. Additional epigenetic research should be conducted to identify additional gene targets in macrophages. The Assay for Transposase Accessible Chromatin with sequencing (ATAC-seq) is currently the most efficient way to accurately profile the open chromatin in macrophages and

monocytes. ATAC-seq requires less time, less money, and fewer cells while providing equivalent genome-wide coverage to DNAse-seq [28].

ATAC-seq

The Assay for Transposase Accessible Chromatin with sequencing (ATAC-seq) works in a manner similar to DNAse-seq [27, 28]. ATAC-seq uses a hyperactive Tn5 transposase which dimerizes and inserts into accessible open-chromatin. This insertion induces a "tagmentation" event which concurrently fragments the DNA at the insertion site and "tags" each fragment with a partial Illumina adapter (Figure 1- 3). The Illumina adapters are then extended to full length using PCR at which point the fragments may be sequenced using an Illumina platform.

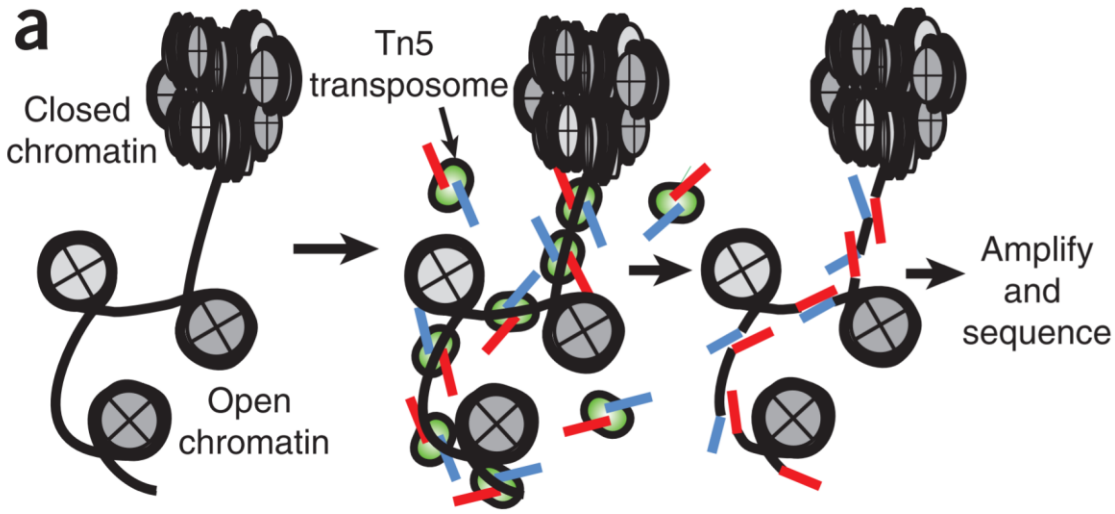

Figure 1- 3: Diagram of the "tagmentation" process. Open chromatin is accessible to transposase (green) insertion while closed chromatin remains protected. The red and blue bars represent Illumina primers [28].

ATAC-seq data provides information about chromatin density as well as transcription factor occupancy. Although a region may have open chromatin it is possible no transcription factors are present, indicating that the genes in that region are inactive. When coupled with RNA-seq, ATAC-seq gives a detailed picture the epigenetic and transcriptional changes that

occur within the cells under specific stimuli [6, 22]. Specialized bioinformatics tools have been created for the analysis of ATAC-seq data, such as CENTIPEDE [31], while others, Bowtie2 and ZINBA [28], were designed for genome assembly and nucleosome peak-calling, respectively. ATAC-seq will provide an accurate representation of the chromatin state in human monocytes and macrophages under various stimulation conditions. Additionally, we hypothesize the human cell line U-937 exhibits a similar open-chromatin footprint and gene expression pattern as the corresponding human monocytes drawn from peripheral blood.

CHAPTER 2: Macrophage Polarization Experiments

2.1 Theory vs. Reality

The methods section represents the initial plan proposed for this project and as such is written in future tense. As challenges arose, we made minor changes which are described in the results section. These adjustments are written in past-tense.

2.2 Methods

U-937 Chromatin Analysis

To study macrophage activation and polarization, we will initially use the monocyte cell line U-937 before expanding to macrophages derived from peripheral blood monocytes. U-937 monocytes have been purchased from ATCC and will be validated by the University of Arizona Genetics Core. U-937s are cultured in antibiotic-free media consisting of RPMI-1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate, and Fetal Bovine Serum (FBS) to a final concentration of 10%.

To induce differentiation from monocyte to macrophage the U-937s will be stimulated with phorbol 12-myristate 13-acetate (PMA). PMA is added at a concentration of 200nM and the culture is left to incubate for 24 hours in a 37^{o} incubator. After 24 hours the cells are centrifuged and the supernatant is removed. Cells are resuspended in RPMI-1640/FBS media and incubated under the same conditions for another 24 hours. Following stimulation, the macrophages will be divided into two test groups which will be further differentiation using either Interleukin 4 (IL-4) for an M2a phenotype or IL-6 for an M2d, or Tumor Associated Macrophage (TAM), phenotype. Differentiation will be performed using a cytokine concentration of 20 ng/ml for 24 hours [32]. The M2a activation will be confirmed using a combination of anti-CD33, CD163, and CD206 antibodies. M2d activation will be confirmed using anti-CD33 antibodies in conjunction with anti-VEGF antibodies.

ATAC-seq

The Assay for Transposase Accessible Chromatin and sequencing (ATAC-seq) only requires an initial sample size of 50,000 cells which will be collected as described above. The cells will be spun down at 500g and 4^{o}C for 5 minutes. They are washed with cold PBS and pelleted under the previous conditions. Cells are lysed, and nuclei are isolated using a cold lysis buffer consisting of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% IGEPAL CA-630 and immediately spun down at 500g and 4^{o}C for 10 minutes. The isolated nuclei are subsequently transposed at 37^{o}C for 30 minutes. Transposition uses a hyperactive Tn5 transposase which dimerizes and binds to two copies of a short DNA fragment which are ligated to the fragmented ends of the transposon insertion site. The insertion sites are the DNA sequences with no protein bound to them. These DNA fragments are partial Illumina adapters which will be elongated using barcoded PCR primers (Table 2- 1).

Table 2- 1: Barcoded primers are used to extend the partial Illumina adapters following transposition [28].

Barcode	Primer Sequence with Barcode
Primer Sequence 1	5′-AATGATACGGCGACCACCGA-3′
Primer Sequence 2	5′-CAAGCAGAAGACGGCATACGA-3′
Ad2.1_TAAGGCGA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCG GAGATGT
Ad2.2_CGTACTAG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCG GAGATGT
Ad2.3_AGGCAGAA	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCG GAGATGT
Ad2.4_TCCTGAGC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTC GGAGATGT
Ad2.5_GGACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTC GGAGATGT
Ad2.6_TAGGCATG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCG GAGATGT

The ATAC-seq protocol will be implemented on four test groups: unstimulated U-937s, general U-937-derived macrophages, and M2a and M2d polarization groups. Barcoded samples will be quantified using a quantitative PCR-based kit from KAPA Biosystems following which the samples will be pooled in equivalent concentrations for multiplex sequencing. Sequencing will be submitted to the BYU DNA Sequencing Center and sequenced at 150 base pairs, paired-end. Sequencing reads will be analyzed using the Fulton supercomputer at BYU.

Reads will be aligned to hg19 using Bowtie2 with duplicates being removed using Picard. All reads aligning to the + strand will be offset by +4 while those aligning to the – strand will be offset by -5 in order to adjust the read start site to represent the center of the transposon binding event. ZINBA will be utilized to call ATAC-seq peaks using a window size of 300 bp and an offset of 75 bp to identify the dyad of each nucleosome. The distribution of paired-end

sequencing fragment sizes across chromatin states will be computed (http://www.ensembl.org/info/genome/funcgen/regulatory_segmentation.html). Reads will be split based upon size: nucleosome free regions are those of less than 100 bp, reads between 180 and 247 will be considered mononucleosomes, reads between 315 and 473 bp as dinucleosomes, and reads between 558 and 615 bp as trinucleosomes. These reads will be analyzed using DanPos and Dantools using parameters -p 1, -a 1, -d 20, -clonalcut 0 with the nucleosome-free reads acting as background to give effective negative weighting to those reads. Footprinting will be performed using msCentipede which is highly efficient at detecting transcription factor binding sites in small samples [31]. All analyses outlined were taken from the ATAC-seq protocol [28].

Peripheral Blood Monocytes

An application for Institutional Review Board (IRB) approval has been submitted for the collection of volunteer blood samples. Upon obtaining IRB approval and following the preliminary analyses on the U-937 cell line, we will proceed with peripheral blood monocytes (PBMs). One green-top tube coated in sodium heparin, an anticoagulant, will be used to collect whole blood from each volunteer. Blood will be mixed in a 1:2 ratio with PBS and layered onto 3mL Ficoll-1077. Blood will then be centrifuged for 20 minutes at 500g and the buffy coat will be removed. Following removal, PBMs will be isolated from the buffy coat using anti-CD33 magnetic beads purchased from Miltenyi Biotec [33].

To induce differentiation from monocyte to macrophage the PBMs will be stimulated with macrophage colony-stimulating factor (M-CSF). Cells will be cultured at a concentration of 2×10^5/ml in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 20 μM β-mercaptoethanol for 6 days [32]. Further differentiation will occur as

outlined for the U-937 cell line to polarize cells into an M2a phenotype using IL-4 or an M2d or TAM phenotype using IL-6 using a cytokine concentration of 20 ng/ml for 24 hours. M2a and M2d activation will be confirmed as described above for the U-937 cell line; M2a activation will be confirmed using a combination of anti-CD33, CD163, and CD206 antibodies and M2d activation will be confirmed using anti-CD33 antibodies in conjunction with anti-VEGF antibodies. ATAC-seq protocol outlined above will be performed on the four test groups of PBMs and PBM-derived macrophages: unstimulated PBMs, general PBM-derived macrophages, and M2a and M2d polarization groups. Following ATAC-seq, reads will be processed through the same workflow as the U-937 cell line with the addition of a comparison against the ENCODE database at UCSC to identify gene annotations corresponding to our peak data. Comparing the reads to the ENCODE database will isolate potential targets for known immunomodulatory drugs.

After establishing potential drug targets in PBM-derived macrophages as identified through the ENCODE database, mapped U-937 reads will be compared against the ENCODE database as well as against the PBM-derived macrophage reads. This final comparison will be used to verify the accuracy and validity of using U-937s for monocyte and macrophage research. This is important because some researchers use only U-937s when performing macrophage studies while others expend great resources to study macrophages directly when U-937s may present an inexpensive, tractable alternative.

2.3 Results

We performed ATAC-seq on U937 cells to establish a baseline epigenetic profile for the cell line. Every attempt was halted by the presence of mycoplasma, a contaminating bacterium.

Nearly 50% of our reads in every round of ATAC-seq on U937 cells came from the bacterium pushing us to discard the transposed DNA. In an attempt to manage the contamination, we added ciprofloxacin to the media. The contamination was unaffected and resulted in premature cell death both with and without stimulating cytokines. As a result, we abandoned attempts to compare U937 cells with primary monocytes.

We next isolated monocytes from three male and three female volunteers. ATAC-seq was performed and the isolated and transposed DNA was pooled and sequenced on a single lane in a Hi-seq 2500 using 150bp paired-end sequencing (Table 2- 2).

Table 2- 2: Sequence file names and read counts for each sample

Sample	Forward	Reverse	Reads
M_23	Ad2-1_S41_L007_R1_001.fastq	Ad2-1_S41_L007_R2_001.fastq	40,305,084
F_25	Ad2-2_S41_L007_R1_001.fastq	Ad2-2_S41_L007_R2_001.fastq	41,159,731
M_24	Ad2-3_S41_L007_R1_001.fastq	Ad2-3_S41_L007_R2_001.fastq	41,977,656
M_28	Ad2-4_S41_L007_R1_001.fastq	Ad2-4_S41_L007_R2_001.fastq	16,430,636
F_23	Ad2-5_S41_L007_R1_001.fastq	Ad2-5_S41_L007_R2_001.fastq	46,949,527
F_27	Ad2-6_S41_L007_R1_001.fastq	Ad2-6_S41_L007_R2_001.fastq	29,717,034

The FASTQ files were then aligned to the human genome hg19 using BWA both with and without read trimming. Alignment files were subsequently sorted using samtools sort and checked using samtools flagstat.

Table 2- 3: Alignment statistics for sample 1

Untrimmed Alignment samtools.flagstats	Trimmed Alignment samtools.flagstat
80751513 + 0 in total (QC-passed reads + QC-failed reads)	71184937 + 0 in total (QC-passed reads + QC-failed reads)
141345 + 0 secondary	28937 + 0 secondary
0 + 0 supplimentary	0 + 0 supplimentary
0 + 0 duplicates	0 + 0 duplicates
80213292 + 0 mapped (99.33%:-nan%)	70680318 + 0 mapped (99.29%:-nan%)
80610168 + 0 paired in sequencing	71156000 + 0 paired in sequencing
40305084 + 0 read1	35578000 + 0 read1
40305084 + 0 read2	35578000 + 0 read2
77943408 + 0 properly paired (96.69%:-nan%)	69131132 + 0 properly paired (97.15%:-nan%)
80026774 + 0 with itself and mate mapped	70597722 + 0 with itself and mate mapped
45173 + 0 singletons (0.06%:-nan%)	53659 + 0 singletons (0.08%:-nan%)
175080 + 0 with mate mapped to a different chr	97626 + 0 with mate mapped to a different chr
71138 + 0 with mate mapped to a different chr (mapQ>=5)	41809 + 0 with mate mapped to a different chr (mapQ>=5)

It was determined the alignment was virtually identical under both conditions so future alignments were performed without read trimming as a means to reduce resource usage on the supercomputer. Following alignment and sorting, peaks were called on the bam files using MACS2, a program developed for ChIP-seq peak calling. Peaks were called for sample 1 and uploaded to the UCSC genome browser (figures 2- 1, 2- 2). Peaks were also called using DanPos to identify discrepancies between peak-calling software. DanPos peaks for sample 2 are included for comparison (Figure 2- 3).

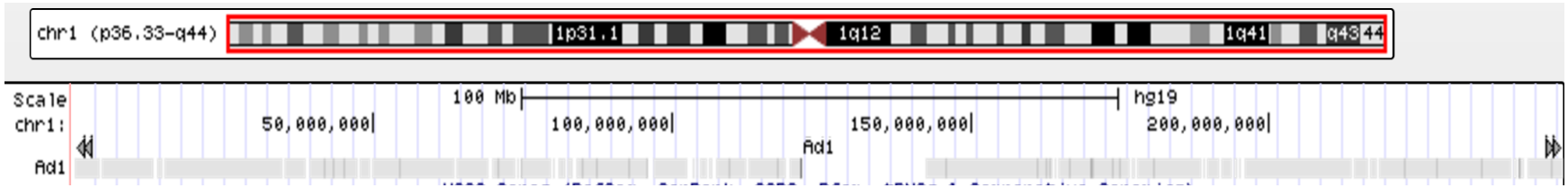

Figure 2- 1: Sample 1 peaks across chromosome 1

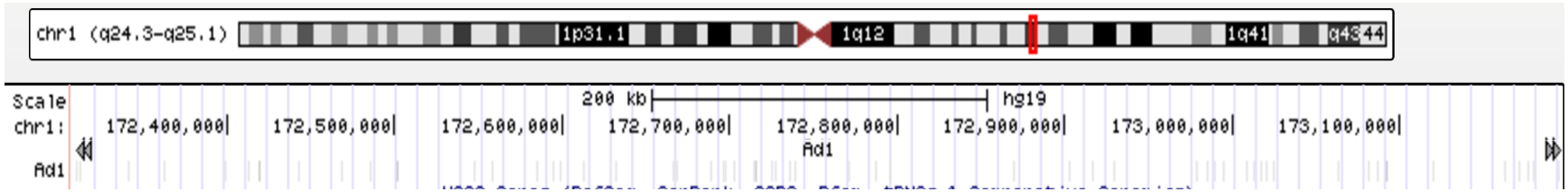

Figure 2- 2: Sample 1 peaks zoomed in across 7 million bp of chromosome 1

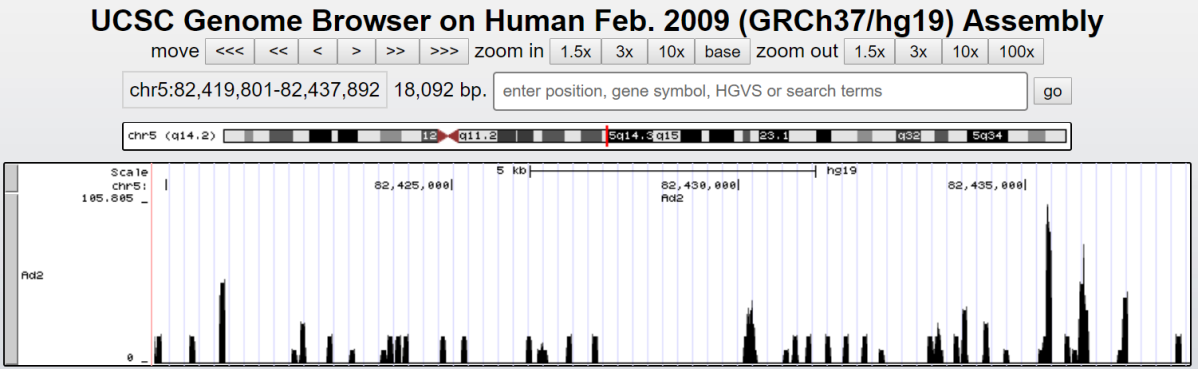

Figure 2- 3: Sample 2 DanPos peaks on chromosome 5

Further bioinformatic analysis became impossible to conduct due to limited resources and errors during the ATAC-seq analysis pipeline. While we were able to generate alignment files and call peaks, we were unable to format the files correctly to view on either the UCSC genome browser or on the integrative genome viewer, IGV. Attempts to reformat the peak files resulted in corrupted files that did not display properly on either genome viewer. We further attempted to refine our alignment files using different alignment tools or peak callers, none of which produced the desired histograms of read coverage. We turned to the official Encode ATAC-seq pipeline, yet despite receiving technical support from the group that wrote the program we were unable to properly install the workflow onto the supercomputer. Furthermore, we were unable to upload most iterations of out peak data due to unknown errors While these errors were being addressed, we continued with the monocyte isolation and macrophage stimulation experiments.

Monocytes isolated from volunteers were stimulated according to the Miltenyi Biotec protocols. Surprisingly, only up to 200,000 cells were recovered, far from the 500,000+ stimulated cells necessary, following each attempt to differentiate monocytes into M1, M2a, or M2d macrophages. In virtually all cases, all macrophages died within four days of isolation. Only on two occasions did the macrophages survive for the entirety of the 10-day stimulation

only to die either during collection or during flow cytometry preparation. We were unable to run flow cytometry on macrophages prior to cell death leading us to attempt U937 cells a second time; however, the cell line failed to survive during the stimulation process. We did not check for mycoplasma the second time, though it is assumed the culture was still contaminated based upon low viability.

2.4 Discussion and Future Direction

Macrophages that have been exposed to varied stimuli exhibit distinct epigenetic programs. In contrast, unstimulated peripheral blood monocytes from different individuals exhibit marked similarities in ATAC-seq landscapes (Figure 2- 4). This baseline histone positioning is beneficial for determining the effects of polarization as it decreases the number of variables involved in histone positioning.

In vitro culture of macrophages represents a distinct challenge to understanding the epigenetic reprogramming associated with monocyte polarization. The process of cell culture must be streamlined to gather the sufficient 500,000+ cells to be used for both flow-cytometry and ATAC-seq. This could potentially be achieved by drawing more blood, though this is a challenge as we were already drawing 50mL of blood and working with more than that is inefficient. If sufficient cells could be harvested, then ATAC-seq could be performed for each stimulation state. With that additional data comes the challenge of analysis.

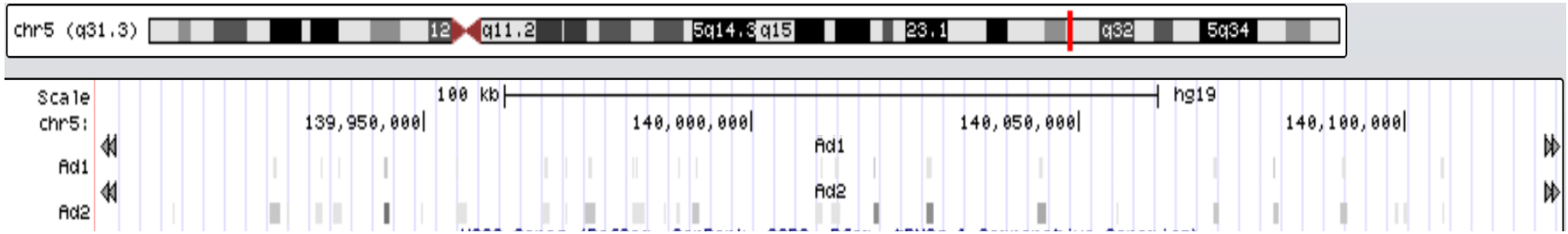

Figure 2- 4: Comparison of peaks between samples 1 and 2. The samples are remarkable similar in most loci, including at the CD14 gene, shown here.

Future data analysis will require the installation and use of the Encode ATAC-seq analysis pipeline. Once properly established, this pipeline should perform the majority of the bioinformatic assessment required to accurately compare peaks across the genome. Macrophage differentiation states theoretically display activation-specific histone and transcription factor landscapes which correlate strongly with differential gene expression.

Differentially expressed genes should be studied to determine potential drug targets to specifically knock down activation-specific macrophages that are involved in the establishment and perpetuation of disease.

CHAPTER 3: Exposome: How Exposure Order Dictates Innate Immune Training

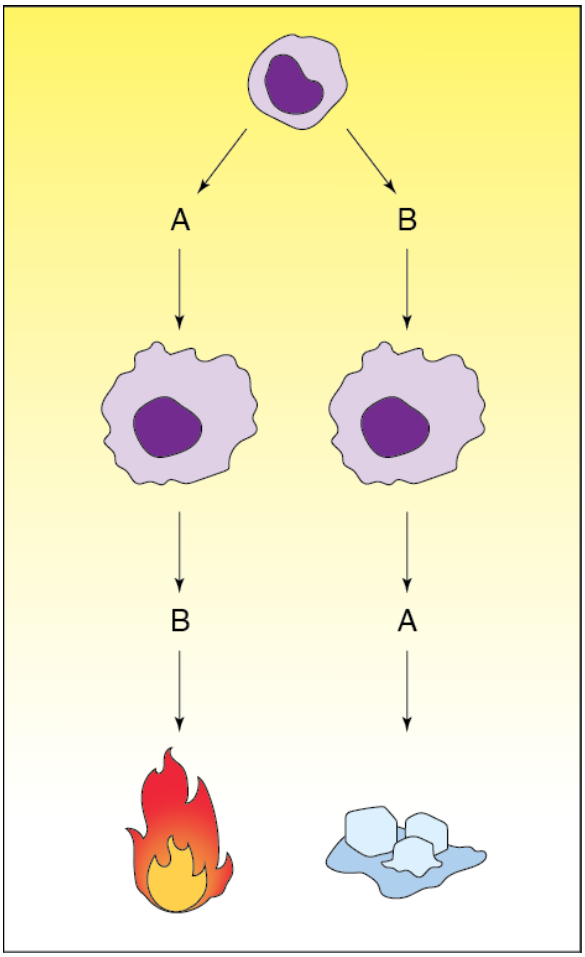

Figure 3- 1: Graphical abstract of innate immune training

3.1 Introduction

Innate immunity is the first line of host defense against external exposures. While traditionally viewed as primitive and nonspecific, a growing body of clinical and experimental evidence argues the innate immune system develops memory as a result of previous exposures, allowing the innate system to respond with enhanced and broad immunological protection upon exposure to a secondary stimulus [34]. This biological process of innate immunity has been termed trained immunity [35]. Trained immunity shares many phenotypic and epigenetic characteristics with adaptive immune memory; however, one of the starkest distinctions is the propensity of trained immunity to develop against heterologous stimuli. Innate memory is not antigen specific and is often protective against unrelated organisms, such as when vaccination with Bacillus Calmette-Guerin (BCG) affords protection against fungal or even viral infections [36, 37].

Monocytes/macrophages, dendritic cells (DCs), and natural killer (NK) cells are innate immune cells which all exhibit the ability to recollect a previous foreign encounter and subsequently mount an altered immunological memory response [38]. Exposure to high levels of bacterial lipopolysaccharide (LPS) and other toll-like receptor (TLR) agonists can induce a tolerogenic or "paralyzed" immune response whereas BCG and many other pathogens induce a proinflammatory milieu of gene expression (Table 3- 1). This enhanced immune response is predicated upon extensive metabolic and epigenetic regulation at the levels of histone modification and differential DNA methylation. The metabolic and epigenetic effects of immune training are often maladaptive and result in arthritis, atherosclerosis, or allergies [34].

The epigenetic changes observed are the result of the specific primary "training" exposure the innate cells encountered. This in conjunction with the various secondary stimuli

encountered results in distinct cytokine profiles. Research into the "exposome" looks at the combination of all exposures an individual has encountered during a set period of their life [39]. Many studies have been conducted to understand the effects of environmental exposures on human health, emphasizing the total combination of exposures. However, they do not take exposure order into consideration, yet trained immunity is significantly influenced by exposure order. This review provides insight into the epigenetic and metabolic mechanisms of trained immunity resulting from the exposome. Understanding not only the combinations of exposures, but also their order is critical for implementing early health interventions, especially in low birth-weight children, and is necessary to curb rampant development of inflammatory diseases.

3.2 Myelopoiesis

During hematopoiesis, hematopoietic stem cells (HSCs) in the bone marrow (BM) undergo lineage-specific differentiation into either myeloid or lymphoid progenitors. Lymphoid cell determination is accompanied by increased DNA methylation compared to myeloid cell determination. Consequently, inhibiting DNA methylation promotes myelopoiesis over lymphopoiesis [40]. Lineage determination is established by epigenetic changes in HSCs which adapt to the exposures they encounter, ultimately passing previous encounters to the terminally differentiated daughter cells [41]. The epigenetic marks observed in trained HSCs is consequently found in both NK cells and monocytes [42]. This process is tightly regulated to prevent disease, as a dysregulated myelopoietic shift in HSCs is associated with immune-mediated diseases such as atherosclerosis and diabetes [43]. Myelopoiesis is inducible by various inflammatory stimuli and is a key factor in the establishment of long-term trained immunity which can be perpetuated for months or years and even passed from mother to child [44, 45].

Infection of the BM by pathogens can induce a myelopoietic shift. BCG induces expansion of short-term HSCs and multipotent progenitors (MPPs) in trans by teaching the HSCs to identify MTB [46]. Upon secondary stimulation with MTB, any myeloid progenitor cells derived from trained HSCs exhibit a memory immune response. This phenomenon is also observed during primary infection with *C. albicans* which induces higher monocyte counts as well as protection against a subsequent lethal dose in which the animals experience decreased fungal load [47]. Similarly, training with β-glucan mediates a favorable response to secondary challenge and protects the individual from chemotherapy-induced myelosuppression, offering a strong protective effect against myeloid cell depletion [48]. In fact, extended TLR2 agonist treatment also induces upregulated myelopoiesis, further indicating trained immune responses occur in HSCs, not just in terminally differentiated monocytes and macrophages [49].

Surprisingly, hypercholesterolemia and a high-fat diet (HFD) can also induce a myelopoietic shift in HSCs [43, 50]. Cholesterol and other metabolites can induce DNA hypomethylation in the HSCs which are pushed toward a myelopoietic fate [51]. In this way, obesity and adipose tissue transplant are both capable of generating increased myelopoiesis in wild-type (WT) mice [52]. Blood cell counts following HFD or adipose tissue transplant reveal a significant increase in myeloid cell subsets in addition to increased activation status of those circulating myeloid subsets, while HSCs exhibit increased disposition toward myelopoiesis with an accompanying transcriptional reprogramming of myeloid precursor cells [50, 53]. This myelopoietic shift is regulated by ApoE which may play a significant role in suppressing HSC and bone-marrow myeloid proliferation [54].

In contrast to myelopoietic-driven trained immunity, many tissue-resident macrophage populations are established prior to birth and maintain themselves independent of blood-

monocyte replenishment. Tissue resident macrophages likely undergo innate immune training *in situ* and pass that memory to their progeny [55].

3.3 Triggers of Trained Immunity

Trained immunity has shown a remarkable ability to provide protection against heterologous insults stemming from various primary exposures. Heterologous immunity was observed as early as 1976 when a group vaccinated mice with BCG, a prokaryote, and then challenged those mice with Plasmodium and Babesia, both eukaryotes, and observed that mice vaccinated with BCG are protected against infection by both Plasmodium and Babesia in an anti-body independent manner [56]. Another group found mice vaccinated with BCG exhibit decreased viral titers upon infection with influenza compared to unvaccinated mice [57]. This phenomenon was independent of adaptive immunity and showed the innate immune system is capable of developing relatively long-lived epigenetic memory. Since those initial studies there has been much research into the extent to which innate immune training can elicit memory responses in NK cells, DCs, and monocytes/macrophages.

Natural Killer Cells

Natural killer cells are immune cells whose primary responsibility is the nonspecific killing of virus-infected cells and tumor cells that do not properly display surface proteins. They exist between classical innate immune cells, such as monocytes and DCs, and classical adaptive immune cells, specifically B- and T-cells. NK cells respond to training stimuli, generally inducing epigenetic reprogramming and increased cytokine production, in a manner that strongly resembles adaptive immune cell memory. While naïve CD8+ T cells and naïve NK cells differ greatly in their epigenetic profiles, both effector and memory cells of these two types are

remarkably similar. This indicates that many of the same genes and remodelers are at work within the CD8+ T cell population and the NK cell population, at least during mouse cytomegalovirus (MCMV) infection [58].

Trained NK cells can be transferred from a sensitized mouse to a naïve mouse and the naïve mouse will also experience the same adaptive immune response. This memory resided in a Ly49C-I$^+$ NK subpopulation, which localized in the donor's liver [59]. Memory NK cells expand 2- to 3-fold in the presence of MCMV. The MCMV-trained cells maintain immunological memory for up to 37 days post infection, at which point they return to pre-infection levels, whereas other training stimuli can induce longer or shorter periods of memory induction (Table S3-1) [60]. Not only can the memory response last for longer than one month, it is also preserved through cell division [44]. These conclusions were drawn from studies performed in Rag1 knockout mice stimulated with interleukin-12 (IL-12) and IL-18 to preclude the involvement of adaptive immune cells [44, 61].

Upon encountering a secondary exposure, NK cells produce increased levels of IFN-γ as well as increased cytotoxicity compared to naïve NK cells. This memory response is not only stimulated through MCMV infection, but also through BCG training and interleukin incubation [58, 60, 62, 63]. BCG notably does not increase the number of NK cells in mice, yet when challenged with a lethal dose of the fungus *Candida albicans*, all mice survive, suggesting a role for NK cells in the protection conferred by BCG (Table 3- 2) [63]. Similar results were observed when trained NK cells were challenged with *Toxoplasma gondii*, mycobacteria, leukemia cells, or MCMV. All these challenges resulted in elevated proinflammatory cytokine production, increased cytotoxicity, and improved survival [62-65].

Dendritic Cells

During an innate immune response dendritic cells are responsible for phagocytosing the invading pathogen and presenting it to the adaptive immune system. Like NK cells, DCs straddle the line between innate and adaptive immunity, both in function and in HSC differentiation. DCs exhibit many functional programs similar to monocytes and macrophages and may be partially derived from a common precursor; however, DCs have also been shown to derive from lymphoid progenitors. This may indicate a niche in which DCs exhibit memory similar to both myeloid and lymphoid cells [66]. DCs can adopt proinflammatory or anti-inflammatory profiles based upon their exposure to various fungal and bacterial stimuli in the gut [67]. This presents a mechanism for immune tolerance and surveillance resulting from the diverse exposures encountered in the gut.

C. albicans, or it's β-glucan cell wall, contributes to a tolerogenic immune state in DCs [67]. Similarly, severe sepsis induces immune tolerance as indicated by a significant down-regulation of pro-inflammatory cytokines [68]. In contrast to *C. albicans*-induced tolerance, encounters with mycobacterium tuberculosis (MTB) prime DCs toward a proinflammatory phenotype. The cells and cytokines induced by bacteria are protective against amoeba infection as segmented filamentous bacteria induce increased IL-17a and IL-23 production, in addition to neutrophil and DC migration to the intestine [69]. Much of the pathogen-associated DC proinflammatory memory is reliant on NK cells in the BM which produce IFN-γ as in the case of *T. gondii* [64]. Interestingly, diet also plays a significant role in DC training. DCs isolated from mice fed a high-fat diet (HFD) exhibit significantly increased *Tnf-α, Il6,* and *Nos2* gene expression. Upon secondary stimulation with LPS, HFD-fed DCs show further induction significantly higher than normal diet-fed mice [53].

Monocytes and Macrophages

Monocytes/macrophages are the most extensively studied example of trained immunity as they are inducible for both training and tolerance [70]. Monocytes and macrophages function in a similar manner to DCs, including comparable functional programs and common precursors. Upon traveling from the circulation into tissue, monocytes differentiate into tissue-resident macrophages which perform the action of phagocytosing invading pathogens and presenting them to the adaptive immune system. Each individual TLR agonist elicits a different, unique response which fall into two categories: tolerizing or training (Table S3- 2) [71].

Macrophage Tolerance

Immune tolerance is a state of diminished secondary immune response following a primary stimulation. Individuals who experience LPS toxic shock fall into a tolerogenic immune state which is unable to respond to secondary insult or infection. LPS and other TLR agonists can "overwhelm" the immune cells, driving decreased expression of proinflammatory cytokines and altering TLR surface expression (Table 3- 3) [70, 72]. Interestingly, monocytes from premature infants also exhibit significantly fewer upregulated genes and lower expression of those genes when exposed to TLR agonists, particularly of cytokine genes and protein production, including IL-6, IL-1β, and TNF-α. Monocytes from premature infants additionally display lower levels of phagocytosis of pathogens, which appears to be requisite for cytokine production [73]. Even intense physical training can induce immune cell infiltration into the airways, as well as altered immune function which result in tolerance [74]. Immune tolerance does not only induce altered cytokine profiles; macrophages exposed to LPS also undergo metabolic shifts, switching from oxidative phosphorylation to glycolysis [75].

Macrophages induced by TLR stimulation express lower levels of inflammatory cytokines with ROS production also diminished [76, 77]. Specifically, exposure to LPS abrogates IL-6 induction for 24-48 hours [78], but if the histone mimic I-BET is used macrophages are protected from becoming tolerized to LPS stimulation in the future, implicating specific histone modifications [79]. Significantly, tolerance is established in a dose-dependent manner as exposure to a high dose of LPS induces tolerance to secondary LPS exposure; however, a low dose of LPS trains macrophages to respond strongly to secondary exposure [77].

Using a low dose of TLR agonist can prevent immune tolerance as can pre-incubation with inflammatory signals. Pre-treatment of cells with IFN-γ prevents tolerization of primary human monocytes and restores TLR4-mediated induction of various proinflammatory cytokines, including IL-6 and TNFα [80]. Some antimicrobial peptide genes also remain inducible despite LPS-induced tolerance while non-tolerized genes respond to secondary stimulation much faster and to a higher degree than in naïve macrophages [78]. This implicates either the order or the combination of exposures in determining whether macrophages become primed or tolerized.

Macrophage Training

Macrophages have been used as an experimental model for a wide variety of training stimuli which, much like immune tolerance, is both dose and training/resting time dependent [81]. The first characterized training stimulus was BCG vaccination which induces large-scale epigenetic reprogramming and significantly alters expression profiles [36]. BCG training induces increased production of TNF-α and IL-1β two weeks and three months post-vaccination and LPS induced cytokine production remains significantly higher one-year post-vaccination [82]. Additionally, children vaccinated with BCG exhibit increased levels of cytokine production upon stimulation with purified protein injection even though immune cell counts were not significantly

increased [83]. These affects can be potentiated or abrogated by the addition of estradiol or DHT, neither of which induced cytokine training by themselves. They merely depress or exaggerate observed phenotypes [84].

Surprisingly, different batches of BCG can have slight variations in their effects. The vaccines that exhibited impaired bacterial growth rate were the most effective at eliciting a secondary response. These slow-growth vaccines tended to induce stronger immune responses in isolated monocytes [85]. Furthermore, monocytes incubated with killed (γ-irradiated) BCG showed similar cytokine expression profiles as live BCG-incubated monocytes. However, *in vivo* cytokine production by innate cells was dramatically reduced compared to live BCG vaccination [86]. This indicates live, slow growth BCG vaccines provide the greatest protection to individuals. Additionally, BCG vaccinated individuals exhibit higher rates of seroconversion when exposed to the H1N1 influenza vaccine and individuals who receive both BCG and influenza vaccines experience significantly altered effects compared to just influenza vaccination [87].

C. albicans and its β-glucan cell wall component are also potent monocyte/macrophage training stimuli [72]. Priming with β-glucan results in increased cell viability in both mouse and human monocyte-derived macrophages [88]. As a result, monocyte counts in the BM are increased following low-virulence *C. albicans* infection of mice. When administered to mice prior to lethal injection of *C. albicans* the mice exhibited increased survival times and decreased fungal loads within organs [47, 89]. In a separate study, mice were injected with a low dose of live *C. albicans*. Seven days later the mice were challenged with a lethal dose and fungal loads, cytokine levels, and survival rates were measured. WT mice were protected from the lethal dose as were RAG1-deficient mice, which lack T and B cells. However, CCR2-deficient mice, which

lack monocytes, were not protected from the lethal dose following preinfection. β-glucan, which makes up the cell wall of *C. albicans*, primes production of proinflammatory cytokines in monocytes (Table 3- 4) [90].

Macrophages also develop memory during an encounter with *Plasmodium falciparum*, the causative agent of malaria, which primes an LPS-inducible proinflammatory response in monocytes [71]. Children who exhibit a high level of IFN-γ upon monocyte stimulation with purified malarial proteins experience lower rates and severity of reinfection [91]. This may be similar to macrophages isolated from mice infected with a γ-herpesvirus. Macrophages from infected mice exhibited bactericidal activity, rapidly killing *L. monocytogenes* after uptake. Significant protection against *L. monocytogenes* was observed for up to three months [92]. This phenomenon requires live organisms as heat-killed organisms do not typically produce such an effect [93].

Several inflammatory diseases are linked to inappropriate innate immune activation [43, 94, 95]. This is predominantly induced by oxidized low-density lipoprotein (oxLDL), though glucose and other metabolites also contribute [94, 96, 97]. OxLDL, but not LDL, is responsible for monocyte training in vivo and while oxLDL does not promote cytokine production on its own, it does result in increased proinflammatory cytokine production upon restimulation [50, 94]. Similarly, pre-incubation with glucose causes monocytes to adopt long-term memory. Monocytes pre-incubated with high levels of glucose exhibit a much stronger response to secondary stimulation compared to monocytes that were not pre-incubated [96]. Even fumarate induces trained immunity via epigenetic changes, promoting a similar profile to β-glucan stimulation [97], as does high levels of blood uric acid [98].

Monocyte/macrophage training is strongly associated with both epigenetic and metabolic changes. Trained cells exhibit increased glycolytic activity and altered oxidative phosphorylation due to changes in transcription factor (TF) binding [99]. This metabolic switch toward the increased glycolysis induced upon training is dependent on both training and resting time [81]. Similarly, β-glucan stimulation induces a shift in metabolism toward glycolysis and oxygen consumption also increasing upon exposure to *C. albicans*; however, β-glucan alone induces a shift away from oxidative phosphorylation [100].

3.4 Epigenetics

Epigenetic regulation is the mechanism by which different cells and tissues in an organism perform cell-type specific programs despite having virtually identical DNA in every cell. This is how each organ in the body establishes distinct phenotypic traits and is how trained immunity is regulated as well. Trained immunity is dictated by changes in chromatin accessibility due to differentially methylated DNA and changes in histone tail modifications, both of which result in chromatin remodeling. Chromatin remodeling via these modifications modulates the enhancement or repression of immune cytokine production [101]. Additionally, TF binding is a significant feature of epigenetic changes, lineage-specific activity, and cellular memory responding to environmental stimulation [102]. These three mechanisms work in concert to drive changes in cell fate and identity as cells do not adopt new phenotypes or terminal differentiation without dynamic chromatin changes [103].

Chromatin accessibility and TF regulation are heavily influenced by external exposures. These external exposures can include microbes or their ligands, metabolites such as oxLDL, and reactive oxygen species (ROS) produced during exercise or inflammation [46, 50, 74]. Exposed

cells can even influence neighboring cells as observed when media from exposed cells is transferred to nonexposed cells. The latter also exhibit altered cytokine production, indicating there are soluble molecules secreted from exposed cells which can alter the epigenetic programming of nonexposed HSCs [76]. This cascade of changes can be perpetuated across cell divisions and even from mother to child, despite the original stimulus being removed [44, 45]. The heavy influence of the epigenome presents a mechanism by which innate immune training is passed down from mother to child to some degree [104].

The Human Early Life Exposome (HELIX) project is a study aimed at measuring and correlating the effects of early-life exposures on human health [105]. The EXPOsOMICS project utilizes high-throughput sequencing and "omics" experiments to build models of lifetime exposures and their effects on human health by measuring epigenetic, metabolic, lipidomic, and proteomic changes [106].

Histone Modifications

Distinct cell types arise from virtually identical genetic material, thus implicating fundamental changes in gene expression and DNA accessibility to control cell fate and phenotype. Eukaryotic DNA is organized into nucleosomes, a combination of histone octamers and DNA. Histone octamers are composed of two copies of each of H2A, H2B, H3, and H4 subunits around which is wrapped ~147 bp of DNA. Each histone subunit has a free N-terminal tail which can receive enzymatically-mediated post-translational modifications. Addition of these moieties results in architectural changes to chromatin as well as transcriptional reprogramming. When the chromatin is condensed into heterochromatin it becomes largely inaccessible to transcription while loosening the chromatin into a euchromatic state either poises the DNA for transcription or allows genes encoded thereon to be actively transcribed. Amid the vast repertoire

of potential histone modifications, the two most extensively studied and broadly characterized are lysine acetylation and methylation. These dynamic modifications are executed by various histone acetyltransferase (HAT) and histone methyltransferase (HMT) proteins [94, 107].

Histone modification is crucial for virtually all instances of trained immunity as inhibition of the proteins involved in modification abrogates training. The nonspecific inhibition of histone methyltransferases with 5-deoxy-5-methylthioadenosine (MTA) or inhibition of histone acetyltransferases with epigallocatechin-3-gallate (EGCG) drastically inhibited the training of monocytes [72]. Additionally, the combinations of various histone tail marks can epigenetically prime or poise enhancer and promoter elements for expression. These histone modifications respond rapidly to environmental stimuli, altering chromatin and gene expression profiles to adapt to injuries or pathogen insults with significant epigenetic modification and remodeling observed between resting, tolerized, and trained immune cells [108, 109].

Histone Acetylation

Histone acetylation by HAT proteins is generally found on histone 3 at lysines 9 (H3K9ac) and 27 (H3K27ac) and is generally associated with euchromatin and an enhanced transcriptional program [110]. Acetylation of lysine neutralizes the positive charge donated by the amino acid, thus decreasing the interaction with the negatively charged DNA backbone [111]. As the histone tail is loosened from the DNA, chromatin remodeling complexes with specialized bromodomains can bind to acetylated lysine tails and move or evict nucleosomes from promoter or enhancer regions [112]. Additionally, bromodomain proteins such as the bromodomain and extra-terminal (BET) domain-containing family of proteins have the ability to perpetuate the acetyl marks to neighboring nucleosomes.

BET governs the assembly of chromatin complexes at sites involved in inflammation by aiding in complex formation at acetylated histones [79]. Significantly, histone acetylation is required for innate immune training as inhibition of HAT proteins via EGCG drastically inhibits the training of monocytes [72].

Histone Methylation

Histone methylation is the addition of between one and three methyl groups to specific amino-acid residues on histone tails. Methylation at lysine residues induces tighter DNA-protein interactions which block TF binding. Additionally, chromodomain containing proteins have the ability to perpetuate methyl marks to neighboring nucleosomes, allowing the silencing signal to be spread along the chromatin. ChIP-seq analysis of histone tail marks shows differential epigenetic profiles between trained and non-trained immune cells and so can be used to identify the epigenetic state of a cell type [38, 113]. Similarly, ChIP-seq of the coactivator p300 is important for the identification of active compared to poised enhancers [114, 115]. While methylation is generally associated with chromatin condensation and decreased gene expression, this is not always the case.

The trimethylations of H3K27, H3K9, and H3K79 are linked to repression whereas monomethylation of H3K27, H3K9, H4K20, H3K79, and H2BK5 are all linked to gene activation, [116-120]. Histone methylation is generally highly dynamic and can produce vastly different effects based on location and the number of methyl marks [121, 122]. H3K4me1 is associated with enhancers in virtually all cells, H3K4me2 is associated with enhancers in macrophages, and H3K4me3 is associated with active promoters [123, 124].

Innate Immune Training is Dependent on Histone Modification

Many latent enhancers are open to stimulation-specific transcription factors that can remain in place for an extended period of time. These latent enhancers become poised to respond quickly to a secondary exposure [36, 124]. Genes associated with H3K4me1-bound enhancer sites trend toward low expression whereas enhancer sites also bound with H3K27ac are upregulated. H3K27ac(-) enhancers are considered poised, while H3K27ac(+) enhancers are active [110, 113]. This is significant in trained immunity as prolonged exposure to training stimuli is unnecessary for long-term memory [110]. Moreover, the dose and nature of the training stimulus can produce opposing histone profiles [78].

Immune training with TLR agonists tends to generate an immunotolerant phenotype with accompanying histone modifications. Severe sepsis in mice causes a loss of H3K4me3 at tolerized genes, resulting in a repressive state for histones at promoters of proinflammatory genes [68, 78]. Coincident with differential histone methylation, histone deacetylases also contribute to immune tolerance [125]. However, immune tolerance can be blocked either by addition of MTA or proinflammatory signals such as IFN-γ or β-glucan, leading instead to inflammatory profiles [37, 80, 108, 126].

Upon encounter with pathogenic or inflammatory metabolic stimuli, innate immune cells undergo rapid epigenetic reprogramming at the level of histone modifications [90]. Monocytes trained with BCG or malaria exhibit an increase of H3K4me3 and a decrease of H3K9me3 at inflammatory cytokine promoters which can last for weeks or months after the stimulus is removed [86, 127, 128]. Hyperglycemia, mevalonate, and oxLDL all similarly incite a strong proinflammatory response, with enrichment of activating methyl and acetyl marks at H3K4 and

H3K9 [42, 94]. These training profiles are remarkably similar, indicating the same underlying mechanism of histone modification for innate immune training [38].

DNA Methylation

The most fundamental level of epigenetic regulation comes in the form of differential DNA methylation. DNA methylation is generally associated with decreased gene expression while DNA demethylation, including the intermediate 5hmC, are associated with increased expression. Correspondingly, as genes experience demethylation they also exhibit increased levels of activating histone marks [129]. These demethylation events are closely tied to immune cell activation and memory from plants to humans. Furthermore, they are evolutionarily conserved in both adaptive and innate immunity [101, 130]. DNA methylation can rapidly respond to infection, causing cytokine genes to be demethylated in response to biotic stressors [130]. Interestingly, infection-induced demethylation is almost exclusively found in distal enhancer elements, not at promoter regions. This active demethylation is associated with extensive epigenetic remodeling and is strongly predictive of changes in expression levels of nearby genes [131].

Activating DNA demethylation is not coupled with DNA replication or cell division [101, 129, 132]. Under steady-state conditions, Tet proteins catalyze the conversion of 5mC to 5hmC, which can then be removed via demethylases. Interestingly, 5hmC is associated with putative regulatory elements marking CG islands near the promoters of expressed, cell-type specific genes, indicating DNA demethylation is important for lineage-specific gene expression [133-135]. During inflammatory signaling, Tet2 is recruited to cytokine genes in response to cellular stimulation and is involved in regulating immune cell differentiation [132, 134].

Differential DNA methylation can respond quickly to environmental exposures, resulting in dramatic transcriptional adaptation via DNA-binding factors and Tet proteins [132, 136]. These demethylases and methyltransferases are inducible by metabolic components or infectious stimuli. Cholesterol blood lipid profiles positively correlate with global DNA methylation, with LDL cholesterol showing a strong, positive correlation [51]. Macrophages infected with live pathogens exhibit dramatic changes in DNA methylation patterns whereas treatment with heat-killed organisms does not produce such an effect [93]. Ultimately, many inflammatory signals induce hypomethylation, promoting myelopoiesis over lymphopoiesis [40].

Transcription Factors and Enhancers

Following chromatin remodeling many transcription factors are able to associate with the DNA to prime expression. Of particular importance are the TFs involved in cytokine production and those involved in metabolic switching that accompanies immune cell activation which can be assayed to determine cell fate and phenotype [102, 107]. Inflammatory stimulation increases histone acetylation and p300 recruitment [126]. Inducible p300 binding is an efficacious target for retrieving macrophage-specific inflammatory enhancers [115, 121]. Additionally, cell-type-specific factors can induce or restrict key transcription factor activity at both structurally and functionally distinct classes of enhancers. These cell-lineage-specific factors and general transcription factors can work in concert to prime enhancers throughout the genome to respond to specific stimuli [137].

Transcription factor binding additionally can create regions of low DNA methylation and may indicate a mechanism by which innate immune cells can induce DNA methylation or demethylation during training. This is the result of stimulus- and cell-specific transcription factor binding within immune cells or in HSCs [41, 136]. Epigenetic changes are observed in several

important signaling pathways due to pathogen-associated immune training [36, 127, 138]. Furthermore metabolite-induced training is also strongly dependent on exposure-specific TFs such as MyD88 and mTOR [50, 53, 98].

Many genes related to glycolysis are significantly altered via Hif-1a, the master regulator of glycolytic gene expression in the cell [99, 139]. Of note, the TFs of the STAT family are strongly associated with innate immune training. STAT1 induction is associated with active histone modification and is crucial for *Candida*-induced trained immunity [119, 140]. Similarly, STAT4 is significant during the expansion of NK memory cell populations which is also dependent on NKG2D and DNAM-1 [62, 65].

Transcriptomics

Changes in gene expression levels are closely associated with changes in chromatin accessibility [70]. This process is intimately linked to chromatin marks and to specific exposures. The various histone tail marks and TFs discussed previously often directly correlate with transcription levels [113, 116-118, 141]. Measuring transcription levels in conjunction with mapping the chromatin landscape provides a more complete picture of the epigenetic mechanisms of trained immunity. As the chromatin relaxes, the DNA becomes more accessible to DNA-binding proteins such as TFs which activate expression of cell-type- or stimulus-specific genes [124, 129]. In many cases, gene activation is poised long-term even after the initial stimulus has been removed as with BCG in the BM [46, 82, 110]. Expression does not always increase, but accessibility does, ultimately allowing for the rapid induction of proinflammatory gene expression upon secondary insult [36, 46]. This is commonly observed in HFD-fed mice which show long-term transcriptomic reprogramming that is not reversed with short-term diet changes [50, 53].

As innate immune cells become activated or tolerized they develop distinct epigenetic profiles [108]. In some instances of immune training those changes are highly correlated as in β-glucan and mevalonate [38, 108]. RNA-seq shows cells injected with β-glucan demonstrate that pathways involved in innate immune function and pathways of cell metabolism, including glycolysis, cholesterol biosynthesis, and especially the mevalonate pathway, are overrepresented in the upregulated genes [48, 90]. Consequently, upon secondary exposure to inflammatory signals, macrophages begin producing inflammatory cytokines and ROS in response to the perceived attack [45, 64, 142].

In contrast, tolerized cells adopt different epigenetic profiles recalcitrant to inflammatory stimulation, with an overrepresentation of anti-inflammatory gene expression and an underrepresentation of inflammatory cytokine expression [73, 76, 79]. Concordantly, pathways involved in lymphocyte development and function are overrepresented in the downregulated genes [48, 90]. Remarkably, monocytes from premature infants exhibited significantly fewer upregulated genes and lower expression of those genes when exposed to inflammatory stimuli. This was particularly true of cytokine gene transcription and protein production [73]. In addition, genes associated with electron-transport and glycolysis are downregulated in tolerized cells [99, 100, 139]. Together, the epigenetic landscape and transcriptomic profile of innate immune cells dictate the memory and activation of trained immune cells.

3.5 Metabolism

Innate immune training is accompanied by dramatic shifts in cellular metabolism. Trained monocytes display high glucose consumption, lactate production, and NAD+/NADH ratio changes, reflecting a shift in the metabolism of trained innate cells. Human primary

monocytes trained with β-glucan are primed to express increased levels of *HIF1a* and subsequently *mTOR* which induces a rapid shift toward glycolysis, accompanied by decreased oxygen consumption [100, 143]. Conversely, *C. albicans* training induces a glycolytic shift, but oxygen consumption is dramatically increased [100]. BCG similarly induces monocytes to switch metabolism strongly toward glycolysis and other genes involved in metabolism and lipid biosynthesis; however, oxygen consumption also increased compared to non-trained monocytes [108, 127].

Sterile exposures similarly generate epigenetic reprogramming in inflammatory pathways. Glucose metabolism and mTOR signaling are crucial in mevalonate-induced trained immunity as well as during fumarate accumulation [38, 97]. LDL cholesterol is also a potent mediator of inflammatory signaling within innate immunity, resulting in proinflammatory cytokine production during secondary exposure [51, 144]. Associated with obesity, specifically high cholesterol, HSCs shift toward increased myelopoiesis and inflammatory cytokine production [43, 52]. Uric acid levels can likewise induce mTOR activation, even in the absence of other inflammatory mediators [98].

Trained immunity is heavily dependent on a metabolic switch toward glycolysis through the TF Hif-1a as is it the most readily available energy source [99, 139, 145]. Hyperglycemia, therefore, stimulates the immune system to adopt an inflammatory expression profile by stimulating increased glycolysis [42, 96]. These effects play key roles in both the beneficial and maladaptive roles of trained immunity with has life-long consequences on human health [34, 105].

3.6 Infection survival

Trained immunity provides both the context and the mechanism by which exposure to an organism elicits improved survival rates in a T- and B-cell independent manner. Even insects are able to mount trained immune protection against a subsequent lethal dose [146, 147]. Likewise, pre-treatment of mice with *C. albicans* has a remarkable ability to elicit decreased bacterial and fungal loads in the host, even when a normally lethal dose is administered [37, 47, 89, 90, 148]. This phenomenon is also observed following BCG vaccination, though remarkably it provides protection against unrelated pathogens as well [149-151]. Individuals vaccinated with BCG experienced lower titers of parasites, bacteria, and fungi [152]. Most surprising is the ability of BCG vaccination to train the innate system against viruses [36, 57, 153].

Conversely, training with various viruses also primes the innate system against bacterial infection. Macrophages from MCMV infected mice exhibited bactericidal activity, rapidly killing *L. monocytogenes* after uptake. Surprisingly, MCMV latently infected mice were also resistant to *Y. pestis [92]*. Similarly, mice show improved resistance and pathogen clearance following pre-treatment with LPS, *C. albicans*, or various parasites [107, 148, 154]. This mechanism also protects individuals from malaria, as children who are exposed to purified malarial proteins experience lower rates and severity of reinfection resulting from high levels of proinflammatory cytokine production [91, 155].

Inflammatory/Disease Phenotypes

While trained immunity can provide incredible benefits through induction of heterologous protection, there is always the risk of inflammatory disease. Environmental factors, specifically in terms of the exposome, are important for the development of diseases such as atherosclerosis and other diseases due to the propensity of trained monocyte-derived

macrophages to infiltrate tissue [43, 53, 95]. Obesity and hypercholesterolemia are associated with increased myelopoiesis which induces trained macrophages to release proinflammatory cytokines to sterile signals [43, 50, 52]. This leads to high uptake of oxLDL and an inappropriate inflammatory environment that damages host tissue [94].

Environmental exposures can be similarly destructive, particularly in the gut or lungs. Intense exercise and air pollution negatively affect air capacity and can cause airway acidification and innate cell infiltration [74, 156]. As a result, individuals are more susceptible to reactive airway disease, asthma, or infection resulting from altered physiological function [38, 156]. Similarly, improper inflammation can cause disease in the gut ranging from Crohn's disease to cancer [81, 157, 158].

Heterologous Immune Protection

What makes trained immunity distinct from adaptive memory is its ability to consistently respond to heterologous environmental exposures [138, 159]. Unlike the adaptive immune system which generally confers antigen-specific protection, innate immune training coordinates a general inflammatory state that can respond strongly to non-specific targets [158, 160, 161]. It is predictable that BCG would protect against MTB, yet it consistently demonstrates training against unrelated bacteria such as *Staphylococcus aureus* or *Escherichia coli* (Table 3- 5) [82]; viruses, including some strains of influenza [57, 87, 162], some herpesviruses [92, 153], and even Yellow Fever [36]; many eukaryotic parasites ranging from Plasmodium to Babesia [56, 63]; and even cancer [157].

Microbes found within the gut of individuals act as strong trainers of innate immunity. Segmented filamentous bacteria can cause immune cell migration to the intestine and stimulate protection against amoeba infection [69]. Similarly, *C. albicans* in the gut contributes to a

tolerogenic immune state which protects the host from excessive or destructive inflammation [67, 148]. Conversely, exposure to environmental stimuli trains the innate system in opposition to the induction of a tolerogenic state [45, 88, 142, 163]. Training with several eukaryotic parasites encourages a proinflammatory profile against many bacterial, fungal, and parasitic microbes [71, 154]. Interestingly, when individuals encounter multiple exposures, they display unique expression programs most strongly influenced by the latest contact [159].

Sex-dependent Differences

It is important to be aware of sex-associated differences in immunity when studying vaccine development or immunostimulatory therapies [104]. In the case of DPT vaccination, infant mortality significantly increased, particularly among female recipients [158]. This is in contrast to the sex-dependent benefits of BCG and MV in which females have lower mortality rates than males [149]. Interestingly, sex-associated differences with BCG are more pronounced in males early on but wane quickly. Females exhibit changes later and for a longer period. Furthermore, other live-attenuated vaccines show greater benefits in females whereas detrimental effects of non-live vaccines are also more exaggerated [84, 150]. These sex-dependent differences cannot be discounted when studying trained immunity or exposomics as the effects of exposures may be beneficial in one sex while detrimental in the other.

3.7 Trained Immunity Mechanisms

Innate immune training is coordinated by exposure-specific epigenetic changes which induce altered transcriptomic profiles. Innate cells encounter either sterile or pathogenic stimuli which generate distinct inflammatory profiles in a T cell independent manner [92]. Stimulation with pathogens often primes immune cells to release stimulatory cytokines which in turn train

other cells [64, 128]. However, this phenomenon is highly dependent on local immunity [57]. In the gut, exposures to pathogens tends to induce immune tolerance locally while inflammatory training to the same pathogen can still occur elsewhere in the host [164, 165]. Of particular note is when stimuli are found within the circulatory system. Many sterile inflammatory mediators such as glucose and cholesterol can induce organism-wide changes [165]. This provides a mechanism by which environmental exposures can establish trained immunity locally or globally.

Innate immune cells can be sensitized by cytokines produced either by themselves or by other WBCs, causing the chromatin to relax and adopt acetylated histones at promoter and enhancer regions [81]. Pre-treatment with inflammatory cytokines prevents TLR-inducible tolerization in NK cells and macrophages [80, 140]. Enhancer landscapes can be primed by other environmental cues or developmental programs, both of which lead to rapid and flexible responses to stimuli and differentiation [115, 137]. During this training, H3K9me may be reduced at key inflammatory cytokine genes, allowing for a more rapid secondary immune response upon restimulation [119, 122]. This provides a mechanism by which memory is established and maintained via cytokine exposures.

During the course of infection, pathogenic components are frequently shuttled to the bone marrow where they train HSCs. Upon primary encounter, HSCs undergo extensive differential DNA methylation due to transcription factor binding, particularly near the promoters of expressed, cell-type specific genes [135, 136]. Demethylation is mediated by Tet proteins which catalyze the conversion of 5mC to 5hmC, which is subsequently removed by demethylases [132, 134]. Interestingly, 5hmC itself acts to prime gene expression at cell-type-specific genes [133-135]. However, training does not necessarily occur in the bone marrow or blood but may be

perpetuated in the tissue [55]. This indicates a mechanism by which immune training can be maintained far longer than the life of any individual cell population.

Upon secondary exposure, trained innate cells respond more quickly and more aggressively than during primary stimulation [126]. This immune cell activation is associated with significant metabolic reprogramming through Hif-1a [75, 99, 139]. Other pathogen surface markers can likewise promote metabolic switching via TLR stimulation [100]. By shifting metabolism toward higher glycolytic activity, cells ramp up production of cytokines and ROS involved in pathogen clearance [81, 99]. This is dependent on the TF STAT1, as individuals with mutated STAT1 are unable to mount trained immune responses [140]. This provides a mechanism by which immune training can be detected by measuring cellular metabolism.

3.8 Exposome

The exposome is the compilation of everything an individual has encountered throughout their life [39]. The exposome varies spatiotemporally and is highly dynamic and diverse thus providing another mechanism by which trained immunity or immunotolerance may be established [166]. Studies of the exposome are relatively sparse, yet the effects on human health are extremely dramatic. Two major projects aimed at unraveling the exposome are The Human Early Life Exposome (HELIX) project and the EXPOsOMICS project. The HELIX project is a study aimed at measuring and correlating the effects of early-life exposures on human health [105]. The EXPOsOMICS project utilizes high-throughput sequencing and “omics” experiments to build models of lifetime exposures and their effects on human health [106, 167].

Before an individual is born, they show evidence of epigenetic reprogramming because they are exposed to many stimuli encountered by their mother while also inheriting maternal

epigenetic marks. Following their birth, each individual encounters environmental pollutants, pathogens, and allergens unique to them [168, 169]. Even fluctuations in diet can cause long-term variations in health outcomes, variations that even exist between family members in the same household [144, 169]. Furthermore, cohabiting individuals maintain uniquely identifiable external microbial clouds and internal microbiomes [168, 170, 171]. The combination of lifetime exposures establishes serious consequences in the development of inflammatory diseases [95, 172].

Exposure to many airborne antigens can induce inflammatory airway diseases such as asthma. Individuals who walk along high-traffic streets experience airway acidification and immune cell infiltration, similar to that induced by intense exercise [74, 156]. Unexpectedly, other airborne antigens such as dog-associated house dust, can provide protection of airways. While some of this protection is attributable to changes in the gut microbiome, the mechanism by which one person develops allergies to dog-associated dust while their sibling is protected remains unclear [173]. Because the training stimulus may be potentiated or abrogated by a secondary stimulus, it is reasonable to conclude that the order of exposures may actually be more important than the combination of exposures. This has been demonstrated in childhood vaccination as well as in animal models, many of which demonstrate significantly altered inflammatory profiles based solely upon the order of exposures received [159].

3.9 Conclusions

Trained immunity is a well-established phenomenon found in organisms ranging from plants to humans. Much of the research performed has focused solely on either the primary or the secondary stimulus, illustrating the extent to which the combination of exposures determines

epigenetic reprogramming; however, in many cases the order of exposures is likely more significant than the combination of stimuli can adequately explain. The most dramatic example is when primary LPS and secondary LPS + IFN-γ are inverted (Table 3- 6). A similar effect is observed when primary LPS and secondary β-glucan are likewise inverted. These examples demonstrate a proclivity for the secondary exposure to induce a stronger influence than the primary exposure.

Further research conducted on the exposome should take order into consideration as emphasis on solely one stimulus or the other paint an incomplete picture of immune training. This may be particularly substantial in airway diseases, such as asthma or allergies, wherein some individuals develop allergies to pollen while others do not [74]. This may be partially attributable to whether the individual's immune system was primed prior to exposure to said allergen. Understanding exposure order could significantly benefit atopic individuals.

It is worth reiterating that vaccine order can dramatically affect mortality rates in newborns [159]. Accordingly, vaccination schedules should be reevaluated as specific vaccines can induce either heterologous immune protection or innate immune tolerance. By reordering vaccine administration, physicians can potentially reduce childhood sepsis and airway infections without necessitating additional vaccine development. Animal studies and, eventually, human trials may be beneficial in reducing childhood mortality, thus reducing global healthcare costs through non-specific prevention of infections.

Further vaccine development must also take into consideration heterologous innate immune effects. While trained immunity presents a compelling avenue for novel vaccine development, care must be taken to prevent detrimental immune tolerance. Further research should be conducted to better understand exposure order. Inverting BCG vaccination with its

various secondary exposures would be an important first step as it represents an existing vaccine currently in deployment. By altering administration of primary and secondary exposures, researchers will better explain the extent to which exposure order can reprogram the innate immune system. Ultimately it is not the individual components that dictate the outcome of the innate immune response but the combination, order, and potency of the stimuli that decides the fate of the system and even the survival of the host organism.

Table 3- 1: Secondary exposures with primary BCG

Primary Ex.	Secondary Ex.	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
BCG	LPS					
	Pam3CSK4					
	PPD (TB test)					
	C. albicans					
	S. aureus					
	MTB					
	Estradiol (female)					
	Estradiol (male)					
	DHT (female)					
	DHT (male)					
	Yellow Fever					
Influenza H1N1	LPS					
	C. albicans					
	S. aureus					
	MTB					
BCG + Influenza (trivalent)	LPS					
	C. albicans					
	S. aureus					
	MTB					

Table 3- 2: Primary exposures with secondary *C. albicans*

Primary Ex.	Secondary Ex.	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
BCG	C. albicans					
Influenza H1N1						
BCG + Influenza						
C. albicans						
β-glucan						
S. cerevisiae						
Chitin						

Table 3- 3: Primary exposures with secondary LPS

Primary Ex.	Secondary Ex.	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
BCG	LPS					
Influenza H1N1						
BCG + Influenza						
C. albicans						
C. albicans hyphae						
β-glucan						
P. falciparum						
Pam3CSK						
LPS						
S. cerevisiae						
Chitin						

Table 3- 4: Secondary exposures with primary *C. albicans*

Primary Ex.	Secondary Ex.	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
C. albicans	LPS					
	C. albicans					
	S. aureus					
	MTB					
	E. coli					
	Pam3CSK4					
C. albicans hyphae	LPS					
	E. coli					
	S. aureus					
B-glucan	LPS					
	C. albicans					
	S. aureus					
	MTB					
	E. coli					
	Pam3CSK4					

Table 3- 5: Primary exposures with secondary *S. aureus*

Primary Ex.	Secondary Ex.	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
BCG	S. aureus					
Influenza H1N1						
BCG + Influenza						
C. albicans						
C. albicans hyphae						
β-glucan						
Chitin						

Table 3- 6: Inverted exposures show order dependence

Primary Ex.	Secondary Ex.	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
LPS	B-glucan					
B-glucan	LPS					
IFN-g + LPS	LPS					
LPS	IFN-g + LPS					

Table S3- 1: Secondary exposures with various pathogenic stimuli

Primary Ex.	Secondary Ex.	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
MCMV	L. monocytogenes					
	Y. pestis					
Herpesvirus	L. monocytogenes					
	Y. pestis					
P. falciparum	Pam3CSK4					
	LPS					
Pam3CSK4	Pam3CSK4					
	LPS					
	C. albicans					
LPS	LPS					
	B-glucan					
	Pam3CSK4					
	IFN-g + LPS					
IFN-γ + LPS	LPS					
S. cerevisiae	LPS					
	C. albicans					
	Pam3CSK4					
Chitin	LPS					
	C. albicans					
	Pam3CSK4					
	S. aureus					
	E. coli					

Table S3- 2: Primary exposures with secondary Pam3CSK4

Primary Ex.	Pam3CSK4 Secondary	IFN-γ	TNF-α	IL-1β	IL-6	IL-10
BCG	Pam3CSK4					
C. albicans						
β-glucan						
P. falciparum						
Pam3CSK4						
LPS						
S. cerevisiae						
Chitin						

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