TWO APPROACHES TO BETTER UNDERSTAND MALARIA INFECTIONS: COMPARISONS OF *PLASMODIUM* GENOTYPING TOOLS & INVESTIGATIONS INTO THE ROLE OF NK CELL IL-10 SECRETION IN HUMAN MALARIA DISEASE

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ABSTRACT

Malaria is a complex infectious disease and clinical outcomes are dependent on an array of host and parasite factors that are only partially understood. Virulence on the part of the pathogen can stem from antigenic variation and the vast genetic diversity of parasite populations. Multiple genotyping tools exist for characterizing parasite genetic diversity which are useful for epidemiological studies and tracking multi-drug resistant strains. Herein we describe a detailed comparison of two genotyping techniques and describe an R code that can be used to further optimize a 24 single nucleotide polymorphism (SNP) assay to differentiate parasite isolates.

Host contributions to malaria infection outcomes are largely influenced by the prior experience of the immune system and components of the early inflammatory response to infection. Early NK cell IL-10 secretion in mouse models has been shown to abrogate immunopathology that leads to severe disease and death. The results presented herein highlight a potentially similar protective role of NK cell IL-10 secretion in human malaria disease.

In summary, our efforts highlight the utility of new genotyping techniques for determining *Plasmodium* genetic diversity. Using samples from Malian donors we also broaden the known biological role of NK cell IL-10 secretion in human uncomplicated malaria infections.

This work is dedicated to the individuals who have felt the brunt of malaria disease and to the families of those whose immune systems were unable to overcome this disease.

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LIST OF ABBREVIATIONS

4-1BB	Tumor necrosis factor ligand superfamily member 9
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
CI	Confidence interval
CM	Cerebral malaria
CMV	Cytomegalovirus
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DNAM-1	DNAX-accessory molecule-1
ECM	Experimental cerebral malaria
GPI	Glycosylphosphatidylinositol
HbS	Sickle hemoglobin
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
iRBC	Infected red blood cell
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
KIRs	Killer immunoglobulin-like receptors
LAMPs	Lysosomal-associated membrane proteins

LFA-l	Leucocyte function-associated antigen-I
LPS	Lipopolysaccharides
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
Msp	Merozoite surface protein
NCRs	Natural cytotoxicity receptors
NKG2D	Natural killer group member D
PAMPs	Pathogen-associated molecular patterns
PbA	*Plasmodium berghei*
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PI	Propidium iodide
PRRs	Pattern recognition receptors
RIFIN	Repetitive interspersed family
RNA	Ribonucleic acid
SMA	Severe malaria anemia
SNP	Single nucleotide polymorphism
STAT3	Signal transducer and activator of transcription 3
TCRs	T cell receptors
Tfh	T follicular helper

Th cells	Helper T Cells
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
WHO	World Health Organization
WT	Wild-type

CHAPTER 1: Introduction

Overview of Malaria

Malaria infections are caused by parasites of the genus *Plasmodium* which are transferred by *Anopheline* mosquito vectors. According to the latest World Malaria Report, there were an estimated 241 million malaria cases and 627,000 deaths due to malaria in 2020. The vast majority of malaria transmission occurs in low- and middle-income countries in sub-Saharan Africa and the populations most at risk for malaria death are children and pregnant women. Malaria represents one of the greatest global health burdens, and the economic impacts both direct, through healthcare or burial expenses, and indirect through lost workdays, absence from school, etc, are staggering. The Center for Disease Control estimates direct costs due to malaria to be at least US $12 billion per year, and the economic costs from indirect expenses to be many times that [1].

The burden malaria has placed on humankind has existed for thousands of years. Evolutionary studies indicate that the parasite originated about 100,000 years ago [2,3] leading to the co-evolution of the parasite and host defense mechanisms [4]. One of the best-studied examples of this selective pressure placed on the human genome is sickle cell trait. Homozygous mutations of the beta globulin gene (HbS) result in vascular complications leading to early death. Heterozygous mutations result in sickle cell trait whereby physiological changes, like hemoglobin S polymerization and alterations in the erythrocyte surface, disrupt *Plasmodium* growth and confer partial protection against fatal malaria [5]. This trait has largely been selected for in populations in malaria-endemic countries, where some regions report upwards of 20% prevalence [6,7]. As part of this co-evolution, it is likely that *Plasmodium* protozoa have developed a variety of mechanisms for escaping or subverting the host immune defense. A better understanding of mechanisms would inform vaccine and treatment efforts. A recently approved vaccine, the RTS,S/AS01 (Mosquirix™) vaccine, may improve malaria infection outcomes. Its limited efficacy (about 30-50% efficacy in preventing severe malaria) [8] and continuing anti-malarial drug resistance indicate a need for continuing efforts to understand the complexities of host-pathogen interactions.

***Plasmodium* Life Cycle**

There are 5 species of *Plasmodium* protozoa that can infect humans: *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi*. Of these, *P. falciparum* causes the greatest number of deaths. Transmission and subsequent malaria infection is initiated when *Plasmodium falciparum* parasites in the sporozoite stage are transferred from the salivary glands of a female *Anopheles* mosquito into a host during a bloodmeal (Figure 1). Most of these sporozoites are destroyed by dermal macrophages but the few that make it into the bloodstream migrate to the liver where they invade hepatocytes [9]. During this stage of infection—the liver stage—the infection is clinically silent, there are no symptoms yet present in the host. Within the hepatocyte, the parasite undergoes multiple rounds of mitosis and meiosis to form a syncytial cell called a schizont that contains tens of thousands of nuclei. These differentiate to form haploid daughter cells, merozoites, that are then released into the blood stream. The blood stage of infection then begins with merozoites invading host erythrocytes and then differentiating into trophozoites. These "ring stage" parasites, named for their appearance by microscopy, grow substantially before undergoing multiple mitotic divisions to form a multi-nucleated schizont, which then erupts from the infected red blood cell (iRBC) as daughter merozoites which then invade more RBCs, continuing the cycle. During the blood stage of infection, some merozoites differentiate into sexual forms referred to as male and female gametocytes. It is these forms that are taken up by *Anopheles* mosquitos during a blood meal and allow for the spread of infection to other individuals (Figure 1).

The blood stage of the *Plasmodium falciparum* life cycle can become highly synchronous, occurring once every 48 hours, and the iRBC rupture and immune response is responsible for inducing fever and flu-like symptoms in many individuals. Additionally, as parasites mature within erythrocytes, they metabolize hemoglobin and induce changes to the architecture of iRBCs, such as inducing the expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). This protein serves as a ligand for endothelial cell adhesion proteins and facilitates adherence of iRBCs to the host endothelial vasculature. The subsequent sequestration of iRBCs within capillaries and post-capillary venules prevents iRBCs from circulating to the spleen where they would be more easily detected and destroyed by macrophages or be destroyed due to their altered deformability. This adaptive trait distinguishes *P. falciparum* from other plasmodia that either lack the ability to sequester or do so much less efficiently and to a lesser extent [10]. Parasite sequestration occurs in multiple organ systems including the brain, lung and gastrointestinal tract. Postmortem observations as well as animal models of severe malaria disease have demonstrated associations between parasite sequestration and pathological events such as activation of coagulation cascades, disruption to the blood brain barrier, and immune evasion, although the nuances of these pathways are still being elucidated [10,11,12].

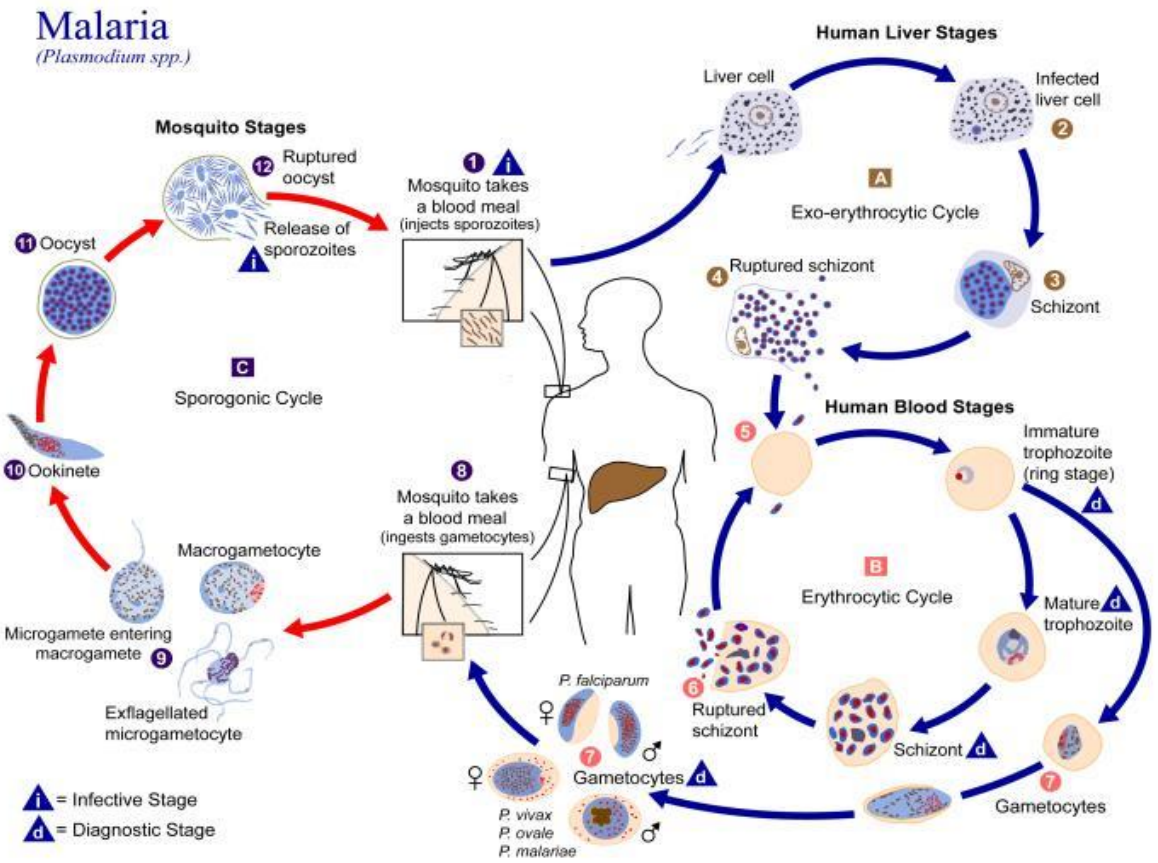

FIGURE 1: Life cycle of Plasmodium falciparum. Reprinted from Centers for Disease Control and Prevention. (2020). Global Health, Division of Parasitic Diseases and Malaria. Retrieved from https://www.cdc.gov/malaria/about/biology/.

Clinical Features of Malaria

Not all *Plasmodium* infections lead to the development of clinical symptoms. Most infected individuals maintain an asymptomatic state [13,14]. Cases of uncomplicated malaria fall in the middle of the clinical outcome spectrum and present with systemic symptoms such as fever, chills, muscle and joint pain, abdominal pain, nausea, vomiting and fatigue. At the severe end of the infection outcome spectrum, some individuals develop life-threatening complications such as respiratory distress, severe malaria anemia (SMA) and cerebral malaria (CM). This last syndrome largely affects children under the age of 6, and is characterized by high fevers, brain swelling, coma, convulsions and an average case fatality rate of ~20% for individuals that receive acute care and anti-malarial treatment [15,16,17].

The symptoms experienced during uncomplicated malaria disease are largely attributable to systemic inflammation mediated by the interaction of the host immune response with iRBCs. During the initial blood stage of infection, host macrophages engulf iRBCs, free merozoites and ruptured schizonts which leads to the early release of inflammatory mediators like TNF-α. This molecule, along with others like IFN-γ, contribute to an inflammatory cascade responsible for fever development [12].

In addition to inducing systemic inflammation, *Plasmodium* parasites act locally to induce pathology within specific host tissue compartments such as the bone marrow, gastrointestinal tract, kidneys and brain. During malaria infection, iRBCs may reside within host bone marrow [18,19]. It has been postulated that hemozoin, a parasite by-product of hemoglobin digestion, may have a role in suppressing erythropoiesis by dampening human monocyte function [20]. Hemozoin has also been shown in mouse models to accumulate within bone marrow, inducing inflammatory responses and an overstimulation of osteoclastogenesis thereby promoting bone resorption and growth retardation [21]. Gastrointestinal (GI) symptoms like abdominal pain, nausea and diarrhea are common symptoms of uncomplicated malaria and have been linked with pathological changes like increased intestinal permeability, leakage of iRBCs into the intestinal lumen and dysbiosis of intestinal microbiota [18,22]. Studies have shown that parasitized RBCs sequester within the GI tract and host inflammatory responses (especially increased histamine release from mast cells) contribute to epithelial breakdown [18,23]. The gut-vascular barrier is similar to the barriers found in other organs, like the blood brain barrier [24]. Studies of the molecular mechanisms of CM disease have revealed that blood brain barrier dysfunction, along with the upregulation of pro-inflammatory cytokines (especially TNF-α and IFN-γ), impaired vasoregulation and dysregulation of coagulation cascades play a key role in disease progression [18,19]. Within the kidneys, parasite sequestration also leads to microvascular dysfunction and endothelial activation, which have been shown to exacerbate host inflammatory responses that damage kidney tissues and lead to the development of acute kidney injury [25]. These and other studies highlight the understanding that host immune processes are instrumental in modulating pathology during disease, thereby influencing clinical outcomes following malaria infection [4,28,29].

Genetic diversity of *Plasmodium* infections and genotyping tools

In addition to host immunopathology, malaria parasite biology can also contribute to the severity of malaria infection [30]. The persistence of *Plasmodium* parasites and the course of malaria disease are influenced by factors such as parasite biomass (which is related to parasite replication and invasion rates), parasite virulence factors like variants of the cytoadhesive protein PfEMP1 (encoded by var genes), and expression of immune inhibitory receptors (such as RIFIN proteins) that mediate immune evasion [31,32,33,34].

Genome variation in the eukaryotic pathogen underpins many of these pathogenic features [35]. For instance, much of the parasite's success in immune evasion is attributed to antigenic variation— a process which involves periodically changing gene expression. Because of this many of the genes encoding virulence factors, especially var genes, are highly polymorphic [36]. Genetic diversity drives the evolution of mutations that lead to antimalarial drug resistance [37,38]. Similarly, extensive genetic diversity in the surface proteins employed as vaccine antigens can negatively influence vaccine efficacy [39].

Whole genome sequencing studies of multiple parasite isolates indicate highly genetically diverse populations [40,41]. This degree of genetic diversity can be leveraged to benefit several different purposes, for instance epidemiological studies, monitoring of drug resistance, and evaluating the effect of clinical trials of malaria therapeutics. Continuously emerging drug-resistant *Plasmodium* strains necessitate continued efforts in developing and testing novel antimalarials. During these trials, it is not uncommon for patients who are given a novel therapy to return to the clinic with another case of malaria infection. For such cases the question arises, was this a drug failure (recrudescence), or did the drug work, the patient cleared their initial infection, but then was exposed to a new infection (reinfection)? As most drug efficacy trials take place in malaria endemic regions, reinfection is a common occurrence. Current World Health Organization guidelines for conducting anti-malarial drug efficacy clinical trials recommend genotyping *Plasmodium falciparum* genes msp1 and msp2 to distinguish recrudescence from reinfection. This method was first established in 1994 as one of the first easily field deployable tools for distinguishing between (i.e. genotyping) malaria parasite isolates [42]. This technique uses a nested polymerase chain reaction (PCR) followed by gel electrophoresis to identify length polymorphisms in 5 highly polymorphic alleles (K1, MAD20, and RO33 for msp-1; IC and FC27 for msp-2). Because *Plasmodium* isolates are haploid during the blood stage of infection, each different allele detected by msp genotyping denotes a genetically different parasite isolate. During clinical drug trials, blood samples are taken from participants at baseline (before treatment), and at readmission if any participants experience malaria symptoms again in the weeks following the initial treatment. After msp genotyping, if the baseline alleles match the subsequent parasitemia alleles, this indicates an identical parasite isolate and therefore a drug failure (recrudescence). However, if the baseline alleles do not match, this indicates the presence of a new infection (reinfection).

One of the downsides to this genotyping system however is that gel electrophoresis interpretations are not always consistent or clear [43]. A meta-analysis using antimalarial clinical trial data showed substantial variation in the interpretation of msp genotyping results and that this was statistically related to the polyclonality of infections (that is, an individual, especially in high transmission areas, can host multiple parasite isolates simultaneously) [44]. In the presence of only one parasite isolate, one allele per marker is observed. But in polyclonal (also referred to as "mixed") infections, multiple alleles can be detected at one or more than one marker, complicating result readouts. Clinical trials vary in how they interpret a mixed result, in which some but not all of the alleles present in the recurrent parasitemia sample were present at baseline.

One way to improve the resolution of genotyping results is to increase the number of polymorphic alleles analyzed. Daniels, et al, set out to do this by analyzing over 112,000 SNPs identified by whole genome sequencing of *Plasmodium falciparum* isolates. A panel of 24 SNPs was designed based on features such as independent segregation, high minor allele frequency and broad distribution across the genome. This 24 SNP "barcode" provides a unique genetic signature or fingerprint for each infection [43]. Our lab set out to report the utility and performance of this novel genotyping technique compared to gold standard msp genotyping in determining recrudescence from reinfection. Additionally, other labs have used the 24 SNP barcode to evaluate the genetic diversity of *Plasmodium* isolates in different geographic locations and track the development of drug-resistant strains [45,46]. Such studies provide insights into parasite evolution and may allow us to identify parasite genotypes or adaptive features that are particularly prevalent, for instance the ability to overcome drug therapies or evade the host immune response.

Overview of Innate Immunity to *Plasmodium*

Humans have evolved a variety of mechanisms to defend ourselves from invading pathogens. If skin or mucosal barriers are breached, by the bite of a pathogen-carrying insect for instance, backup lines of defense come into play. These defenses can be divided into two branches: the innate and the adaptive immune responses. Innate immunity is a rapid, generic immune response initiated by cells such as phagocytes (macrophages and neutrophils), basophils, eosinophils, dendritic cells, natural killer cells and innate lymphoid cells. Innate immunity to pathogens relies on the recognition of pathogen-derived components, also known as pathogen-associated molecular patterns (PAMPs) which are recognized by immune cells through pattern recognition receptors (PRRs). Examples of PAMPs include components of bacterial cell walls, like lipopolysaccharides (LPS) and peptidoglycan, fungal glucans and double-stranded RNA--a hallmark byproduct of many viral infections. Malarial PAMPs include glycosylphosphatidylinositol (GPI)—an anchor protein involved in erythrocyte invasion—as well as *Plasmodium* DNA and RNA [9]. PRRs are also capable of recognizing endogenous factors called damage-associated molecular patterns (DAMPs) released during an infection. Examples of DAMPs are degraded hyaluronic acid, and proteins like heat shock protein 70 and high-mobility protein group 1 which are expressed in response to stress [47]. Malarial DAMPs include hemozoin—a polymer of heme that is a byproduct of hemoglobin digestion by parasites—and uric acid that is formed during purine metabolism by *Plasmodia* [9]. Recognition of PAMPs and DAMPs by PRRs induces various inflammatory cytokines and chemokines. These are small proteins involved in cell–cell communication and recruitment which both coordinate the early host response to infection and activate the adaptive immune response.

Overview of Adaptive Immunity to *Plasmodium*

The adaptive immune response is characterized by an antigen-specific response and the generation of immunologic memory whereby a re-exposure response elicits a more rapid and stronger immune response against pathogens previously encountered. The cells of the adaptive immune system include T cells, which proliferate in response to specific antigen presentation as discussed below, and B cells which differentiate into plasma cells to produce antibodies. The process of antibody formation and clonal expansion of T cells in response to a specific pathogen takes several days, a distinguishing feature from the rapid "first line response" of the innate immune system.

Major players of the adaptive immune response are T cells. These lymphocytes express unique antigen-recognizing surface receptors called T cell receptors (TCRs). The function of TCRs is to recognize foreign or tumorigenic antigens. Each individual T cell expresses a unique TCR and can undergo rapid proliferation and differentiation given an activating interaction with an antigen presenting cell (APC). Antigen presenting cells are immune cells that process and present antigens for recognition by lymphocytes such as T cells. Examples of APCs include dendritic cells (DCs), Langerhans cells, macrophages, B cells, and some fibroblasts and endothelial cells [48]. During the liver stage of malaria infection, infected hepatocytes serve as critical antigen presenting cells, along with DCs presenting sporozoite antigens [49].

Antigen presenting cells (APCs) express a group of cell surface glycoproteins called major histocompatibility complex (MHC) molecules. There are two classes of MHCs. MHC type I, which is found on all nucleated cells, and MHC II which is present on specific cells of the immune system like dendritic cells and macrophages. MHC class I molecules present endogenous (intracellular) peptides, like proteins made by viruses during intracellular replication, while class II MHCs present exogenous (extracellular) proteins that have been phagocytosed and proteolytically processed by the APC [48]. During an adaptive immune response to an infection, MHC-antigen complex activates a matching unique TCR, stimulating the T cell to release cytokines, proliferate and differentiate. T cells differentiate primarily into one of two subtypes, CD8+ or CD4+ T cells. CD8+ T cells are MHC I restricted, that is they only respond to antigen presented via MHC I molecules and have effector functions that include the direct cytotoxic lysis of target cells. CD4+ T cells—or helper T cells (Th cells)—are MHC II restricted and mediate and optimize the immune response in a variety of ways. There are several types of Th responses, for instance Th1, Th2, Th17, that generally exert their effects over other cells of both the innate and adaptive immune responses through the secretion of cytokines (like IFN-γ secreted during a Th1 response to activate macrophages during a bacterial infection). CD4+ T cells can also differentiate into other subsets, such as T follicular helper (Tfh) cells which play a critical role in the formation of germinal centers and facilitating the proliferation of B cells and antibody formation.

During the liver stage of *Plasmodium* infection, cytotoxic CD8+ T cells eliminate infected hepatocytes following recognition of parasite antigens presented on MHC class I molecules. Sporozoite invasion of hepatocytes is hindered by CD4+ T cell-dependent antibody responses. During the blood stage of infection, iRBC's—which lack functional MHC expression—are indirectly targeted by CD4+ T cells, especially Th1 cells that secrete IFN-γ which in turn enhances the phagocytic abilities of monocytes and macrophages, and Tfh cells that aid in the formation of memory B cells and long-lived antibody-secreting plasma cells [50].

While adaptive immune responses are essential for limiting *Plasmodium* replication and clearing an infection, other components of the adaptive immune response, especially cytotoxic CD8+ T cells, play key roles in the pathogenesis of severe disease. Several studies performed in a mouse model of CM have shown that parasite-specific CD8+ T cells directly interact with endothelial cells of brain vasculature during severe malaria and, through an IFN-γ dependent manner, induce fatal blood brain barrier breakdown and subsequent cerebral edema and neuronal death [51,52]. A study utilizing tissue samples from children who died of CM gave definitive evidence of effector CD8+ T cell adherence to the cerebral vasculature during CM, mirroring what has been observed in mouse models [26]. Adjunctive therapies targeting CD8+ T cells are currently undergoing Phase I clinical trials [53].

One novel feature of immune resistance to *Plasmodium* infection is the length of time it takes to develop clinical immunity when compared to the rapid acquisition of immunity to many viral infections (like polio, measles, and rubella for instance). Even in areas of high transmission, where children may be exposed to hundreds of infectious mosquito bites each year, individuals remain susceptible to the most severe forms of malaria until about the age of 5-10. Even then they are still susceptible to cases of uncomplicated malaria until reaching early adulthood when such cases become rarer (Figure 2) [54]. Adults who have acquired immunity to severe disease are not immune to infection and can serve as asymptomatic infectious reservoirs for malaria transmission. Having an immune response that provides protection against the clinical symptoms of malaria is referred to as "clinical immunity". Complete 'sterilizing' immunity to infection is rarely if ever achieved. From this it can be inferred that the acquisition of immunity to malaria in humans is a complicated and only partially understood process [4].

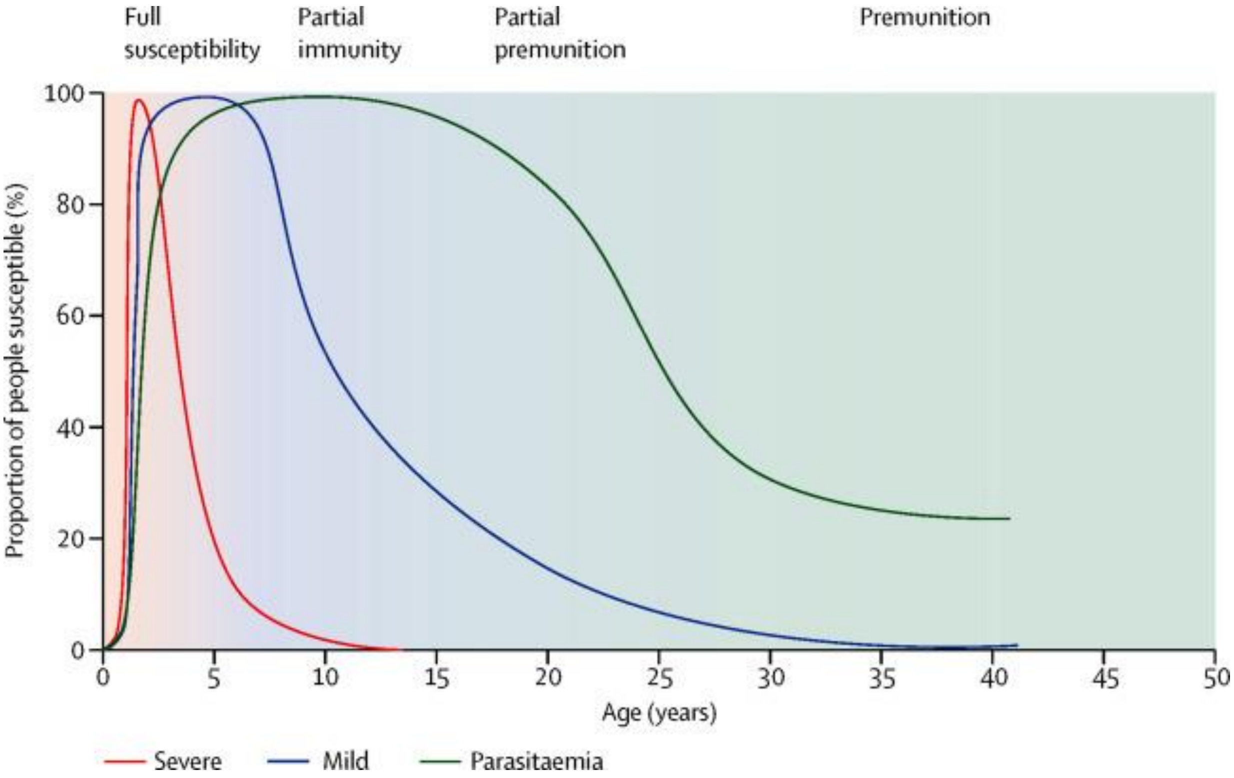

FIGURE 2: Relation between age and malaria disease severity in an area of moderate malaria transmission intensity. Note. Reprinted from "Malaria" by White, NJ, 2014, Lancet, 383, p. 724.

Cytokines as important immunoregulators during malaria infection

In most disease processes, the optimal host immune response is robust enough to clear pathogens without being overwhelming to the point of development of auto-immunity, chronic inflammation or immune-related tissue damage (immunopathology). During a malaria infection, the usual host immune response to malaria is a balance between protection and pathology. Phagocytosis and direct cytotoxic killing of *Plasmodium*-infected cells limit parasite growth, decreases parasite burden and leads to host protection. Other immune mechanisms involved in this process, such as the release of inflammatory cytokines, such as TNF, interleukin (IL)-1β and IFN-γ, can contribute to an overexuberant immune response leading to aberrant inflammation and damage to host tissues [12,26,55]. The outcome of a malaria infection depends at least in part on the balance of an immune response strong enough to limit infection, but not so robust as to cause damage to the host [10].

Pivotal players in mediating the strength and duration of an immune response are cytokines which include chemokines, interferons (IFN), interleukins (IL), lymphokines and tumor necrosis factor (TNF). These small proteins can be secreted by numerous cell types and catalyze cell signaling events. They provide a means for "crosstalk" between different cell types which allows cells to interact with their environment. Cytokines can be broadly categorized as pro- or anti-inflammatory in nature. This promoting or dampening of downstream inflammatory pathways is what allows cytokines to regulate the immune response between protection against pathogens vs immunopathology. Cytokines influence not only the pro-vs anti-inflammatory balance but can also promote or inhibit the production of additional cytokines thereby also affecting the robustness and duration of the immune response.

For studying the role of cytokines in malaria disease progression, several mouse models have been utilized. One of the most prominent is the experimental cerebral malaria (ECM) mouse model of CM, which shares many clinical and molecular characteristics with the human disease. In this model, mice are infected with the parasite strain *Plasmodium berghei* ANKA (PbA) which, like *P. falciparum*, sequesters within the microvasculature of multiple organ systems and induces severe disease, brain swelling, blood brain barrier dysfunction, and fatal brainstem herniation as is seen in human CM [51,56,57]. Notably, mice inoculated with PbA typically die from these complications within 21-30 days post-inoculation [58].

Several key cytokines have been implicated in malaria protection and/or pathogenesis in both human and mouse models. The conclusions of studies of malaria and the cytokines implicated in severe disease are complicated by factors like differing transmission intensities, rates of pathogen coinfections, age of participants or mouse models used. In general though, high levels of IFN-γ, TNF-α and IL-1β (cytokines considered to be pro-inflammatory) have been shown to correlate with malaria complications like hypoglycemia, anemia and neurological complications like CM, in both humans and mouse models [4,55,59,60,61]. Complications of severe disease have also been linked to reductions in the anti-inflammatory cytokine IL-10, which has emerged as an important immunoregulatory molecule in malaria. IL-10 suppresses inflammation by dampening the production of pro-inflammatory cytokines and downregulating MHC-II expression on antigen presenting cells. Additionally, it can promote humoral immunity by promoting B cell differentiation, proliferation and survival and antibody production [62,63,64]. In several mouse models IL-10 has been shown to play a protective role against development of severe anemia and neurological complications especially CM [60,65]. In an elegant set of experiments, Burrack et al. utilized the ECM mouse model to show that treatment with an IL-15 complex rescued mice from fatal disease. Mechanistic investigations found that IL-15 complex induced NK cells to produce IL-10, and this release was necessary and sufficient for rescue from fatal disease [66]. Additionally, the authors showed that IL-15 treatment decreased the activation of and IFN-γ production by $CD8^+$ T cells within the brain vasculature during ECM, suggesting that $CD8^+$ T cells may be mediated by NK cell derived IL-10 [66]. However, the role of NK cell IL-10 release during human malaria disease remains unclear and is a highlight focus of this thesis.

Role of NK cells in malaria defense

Natural killer (NK) cells are granular lymphocytes that constitute a major component of the innate immune response. They primarily develop within the bone marrow. Under homeostatic conditions NK cells are present in blood—where they comprise about 5-20% of circulating lymphocytes—and organ tissues such as the liver, spleen and uterus, but they can migrate to inflamed tissues in response to chemoattractants [67]. Human NK cells are identified as $CD3^-$ $CD56^+$ lymphocytes. They are known to defend against intracellular pathogen infections and contribute to tumor cell eradication. The balance regulating self-tolerance on one hand and the destruction of target cells on the other is mediated by receptors that, when engaged, initiate either inhibiting or activating signaling events. Normal healthy cells extracellularly express major histocompatibility complex (MHC) molecules that are recognized by NK cell inhibitory receptors like killer immunoglobulin-like receptors (KIRs) and the heterodimer CD94/NKG2A, contributing to NK cell self-tolerance. Tumor and virally infected cells often downregulate MHC I, which leads to a lower inhibitory signal. Additionally, cellular stress, as is seen during an intracellular infection, causes cells to upregulate proteins like MICA/MICB and CD155 that serve as ligands for NK cell activating receptors [68]. NK cell engagement with these activating ligands, in the presence of reduced or absent inhibitory signaling, tips the scales toward NK cell activation. Depending on the downstream signaling mechanisms from this interaction, activated NK cells may secrete cytokines and/or chemokines and degranulate to kill a target cell.

Degranulation is the release of secretory lysosomes containing cytolytic molecules. Formation of an immune synapse between the NK cell receptor and its ligand triggers intracellular cytoskeletal organization that promotes a polarized release of secretory granules. During NK cell degranulation, as the lysosome fuses with the outer plasma membrane of the NK cell, lysosomal-associated membrane proteins (LAMPs) like CD107a which were expressed on the inner lysosomal compartment become transiently expressed on the outer surface of the NK cell. These proteins are often used as an indirect measurement of cytolytic activity. The cytolytic components of secretory lysosomes that are released during degranulation include perforin and granzymes, which mediate target cell killing. Perforin plays a key role in pore formation on the target cell membrane and delivery of granzymes promote apoptosis through several mechanisms including caspase proteolysis and activation within the target cell. The cytotoxic degranulation of an NK cell can be promoted by engagement of Natural Cytotoxicity Receptors (NCRs) with a target cell in a process known as natural cytotoxicity. NK cells can also cooperate with the adaptive immune response by performing antibody-dependent cellular cytotoxicity (ADCC). This process is initiated by binding of the Fc Receptor, CD16, on NK cells to an antibody-coated target cell, providing a point of cooperation between innate and adaptive immune responses.

Recent studies have implicated an important role for ADCC-mediated iRBC clearance during malaria infections. Human antibodies to *P. falciparum* antigens PfEMP1 and RIFIN activate primary human NK cell-mediated ADCC, which leads to the selective lysis of iRBCs and the inhibition of parasite growth [69]. However, not all NK cells perform equally well during ADCC. A unique subset of NK cells displaying memory-like properties has been associated with more robust responses. These cells, referred to as "adaptive" NK cells, respond to certain viral reinfections or serial exposures to the same haptens in a memory-like manner, exhibiting significantly higher cytokine production and enhanced cytotoxic responses upon restimulation [70]. Adaptive NK cells have been studied most extensively in murine cytomegalovirus (MCMV) infection. The mouse-NK cell activating receptor Ly49H is required for direct recall responses and subsequent MCMV resistance in mice [71]. Similarly, human cytomegalovirus infection is associated with expansion of NK cells expressing NGK2C—the human counterpart of Ly49H. Adaptive NK cells have also been associated with upregulated CD57 (a marker of NK cell maturation) as well as deficiencies in the adapter protein FcεRγ, SYK tyrosine kinase, and the transcription factor PLZF [71,72]. Recent evidence has shown that individuals living in a malaria-endemic country (Mali) have a high proportion of adaptive NK cells. Hart *et al* have shown that these adaptive NK cells demonstrate enhanced degranulation during ADCC, and that their frequency within total NK cells correlated with lower parasitemia and resistance to malaria [73].

In addition to direct target cell lysis, NK cells are also able to influence the outcome of an infection by secreting various cytokines and chemokines that have downstream effects on other immune cells. One of the most potent cytokines released by NK cells is IFN-γ [55]. Considered to be mainly pro-inflammatory, IFN-γ has varied downstream effects, a few of which include mediating the cytotoxicity of $CD8^+$ T cells, acting as a chemoattractant promoting the migration of neutrophils, as well as modifying protein expression patterns on non-immune cells (ex: inducing ICAM-1 expression on endothelial cells during malaria infection) [55,68].

Natural killer cell activation by most pathogens is dependent on the presence of accessory cells, such as monocytes, macrophages and dendritic cells [74]. These cells can secrete members of the IL-12 family of cytokines such as IL-12, IL-23, and IL-27 and cytokines such as IL-15 and IL-18 [75]. NK cells constitutively express receptor subunits for IL-12, IL-15, and IL-18. Stimulation of NK cells with exogenous IL-12 and IL-18 induces IFN-γ secretion, and IL-15 has shown to play key roles in inducing NK cell proliferation and survival [76,77,78]. In the setting of malaria infection, studies have shown that the *in vitro* activation of NK cells by iRBCs--and the subsequent NK cell–derived IFN-γ response—are dependent on the cytokine production of accessory cells, especially dendritic cells and monocytes [79]. In a mechanism of bidirectional crosstalk, NK cell–derived IFN-γ enhances monocyte phagocytosis and the production of inflammatory cytokines [55].

Another important cytokine produced by NK cells is IL-10. It increases the expression of cytotoxicity-related genes in NK cells and, in combination with either IL-15 or IL-18, is a potent inducer of NK cell proliferation and cytotoxicity [80]. As discussed in an earlier section, IL-10 is also an important immunoregulator for many other immune cells and mostly exerts anti-inflammatory effects. It suppresses monocyte and macrophage functions by inhibiting their expression of MHC class II and costimulatory molecules, and by inhibiting their production of proinflammatory cytokines. It acts on dendritic cells to inhibit chemokine production, preventing their trafficking to lymph nodes where they would otherwise induce Th1 differentiation in naïve T cells. Interleukin-10 also directly dampens the activation of members of the adaptive immune response. For instance, it can act directly on $CD4^+$ T cells to inhibit their proliferation and production of cytokines including IFN-γ and TNF-α [81].

Recently, IL-10 has emerged as an important regulatory molecule in malaria that protects tissues by preventing excessive inflammation [81]. Some of the immunopathologies associated with severe malaria disease have been extensively characterized in several mouse models of malaria infection. One of the most widely used models of experimental cerebral malaria (ECM) utilizes infection with *Plasmodium berghei* ANKA which causes a neurological syndrome that closely parallels cerebral malaria. Some of the features this model shares with the human disease include parasite sequestration in the microvasculature, blood brain barrier dysfunction and fatal cerebral edema [56,57,82]. The pathology in ECM has been shown to be largely mediated by $CD8^+$ T cells [83,84]. However, treating ECM mice with an IL-15 complex (IL-15C) protects mice from ECM and dampens CD8$^+$ T cell activation. Notably, IL-15C mediated survival requires NK cell-derived IL-10 [66].

Human studies attempting to characterize a general role of IL-10 in malaria disease have had conflicting results [80]. Genetic variants of the IL-10 gene that are associated with enhanced IL-10 production were found to increase the risk of developing clinical malaria in young children [80]. Increased levels of circulating IL-10 have been reported in patients with cerebral malaria [59]. Evidence for a protective role exists in severe malaria anemia, where studies have shown that children with severe anemia had lower plasma IL-10 levels than healthy controls and patients with moderate anemia, implying that IL-10 plays a role in preventing severe anemia [85]. The overall actions of IL-10 are likely affected by its cellular source, as well as the timing and robustness of its release. Examining IL-10 release from individual components of the immune response, for instance NK cells, will allow us to better characterize the overall role of this important immunomodulator.

Given this background, in the following chapters I will describe two different approaches to understanding the complexities of malaria disease. In chapter 2 I will present an adaptation of our manuscript that compares *msp* genotyping and SNP barcoding for *Plasmodium falciparum* genotyping. In chapter 3, I switch from the parasite side of infection to the host immune contributions to disease as I present our studies aimed at characterizing IL-10 secretion from NK cells in the context of malaria infection.

CHAPTER 2: Comparison of msp genotyping and a 24 SNP molecular assay for differentiating Plasmodium falciparum recrudescence from reinfection

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Abstract

Background: Current World Health Organization guidelines for conducting anti-malarial drug efficacy clinical trials recommend genotyping Plasmodium falciparum genes msp1 and msp2 to distinguish recrudescence from reinfection. A more recently developed potential alternative to this method is a molecular genotyping assay based on a panel of 24 single nucleotide polymorphism (SNP) markers.

Methods: Performance parameters of these two genotyping methods were compared using data from two recently completed drug efficacy trials. Blood samples from two anti-malarial therapeutic trials were analyzed by both msp genotyping and the 24 SNP assay. Additionally, to conserve time and resources, the statistical program R was used to select the most informative SNPs for a set of unrelated Malawian samples to develop a truncated SNP-based assay for the region surrounding Blantyre, Malawi. The ability of this truncated assay to distinguish reinfection from recrudescence when compared to the full 24 SNP assay was then analyzed using data from the therapeutic trials.

Results: A total of 360 samples were analyzed; 66 for concordance of msp and SNP barcoding methodologies, and 294 for assessing the most informative of the 24 SNP markers. SNP genotyping performed comparably to msp genotyping, with only one case of disagreement among the 50 interpretable results, where the SNP assay identified the sample as reinfection and the msp typing as recrudescence. Furthermore, SNP typing was more robust; only 6% of samples were uninterpretable by SNP typing, compared to 19.7% when msp genotyping was used. For discriminating reinfection from recrudescence, a

truncated 6 SNP assay was found to perform at 95.1% the accuracy of the full 24 SNP bar code.

Conclusions: The use of SNP analysis has similar sensitivity to the standard msp genotyping in determining recrudescence from reinfection. Although more expensive, SNP typing is faster and less work intensive. Limiting the assay to those SNPs most informative in the geographical region of interest may further decrease the workload and the cost, making this technique a feasible and affordable alternative in drug efficacy trials.

Background

In the face of continuing emergence of anti-malarial drug resistance, therapeutic trials of novel drugs, or combinations of known drugs, remain a critical part of a defensive strategy for combating this high-morbidity disease. A challenge with such studies arises in areas where malaria is endemic, such as sub-Saharan Africa, where individuals experience frequent infections. For example, the Chikhwawa district in southern Malawi has an estimated inoculation rate of 183 infective bites per person per year [86]. In such high transmission settings, it is imperative to determine whether infections occurring during the course of therapeutic drug trials are due to a recurrence of the initial infection (recrudescence) or a new infection (reinfection). This is a critical distinction, as recrudescence signifies resistance of the infection to the anti-malarial regimen, while a reinfection merely reflects a high transmission setting.

To distinguish between these two possibilities, each infection is characterized or “fingerprinted” using molecular genotyping of highly polymorphic alleles. Genotypes of the infection at enrollment, prior to the administration of the trial drug, are then compared with the post-treatment infection. Recrudescence is characterized by identical molecular fingerprints at both time points. In reinfection, on the other hand, the molecular fingerprints at the two-time points differ.

Genotyping to distinguish between reinfection and recrudescence is an effective approach because of the wide range of genetic heterogeneity among individual Plasmodium falciparum parasites, which are responsible for the majority of human malaria morbidity and mortality. Genetic diversity within this species is augmented by recombination events during its obligate sexual stage within the mosquito vector. Genetic diversity is particularly evident in high transmission settings like Malawi and other sub-Saharan African countries.

The current gold standard recommended by the World Health Organization (WHO) for distinguishing genetically distinct parasites involves genotyping the merozoite surface protein 1 and 2 (msp1 and msp2) genes to identify length polymorphisms [87,88]. Msp genotyping is widely used and, because of the extensive polymorphism of msp1 and msp2 [89], is highly sensitive and specific. However, it is labor-intensive and time consuming, involving nested polymerase chain reaction (PCR) reactions and gel electrophoresis, and is therefore prone to amplification errors from contamination as well as subjective interpretation of band size on gel imaging.

An alternative method, a Taqman-based 24 single nucleotide polymorphism (SNP) barcode [90] has the potential to replace the cumbersome msp1 and msp2 genotyping [90]. The barcode method is less time and labor-intensive, as it uses only one round of DNA amplification and does not require electrophoresis. The results are relatively easy to interpret, and reactions can be monitored in real time. The particular 24 SNPs incorporated into this assay were chosen not only for their high minor allele frequency but also for their distribution across the 14 chromosomes of the P. falciparum genome, providing a high-resolution platform for distinguishing two closely related parasites [43]. Before the barcode method can be recommended as a replacement for genotyping msp1 and msp2, the two techniques must be compared in terms of their capacity to accurately distinguish recrudescence from reinfection. Performance characteristics of the two assays were compared using samples collected during two recent drug efficacy trials in Malawi.

In addition to comparing *msp* genotyping to the originally designed 24 SNP barcode, we also developed a method to customize the barcode for Malawi. The original 24 SNP barcode was developed using sequences from parasites from West Africa and Southeast Asia. We hypothesized that for drug efficacy studies in a smaller geographical area, a smaller number of SNPs would be sufficient to distinguish recrudescence from reinfection; this would increase the efficiency and decrease the costs of the assay.

In addition to its usefulness in distinguishing recrudescence from reinfection, the 24 SNP barcode is also often used to determine whether an infection represents a single genotypic clone or a mixture of multiple clones. Given that the malaria parasite is haploid during the blood stage in the human host, a single clone will reveal homozygous results at all 24 SNPs, whereas a mixed infection would reveal heterozygosity at one or more of the SNPs. Although identifying mixed infections was not the focus of this study, the truncated assay was also evaluated for this application as this is a common use of the 24 SNP assay.

Materials and Methods

Study area and population. To compare the barcode method versus *msp* genotyping, this study utilized 66 paired samples from cases of recurrent malaria infection during two therapeutic efficacy studies conducted among children age 6 to 59 months in Malawi between July 2011 and November 2012 at Machinga District Hospital and between March and July of 2014 at Machinga, Nkhotakota, and Karonga District Hospitals [91]. Consented, eligible children with fever or history of fever and *P. falciparum* mono-infections (i.e. no evidence of infection with another *Plasmodium* species) with 1000–200,000 asexual parasites/µl were enrolled (day 0) and followed on days 1, 2, 3, 7, 14, 21, 28, and 42 in the earlier study, and through day 28 in the later study. In addition, children were seen on any other day if they were ill. At each visit, blood was collected for thick and thin smears and for molecular testing on filter papers (Whatman 3MM). Patients with microscopically detected parasites on any day after day 3 were deemed to have recurrent parasitemia and samples were tested by both *msp* and SNP genotyping methods as described below to differentiate recrudescence from reinfection. All genotyping was performed blinded to patient information.

For the evaluation of a geographically relevant truncated bar code, samples were obtained from patients (n = 294) admitted to the pediatric research ward at Queen Elizabeth Central Hospital in Blantyre, Malawi from January to June during the years 2009 and 2011. Peripheral blood was collected on FTA cards (Whatman 3MM) at admission and SNP genotyping was performed as described below.

DNA extraction. DNA was extracted from blood spots using QIAamp DNA Mini and Blood Mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions.

Msp1 and msp2 genotyping. A nested PCR was used to amplify the polymorphic repetitive regions block 2 of *msp1* [92] and block 3 of *msp2* [93] of all day 0 and day of recurrent parasitemia samples [94]. Parasite DNA samples from standard laboratory strain 3D7 were included in each genotyping run as positive controls whilst water was included in each run as a negative control. The sequences of the primers and their respective positions in the respective genes are presented elsewhere [95]. In the first nested reaction, oligonucleotide primer pairs corresponding to conserved sequences spanning the polymorphic regions of the two genes were included.

To determine the presence of allelic variants for *msp1* and *msp2*, the product generated in the reaction above was next used for a subsequent round of five separate nested reactions. The five nested PCR reactions used specific primer pairs for MAD20, K1, and RO33 families of the *msp1* block 2, and the FC27 and 3D7/IC families of the *msp2* repeats as listed elsewhere [96]. Amplifications were performed on a BIO-RAD T100TM thermocycler [96] and separated on a 2% gel for both *msp1* and *msp2.*

Full 24 SNP and truncated SNP genotyping. Samples from all cases of recurrent parasite infection were genotyped using the molecular barcode assay as described previously [43]. The positions of the SNPs genotyped were as described in the original paper and annotated in PlasmoDB version 5.0. Parasite DNA samples from 3D7 were included in each genotyping run as positive controls and water was used as the negative control.

A master mix consisting of 2.95 µl of commercial grade nuclease-free water, 0.05 µl of the 40× Taqman SNP assay and 5 µl of the Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) per reaction was added to each well of the 96 well real-time PCR plate pre-loaded with 2 µl (10 ng) of each parasite DNA sample. Parasite DNA samples were then amplified on the Step One Plus real-time PCR instrument (Applied Biosystems, Foster City, CA, USA).

Analysis. Samples were classified as either recrudescence, reinfection, or uninterpretable by each method, as follows. For *msp1* and *msp2* genotyping, the number and size of the electrophoresed bands on the day of recurrent parasitemia and day 0 were compared. Bands less than 500 bp in size were considered the same if they were within 10 bp difference in size. If the number and size of bands representing allelic types on day 0 was equal to the number and size of bands observed on the day of recurrent parasitemia, the recurrent episode of parasitemia was considered a recrudescence (treatment failure). Otherwise, it was considered a new infection. Samples lacking a distinct band in any of the five amplification reactions were re-tested. Samples that twice failed to amplify were considered uninterpretable.

For SNP analysis, PCR amplification results of both the full 24 SNP barcode and truncated barcodes were completed using Applied Biosystem's Proprietary Allelic Discrimination software version 2.2.2. Where the software did not give genotype calls directly, allele calls were made manually by examining both the amplification plot and the multi-component plot. A single allelic difference between day 0 and the day of recurrent infection was considered sufficient to call the infections different (i.e. reinfection) due to the high-resolution power of the allelic discrimination software used. Similarly, if a single allele was homozygous at day 0 but heterozygous at follow up, it was considered a new infection because the addition of a new allele represents a new parasite clone. Conversely, a heterozygous allele at day 0 changing to a homozygous allele at follow up could be due to the persistence of one strain of a mixed infection, with simultaneous clearance of the second strain and, therefore, in the absence of any other allelic differences, the case was designated as recrudescence and not reinfection.

For the SNP genotyping assays, if more than 2/3 of the SNPs (i.e. 17 of the full 24 SNP assay and 5 of the 6 SNPs of the truncated assay) were identical and the remaining SNPs failed to amplify, then the case was labelled recrudescent. Sensitivity analysis showed that variation of this cutoff by 3 SNPs in either direction resulted in the reclassification of only one case (a recrudescent sample being reclassified as uninterpretable). If 1/3 or more SNPs (8 for the full barcode and 2 for the truncated assay) failed to amplify for at least one of the time points of a matched sample, and there were no other SNP differences indicating a reinfection, then testing was repeated. If no additional SNPs were amplified the sample was labelled as uninterpretable.

Samples uninterpretable by either genotyping method were excluded from the final analysis. For the purposes of this study, *msp* genotyping was considered the gold standard, and results obtained from the barcode method were compared to the *msp* method using Stata version 10.1 (Stata, College Station, TX) to assess sensitivity and specificity of the barcode method.

The 24 SNP barcode was optimized for Malawi using the statistical package R (RStudio, Boston, MA). SNP calls were formatted into a 294 × 24 matrix where the 294 rows contained SNP calls from individual samples and the 24 columns represented SNPs 1–24. A function was created that would analyze and compare all pairs of rows (samples) using all possible combinations of 6, 8, 10, and 12 SNPs. The SNP combinations that yielded the maximum number of different rows (i.e. most often correctly identified a sample as being unique from all others) were subsequently labelled as truncated versions of the full 24 SNP assay optimized for our Malawian data set. The R functions specifically used are outlined in Additional File 1. The optimized truncated 6 SNP assay was then used to identify recrudescence and reinfection among the matched samples from the two therapeutic drug trials as described above, and results were compared to both the full barcode and to *msp* genotyping.

Results

Paired samples from 66 children were genotyped to distinguish between recrudescence and reinfection. Of these, *msp1* and *msp2* classified 5 (7.6%), 48 (72.7%), and 13 (19.7%) as recrudescence, reinfection, and uninterpretable, respectively (Figure 3). Using the 24 SNP molecular barcode, 4 (6.1%), 58 (87.9%), and 4 (6.1%) were classified as recrudescence, reinfection, and uninterpretable, respectively, while 6 (9.1%), 55 (83.3%), and 5 (7.6%) were classified as recrudescence, reinfection and uninterpretable by the truncated 6 SNP barcode (Figure 3).

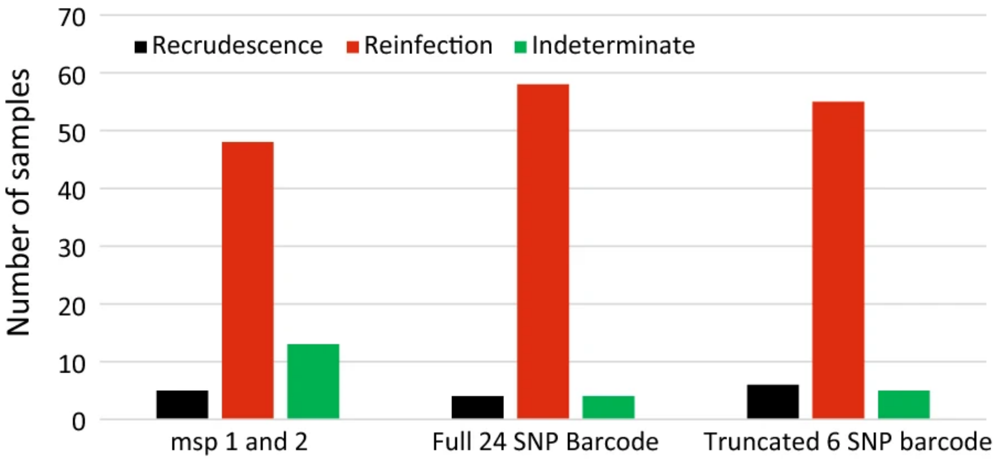


FIGURE 3: Genotyping results by msp, full 24 SNP, and truncated 6 SNP methods.

When comparing the performance of the molecular barcode to the standard practice of *msp1* and *msp2* genotyping as the gold standard for detecting reinfection, sensitivity was 100% (95% CI 90.4–100%), specificity was 75.0% (95% CI 21.9–98.7%), positive predictive value was 97.9% (95% CI 87.3–99.9%), and negative predictive value was 100% (95% CI 31.0–100%) (Table 1).

	MSP		**Total**
	Reinfection	**Recrudescence**	
24 SNP barcode			
Reinfection	46	1	47
Recrudescence	0	3	3
Total	46	4	50
		Estimated value (%)	**95% CI**
Sensitivity		100	90.4–100
Specificity		75.0	21.9–98.7
Positive predictive value		97.9	87.3–99.9
Negative predictive value		100	31.0–100

TABLE 1: Performance of the barcode using *msp1* and *msp2* as the gold standard.

The performance of *msp1* and *msp2* was also tested using the molecular barcode as the gold standard (Table 2). Sensitivity was 97.9% (95% CI 87.3–99.9%), specificity was 100% (95% CI 31.0–100%), positive predictive value was 100% (95% CI 90.4–100%), and negative predictive value was 75.0% (95% CI 21.9–98.7%). The overall correlation between the two methods was high with only one case showing a discrepancy (phi coefficient = + 0.86, p = 0.0002 via two-tailed Fisher Exact Probability Test).

	24 SNP barcode		**Total**
	Reinfection	**Recrudescence**	
MSP			
Reinfection	46	0	46
Recrudescence	1	3	4
Total	47	3	50
		Estimated value (%)	**95% CI**
Sensitivity		97.9	82.5–98.4
Specificity		100	31.0–100
Positive predictive value		100	90.4–100
Negative predictive value		75.0	21.9–98.7

TABLE 2: Performance of msp1 and msp2 using the 24 SNP barcode as the gold standard.

Some SNPs have extremely low variability in Malawian samples and therefore offer little in the way of genetic information. For instance, the vast majority of SNP 2 results are adenosine, whereas SNP 24 is almost always a guanosine (Figure 4). Limited heterogeneity at specific SNPs adds little additional information to the genetic “fingerprint” of a sample. This observation led to the effort to optimize the 24 SNP barcode for Malawi—excluding certain SNPs from the full panel without compromising the barcode’s ability to determine infection uniqueness.

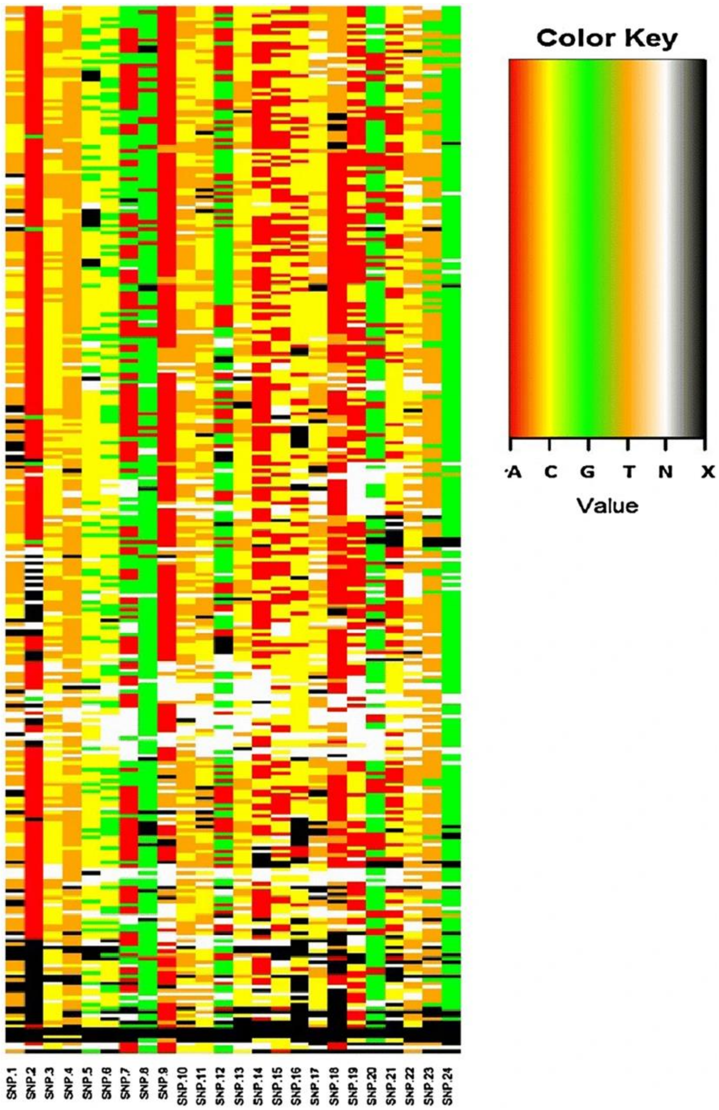


FIGURE 4: Heterogeneity of individual SNP’s among Malawian samples. In the key, values “A”, “C”, “G” and “T” indicate their respective nucleotide. Values labeled “N” represent a heterozygous or mixed finding at the respective SNP, while “X” represents missing (nonamplified) values.

The resulting optimized barcode of only 6 SNPs could, within the data set from Queen Elizabeth Central Hospital, distinguish two unique infections from each other over 98% of the time when compared to the full 24 SNP barcode. Similar test performances were found with a subset of 8, 10, and 12 SNPs (Table 3).

	6 SNP Barcode						8 SNP Barcode		10 SNP Barcode		12 SNP Barcode	
SNP	**3**	**7**	**11**	**15**	**19**	**22**	**4**	**14**	**9**	**10**	**5**	**17**
Accuracy in calling uniqueness	98.5%						99.5%		99.8%		99.9%	
Accuracy in determining heterozygosity	78.0%						79.7%		83.6%		89.3%	
% Amplification Success	98%	97%	95%	95%	97%	96%	94%	93%	96%	97%	93%	90%
Minor Allele Frequency	0.31	0.32	0.44	0.50	0.43	0.49	0.16	0.29	0.19	0.40	0.21	0.40

TABLE 3: Optimized subsets of the 24 SNP barcode.

In contrast to the ability to distinguish unique infections, the truncated bar code did not perform as robustly at identifying mixed infections. The 12 SNP barcode was only able to determine 89.3% of the mixed infections called by the 24 SNP bar code, suggesting that heterozygosity tends to occur at alleles less useful for showing the distinction between genotypes.

To determine how well these theoretically optimized barcodes could differentiate between two unique infections within the same individual (reinfection), they were then used to analyze the matched sample sets from the two therapeutic drug trials. The results were compared to both *msp* genotyping and the full 24 SNP barcode (Table 4).

	6 SNP Barcode						**8 SNP Barcode**		**10 SNP Barcode**		**12 SNP Barcode**		**24 SNP Barcode**
SNP	**3**	**7**	**11**	**15**	**19**	**22**	**4**	**14**	**9**	**10**	**5**	**17**	**Remaining**
Accuracy in identifying reinfection using 24 SNP barcode as the gold standard	95.1%						95.1%		96.7%		96.7%		100%
Accuracy in identifying reinfection using *msp* genotyping as the gold standard	91.8%						91.8%		93.9%		93.9%		93.9%

TABLE 4: Comparison of the truncated barcodes to both the full 24 SNP assay and to msp genotyping.

For comparison of the full 24 SNP barcode and the truncated barcodes, a total of five uninterpretable samples from the original 66 samples were excluded. For comparison of the truncated barcode and *msp* genotyping, 17 samples were excluded because they were uninterpretable by *msp* (n = 13) or by the truncated barcode (n = 5), with one sample overlapping. The overall sensitivity for detecting reinfection using the 6 SNP barcode compared to the full 24 SNP assay was 94.8%, and specificity was 100%.

Discussion

Molecular genotyping is a reliable method of distinguishing recrudescence from reinfection in anti-malarial drug efficacy trials. Although the WHO recommends *msp1* and *msp2* genotyping as a gold standard for distinguishing recrudescence from reinfection in areas with endemic malaria [88], this method is time consuming and labor-intensive [43]. The novel 24 SNP barcode method, which is considerably quicker and less labor-intensive, performed comparably to *msp* genotyping for distinguishing recrudescence from reinfection (sensitivity 100% and specificity 75%).

The barcode method designed by Daniels et al. [43] uses 24 highly polymorphic SNPs that are broadly distributed across the *P. falciparum* genome and not in linkage disequilibrium, to clearly discriminate alleles in mixed genome samples. Daniels et al. demonstrated that no two parasites known to be of independent origin had the same allele signature. In addition, they showed that there was generally good agreement between the barcode and *msp1* and *msp2* genotyping with respect to identifying mixed parasite samples.

This study established that the two methods are comparable in their ability to distinguish recrudescence from reinfection. Both assays have advantages and disadvantages. The *msp* genotyping method resulted in more uninterpretable calls in our hands, with more reactions failing to generate distinct bands. Our levels of uninterpretable results were similar to those in other large-scale studies [97,98]. However, it is significantly less costly to use, with per sample reagent estimates of $3.60 for the *msp* method and $11.45 for the SNP-based assay. Although the ease of the SNP assay allows for higher throughput, when technician salaries as well as the cost of repeating uninterpretable results are included in the per assay estimate, the *msp* method is still cheaper with a cost of approximately $14.36 per sample as compared to $18.66 for the SNP based method. However, truncation of the SNP assay from 24 to 6 locations decreases the overall cost to $10.25 per sample, less than the *msp* method. Furthermore, the SNP assay requires more specialized equipment, with the requirement of a real-time PCR machine. In contrast, the *msp* based assay relies on the more universally available conventional PCR machines and standard gel electrophoresis. The SNP based assay has the advantage of being higher throughput—requiring only one amplification step, whereas two are required in the *msp* method. The SNP method also avoids the step of loading samples onto an agarose gel.

In an effort to minimize time and resources while maintaining the equal discriminatory ability of the assay, the possibility of truncating the barcode was explored. Various studies have demonstrated surprisingly high levels of genetic relatedness among *P. falciparum* isolates from the same geographic region [99,100,101,102]. This suggests that, within a limited geographical region, the 24 SNP barcode might be truncated to eliminate any of the 24 SNPs which might be non-informative. The findings indicate that in areas of northern Malawi the truncated 12 SNP barcode suggested above determines whether a new infection is unique from the last cleared infection (i.e. reinfection) 96.7% of the time when compared to the full 24 SNP barcode. This would decrease time and resources by almost half. Further truncation to a set of the six most informative SNPs would still result in 95.1% accuracy in determining recrudescence from reinfection. The truncated bar code does not, however, perform as robustly when used for determining single from mixed infections, likely due to the increased advantage of detecting low frequency heterogeneity at more sites in the full bar code assay.

A significantly higher proportion of samples were uninterpretable by *msp* genotyping than by SNP barcode. Uninterpretable results seen in the *msp1* and *2* based assays were due to poor amplification or inability to determine amplicon size on gel electrophoresis, while uninterpretable results in the SNP based assay were due to poor amplification. Of the 13 samples that were uninterpretable by *msp* genotyping and 4 uninterpretable by the full barcode, only one sample was uninterpretable by both methods, suggesting assay specific failures rather than general poor quality of DNA. In the analysis of the truncated barcode, this led to a drop in the accuracy of the 6 SNP barcode in determining reinfection when using *msp* genotyping rather than the full 24 SNP barcode as the gold standard—from 95.1 to 91.8%. Using the full 24 SNP panel as the gold standard, the 6 SNP barcode only missed three cases of reinfection within the total 61 samples analyzed in that comparison. Using *msp* genotyping as the gold standard, even though there was only one additional sample disagreement, the drop in total sample number from 61 to 49 led to a 45/49 agreement, i.e. a 91.8% accuracy.

In comparing Tables 3 and 4, it is notable that the truncated barcodes did not perform as well in the analysis using *msp* results as the gold standard. For instance, the 6 SNP assay's accuracy in discriminating unique infections dropped from 98.5 to 95.1%. One possible explanation may be that the 6 SNP optimized barcode was proposed based on a large data set of unique genotypes from a geographic area immediately surrounding Blantyre. This SNP set was then used to analyze samples from Machinga, Nkhotakota, and Karonga district hospitals, which are 120, 375, and 825 km away from Blantyre, respectively. Geographic genotype variance has been shown to be very focal [99,100,101,102]. While it was originally assumed that a barcode optimized using samples from southern Malawi would still perform satisfactorily in analyzing samples from northern Malawi, perhaps a different truncated SNP panel, optimized using a large database generated from unique infections in northern rather than southern Malawi, would perform more accurately for these samples collected at some distance from Blantyre. The R algorithm could be used to determine optimum SNPs for other geographically distinct malaria endemic regions as well.

The major limitation of this study is the small sample size. This limitation is magnified by the fact that there are very few recrudescent infections by either measurement method. This leads to an exceedingly low number of recrudescent infections to be compared by the two methods. An additional limitation is the inability to amplify all alleles in all samples—in both the *msp* and the SNP based methods. Although this non-amplification was not biased towards any specific location, the cutoff of allowing up to seven non-amplified alleles could result in the misclassification of reinfections as recrudescence. Although this limitation would be equally present with either technique, it is of increased concern in the *msp* assay where there was a high percent of non-amplification (19%). Finally, the choice of samples from geographically separated regions for design and validation of the truncated bar code may have resulted in less-than-ideal performance of the truncated barcode.

Conclusions

This study showed comparable results from *msp1* and *msp2* genotyping and the SNP-based barcodes. The major downfall of the SNP-based method—increased cost—could likely be effectively ameliorated with the design of a truncated set of SNPs. In Malawi, a subset of 6 SNPs performed almost as well as the originally designed 24 SNP method; this could substantially decrease the cost without compromising data quality.

Authors' contributions

JF, SM: study design, laboratory work, data analysis and writing of the manuscript. JV: study design, laboratory work, data analysis. AS, GM: laboratory work, data analysis. TT: patient enrollment and characterization, study design and editing of manuscript. DM, JS: study design, editing of manuscript. DS: study design, statistical analysis, editing of manuscript. JG: study design, data analysis, writing and editing of manuscript. KS: study design, laboratory work, data analysis, writing and editing of manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

CHAPTER 3: Investigations into the roles of Natural Killer Cell Interleukin-10 Secretion in Human Malaria Disease

This chapter is not published at the time of dissertation submission. The following study is in collaboration with Geoffrey Hart, PhD, in the Department of Medicine and Sara Hamilton Hart, PhD, in the Department of Laboratory Medicine and Pathology at the University of Minnesota, Minneapolis.

Abstract

Natural killer (NK) cells are important defenders against malaria disease. They directly lyse infected red blood cells and release inflammatory and anti-inflammatory cytokines that can mediate the actions of other immune cells. Recently NK cell-derived IL-10 was shown to play a protective role in a mouse model of severe malaria, experimental cerebral malaria (ECM). Researchers found that treating mice with an IL-15 complex protected against ECM and that NK cell-derived IL-10 was both necessary and sufficient for mediating survival. However, the role of IL-10 secretion from NK cells during human malaria is unclear. Here, we compared the IL-10 release of NK cells from individuals from a malaria endemic country (Mali) to the IL-10 release of NK cells from malaria-naïve (US) individuals. We found that the NK cells of Malian individuals secreted significantly more IL-10 than the NK cells of malaria-naïve individuals. We also described NK cell IL-10 release under different conditions (cytokine stimulation, antibody-dependent cellular cytotoxicity, and natural cytotoxicity). We screened for extracellularly expressed NK cell markers that correlate with IL-10 release, and found that several activating immune checkpoint molecules, adaptive NK cell markers, and proteins associated with enhanced cytotoxicity were upregulated on these cells. Taken together, these findings indicate that NK cell IL-10 release may play a role in protecting against severe malaria disease and implicate a potential dual role for IL-10 releasing NK cells as being both cytotoxic and immunoregulatory in nature.

Background

Malaria is a mosquito borne infectious disease caused by parasites of the *Plasmodium* species. It claims roughly 450,000 lives per year. Over 90% of the world's deaths due to malaria occur among pregnant women and children living in sub-Saharan Africa. Recent headway has been made in the field of malaria vaccine development with the rollout of the RTS,S vaccine, but its limited efficacy in preventing malaria (around 36% in 4 year follow-up studies) highlights a need for better understanding the complexities of developing immunity against malaria infections [103,104].

Natural Killer (NK) cells are some of the earliest responders to malaria infection. These innate lymphoid cells are enriched in the liver, where the initial rounds of *Plasmodium* parasite replication occur following inoculation. They are also abundant in blood, spleen, bone marrow and lymph nodes, but can migrate to sites of inflammation in response to chemoattractants. The functions of NK cells are influenced by signals from their environment that contribute to either activation or inhibition. Healthy host tissues express major histocompatibility complex (MHC) molecules that are recognized by NK cell inhibitory receptors like killer immunoglobulin-like receptors (KIRs) and the heterodimer CD94/NKG2A. Other important NK cell receptors that mediate activating or inhibitory signals include natural cytotoxicity receptors (NCRs), the Fc receptor CD16, DNAX-accessory molecule-1 (DNAM-1), and NK group member D (NKG2D). When inhibitory signals are decreased or absent, the engagement of activating NK cell receptors can promote several effector functions including natural cytotoxicity, ADCC, death receptor mediated-killing and cytokine production. Natural cytotoxicity is initiated by NCR engagement which triggers the polarized release of cytotoxic granules into the immune synapse leading to target cell lysis. A similar process of degranulation, which links innate and adaptive immune responses, is initiated by CD16 binding to the Fc portion of an antibody bound to a target. The release of cytotoxic granules following this interaction is referred to as antibody-dependent cellular cytotoxicity (ADCC). Activated NK cells can also engage in death receptor mediated cell killing through Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which induce apoptosis within a target cell. Lastly, cytokines can also influence the activation state of NK cells, for instance by raising or lowering the threshold required for NK cell degranulation, or by encouraging or inhibiting further cytokine production and secretion from NK cells [78,105,106].

During the early immune response to malaria, which is largely confined to the liver, NK cells are one of the earliest sources of the pro-inflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) [55]. These effector molecules play important roles in initiating anti-malarial immune defenses by promoting dendritic cell activation and maturation and activating CD8$^+$ T cells, which may help inhibit the replication of schizonts in the liver and indirectly control parasitemia by amplifying the early immune response[55,61,66].

During the blood stage of malaria infection, NK cells have been shown to inhibit *Plasmodium* growth by directly lysing infected red blood cells (iRBCs). Several key studies have shown two major mechanisms for this process. The first studies demonstrating NK cell killing of iRBC's *in vitro* indicated that NK cell NCRs recognize the extracellularly expressed iRBC antigen *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP1). This interaction initiated natural cytotoxicity-mediated target cell lysis [107]. More recently, NK cells have also been shown to lyse iRBCs in an antibody-dependent manner, indicating a role for ADCC in inhibiting parasite growth [69].

NK cell mediated ADCC is also an important mechanism for controlling the replication of other infectious intracellular pathogens such as cytomegalovirus (CMV), HIV, Influenza A, dengue, HSV, hepatitis C and SARS-CoV-2 [108,109,110,111,112]. Not all subsets of NK cells are equally adept at ADCC, however. Specialized subsets referred to as adaptive NK cells display memory-like functions and enhanced ADCC capabilities [113]. These cells were originally identified and characterized as unique subsets of NK cells that expand following murine CMV (MCMV) and human CMV infections [114,115]. Characteristic phenotypes associated with adaptive NK cells include high expression levels of NKG2C which is often co-expressed with the maturation marker CD57, along with reduced expression of the signaling adapter Fc receptor γ and transcription factor PLZF [72]. Recently it has been shown in a cohort of individuals from Mali, a malaria-endemic country, that adaptive NK cells that are lacking the Fc receptor γ chain correlate with protection from malaria [73].

NK cells have also been shown to limit malaria-induced pathology in mouse models. The widely used experimental cerebral malaria (ECM) mouse model shares many features with the human disease, including neurological symptoms, parasite sequestration in the microvasculature, blood brain barrier dysfunction and fatal cerebral edema [56,57,82]. Using this model of malaria disease, our lab has previously shown that treating mice with an IL-15 complex (IL-15C) protected mice from death, and that NK cell-derived IL-10 was both necessary and sufficient for IL-15C-mediated survival [66].

The role of NK cell derived IL-10 in human malaria infections has yet to be elucidated. Studying the mechanisms of malaria pathology in humans poses several challenges, namely, ethical considerations and the costs associated with large human trials. One safe approach is to use proxies for protection vs susceptibility to severe disease. For this study we recruited healthy adolescents and young adults from a malaria endemic country (Mali) who, by this age, have had multiple exposures to *Plasmodium falciparum*. Although these individuals continue to get infected, they are typically no longer susceptible to developing severe malaria because of established immunity (see Figure 2). In contrast, individuals who have had no previous exposure to *Plasmodium* species lack parasite-specific antibodies and are particularly susceptible to developing severe disease if infected. For this study, the malaria naïve group was composed of blood bank donors from the mid-western United States. We sought to investigate whether NK cell IL-10 secretion may play a role in protecting against human malaria by comparing IL-10 secretion in NK cells from individuals within these two study groups. We quantified IL-10 secretion from these two donor groups following three different NK cell stimulation methods: cytokine stimulation with IL-15, IL-21 and IL-12, ADCC, and natural cytotoxicity. Additionally, peripheral blood mononuclear cells (PBMCs) from Malian donors were extracted at three different timepoints: pre-malaria infection (samples were taken in early May before the start of the malaria transmission season), during symptomatic malaria infection, and post-infection during the convalescent period. This allowed us to compare IL-10 secretion over the span of a malaria season and over an individual's malaria infection. Finally, we employed a 365 extracellular marker screen for the purpose of immunophenotyping IL-10 secreting NK cells.

Materials and Methods

Human Subjects

Study participants from Kalifabougou, Mali were enrolled in this study as part of an ongoing cohort study of acquired immunity to malaria. Children between the age of 5 and 15 years old were recruited and written informed consent was obtained from parents or guardians of participating children before inclusion in the study. In Mali, and many other malaria endemic countries, malaria transmission is seasonal, corresponding to periods of heavy rainfall that promotes breeding of mosquitoes carrying the disease. In Mali, seasonal transmission of *P. falciparum* malaria occurs from June to December. Blood was collected by venipuncture at 3 different time points for each study participant. The first blood collection was taken during May, before the malaria season began. The second collection was taken when participants presented to their local health clinic with uncomplicated malaria symptoms. Blood smears were performed for malaria diagnosis and any subjects with detectable parasitemia were treated for malaria according to the National Malaria Control Program guidelines in Mali, which recommend artemether-lumefantrine for uncomplicated *P. falciparum* malaria. A final blood collection was obtained 7 days after treatment (convalescent time point). For all three timepoints blood was collected into sodium citrate–containing cell preparation tubes (Vacutainer CPT; Becton Dickinson). Plasma and PBMCs were separated according to the manufacturer's instructions. PBMCs were frozen in fetal bovine serum (GIBCO BRL) containing 7.5% DMSO (Sigma-Aldrich), kept at −80°C for 24 h, and then stored at −196°C in liquid nitrogen. Samples from Mali were shipped on dry ice (−78.5°C) over a course of 3 d using a courier service to Rockville, MD, where they were again stored in liquid nitrogen. The samples were then forwarded, again on dry ice (−78.5°C) overnight using a courier service to Minneapolis, MN where they were stored in liquid nitrogen before being used for experiments. Blood samples from malaria-naïve US controls were obtained from the Memorial Blood Bank in Minneapolis, MN. To isolate and freeze PBMCs, the protocol that is well described in Redihammer et al. was followed [116].

Phenotype and cytotoxicity assays:

In a single day, 12 – 16 PBMC samples, including an internal control described below, were thawed, 3 – 6 PBMC vials (~3 – 8 million cells per vial) at a time, rapidly in a 37˚C water bath. Samples were then transferred to 15-mL conical tubes and 4 mL of pre-warmed Roswell Park Memorial Institute-1640 media supplemented with 10% fetal bovine serum (RP10) was added dropwise to each tube while swirling by hand. The 15-mL conical tubes were then filled with RP10 and centrifuged at 500xg for 10 min. The supernatant was carefully aspirated, and the cell pellets were resuspended in 2 mL of culturing media (Lonza X-VIVO-15, Serum-free hematopoietic cell medium, with L-Glutamine, gentamicin and phenol red, and 20% heat inactivated human male AB serum (Peak Serum)). Cell counts for each sample were obtained by flow cytometry using the following method. A 50µL aliquot of each PBMC sample was diluted in 450 µl PBS1x and 0.5µL of propidium iodide (PI) conjugated to the fluorophore phycoerythrin (PE). After 10 minutes incubating the aliquots were analyzed on a Beckman Coulter’s CytoFLEX cytometer with a violet (405 nm), blue (488 nm), and red (632 nm) laser set up. Live cells were gated as singlet cells that were PI-PE negative and cell counts for each sample were calculated using the following formula:

Total cell count = (live cell events/µL) * (1000 µL/mL)*(10 for dilution factor)*(2mL total sample volume)

Following the schematic in (Figure 6), culture media was then added to each sample to create a concentration of 800,000 cells per mL. For each sample, 1 mL of this cell suspension was plated into each of 3 wells for day 0 experiments. These cells were rested overnight at 37˚C, 5% CO2 before proceeding with cytotoxicity assays and cell staining as described below. To the remaining cell suspension, IL-15 (National Cancer Institute) was added to a final concentration of 20ng/mL. This cell suspension was gently but thoroughly mixed and 1 mL from each sample was plated into another 24 well plate to serve as the "IL-15" day 6 control. To the remaining cell suspension, recombinant human IL-21 (Biolegend) was added to a final concentration of 50 ng/mL. This cell suspension was then plated at 800,000 cells per well and incubated at 37˚C, 5% CO2 for 6 days.

For the day 0 assays, 500,000 cells were transferred to each of 3 individual wells (one for the natural cytotoxicity assay, one for ADCC and one for a negative control) per PBMC sample in a 96 well plate. As stated earlier, Mali participants had 3 PBMC samples each, one for each of three timepoints (pre-malaria season in May, during malaria, and post-malaria convalescent timepoint). For the ADCC and natural cytotoxicity assays, human red blood cells (Memorial blood bank, St. Paul MN) and K562 cells were counted on a FACS machine (CytoFLEX) and resuspended to a concentration of 500,000 cells per 100µL. For the natural cytotoxicity assay, K562 cells were added to sample PBMC's at a ratio of 1:1. For the ADCC assays, the RBC suspension was incubated with rabbit anti-human RBC antibodies (1:1000 dilution) for 20 minutes, then added to 500,000 PBMC's at a 1:1 ratio in the 96 well plate. For the negative control, 100 µL of culturing media was added and all cells were incubated at 37˚C, 5% CO2 for 4 hours. Following the incubation period, a master mix of anti-human CD107a (Biolegend; 1:200 dilution) and IL-10 catch reagent were added per manufacturer's instruction to all 3 wells for each sample, and cells were incubated for 1 more hour at 37˚C in 5% CO2.

For immunophenotyping, cells were first washed with PBS1x then stained with Ghost Dye UV450 (Tonbo) in PBS at 5˚C for 20 min. Using the remaining antibodies listed in (Table 5), PBMC's were then stained a second time in FCS buffer (1X PBS with 2% heat inactivated fetal cow serum and 2mM EDTA) for 20 min at room temperature in the dark. Cells were then washed and incubated in 2% formaldehyde in PBS at 37˚C for 10 min. After fixation, cells were then washed and resuspended in FCS buffer for analysis on a BD Fortessa X-30 H0081 with Blue (488nm), Red (640nm), Violet (405nm), Yellow-Green (561nm) and UV (355nm) spatially-separated laser sources.

Day 6 assays followed the protocol above with the following exceptions. On day 6, IL-12 (3ng/mL) was added to all wells except those serving as the IL-15 only controls. Another assay, "cytokine stimulation", was added to include PBMC's stimulated with IL-15 (20ng/mL), IL-21 (50ng/mL) for 6 days and IL-12 (3 ng/mL) for 5 hours total. The ADCC and natural cytotoxicity assays were performed the same as on day 0 with the exception that IL-12 (3ng/mL) had been added prior to transferring 500,000 PBMC's from each sample to individual wells on a 96 well plate. Also, in order to keep cytokine concentrations the same for the functional assays as for the cytokine stimulation assay, for the functional assays culturing media containing IL-15 (20ng/mL), IL-21 (50ng/mL) and IL-12 (3 ng/mL), was used to resuspend RBC's and K562 cells before they were added to PBMC's at a 1:1 ratio. Sample PBMC's from wells that had been incubated with IL-15 alone were treated the same as were the day 0 negative controls.

Quality control of staining was monitored using the same internal control (PBMC's derived from a single donor) in every batch of samples analyzed.

Antibody	Fluorophore	Clone	Company	Purpose in panel
CD64	BUV737	10.1	BD Biosciences	Exclusion of monocytes
CD3	BUV805	SK7	BD Biosciences	Exclusion of other non-NK cell lymphocytes (ex: T cells)
CD56	BUV563	NCAM16.2	BD Biosciences	General phenotypic marker for NK cells
CD2	BUV395	RPA-2.10	BD Biosciences	Marker specific to NK cells and T cells
IL-10	APC	--	Miltenyi	Identification of IL-10 secreting cells
CD107a	BV421	H4A3	Biolegend	Indirect measurement of cellular degranulation
CD16	BUV496	3G8	BD Biosciences	Fc Receptor essential for ADCC
CD8	BV510	SK1	Biolegend	Identification of cytotoxic T cells and a novel subset of CD8+ NK cells
NKG2C	PEVio770	REA205	Miltenyi	Killer Cell Lectin type Receptor; marker of adaptive NK cells
PD-1	PE	PD1.3.1.3	Miltenyi	Inhibitory immune checkpoint molecule, expressed on both NK and T cells
TIGIT	BV605	A15153G	Biolegend	Inhibitory immune checkpoint molecule, expressed on both NK and T cells
CD45RO	BV785	UCHL1	Biolegend	Memory T cell marker; also expressed on (degranulating?) NK cells
KLRG1	KIRAVIA Blue520	SA231A2	Biolegend	Immune checkpoint receptor on CD8+ T cells and NK cells
CX3CR1	PE-CF594	2A9-1	Biolegend	Chemokine receptor with pivotal role in recruiting NK cells to inflamed tissues
TIM-3	BV650	F38-2E2	Biolegend	Immune checkpoint receptor; "hit" from Biolegend screen
4-1BB	BV711	4B4-1	Biolegend	Inducible co-stimulatory protein involved in T cell proliferation and survival
CRTAM	PerCP-Cy5.5	Cr24.1	Biolegend	T cell associated marker; "hit" from Biolegend screen
CTLA-4	R718	BNI3	BD Biosciences	On NK cells, activating immune checkpoint receptor; "hit" from Biolegend screen
Siglec-7	APC-Cy7	REA214	Miltenyi	Inhibitory receptor expressed on NK cells; "hit" from Biolegend screen
Live/Dead	UV450		TONBO	Exclusion of dead cells

TABLE 5: Antibodies used for immunophenotyping and functional assays.

Data Analysis

FlowJo version 10.8.1 was used for the analysis of raw fcs file data. Gating schemes are outlined below (Figure 5). Four samples, all malaria timepoint samples, had fewer than 500 NK cells and were therefore excluded from analysis, all other samples were analyzed and included. Biolegend screen volcano plots (Figure 11) were made using R 4.1 (R, 2021). The code was adapted from https://biocorecrg.github.io/CRG_RIntroduction/volcano-plots.html (Bonnin, 2020) and is available at https://github.com/jamesdahlvang/volcano.

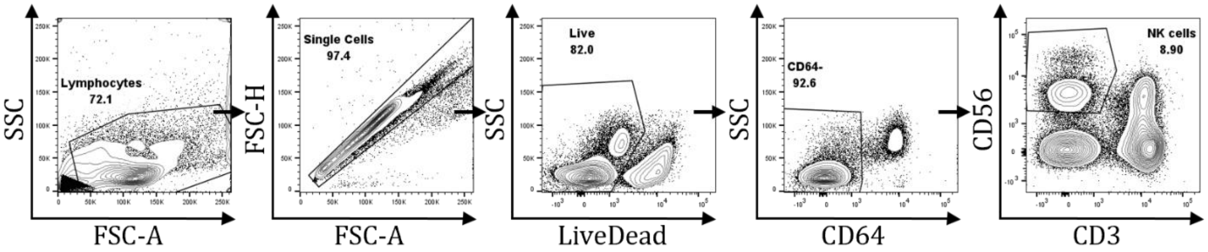


FIGURE 5: Gating strategy for NK cells using a healthy US donor.

Statistical analysis

Data in all graphs is represented as median values, and statistical analysis is described in all figure legends. GraphPad Prism V8 was used for Wilcoxon signed rank tests, Mann-Whitney U tests, and one-way ANOVA post hoc Tukey multiple comparisons tests.

Data availability

Raw data is available from the author upon reasonable request.

Results

NK cells from Malian individuals secrete more IL-10 than NK cells from malaria naïve individuals.

The observation that IL-15 complex treatment could rescue mice from ECM death in a manner that was dependent on IL-10 secretion by NK cells led us to hypothesize that IL-10 release from NK cells may also play a protective role against developing severe disease in humans. We sought to determine whether NK cells from individuals who are more protected from severe malaria symptoms, that is adolescents from a malaria-endemic country, may have a higher propensity to secrete IL-10 during subsequent malaria infections than individuals more susceptible to developing severe disease. In order to test this hypothesis *in vitro*, we collected peripheral blood mononuclear cell (PBMC) samples from malaria-naïve US individuals and from individuals from a malaria endemic country (Mali). Malian participants were enrolled before the beginning of the malaria season and blood samples were acquired at 3 different timepoints for each individual (before the malaria season, during an uncomplicated malaria episode and 2 weeks post-malaria resolution) (Figure 6A). We then exposed cells to inflammatory conditions using a previously published 6 day cytokine stimulation procedure [66]. In addition to cytokine stimulation alone, we also assessed IL-10 secretion by NK cells during ADCC and natural cytotoxicity (Figure 6B).

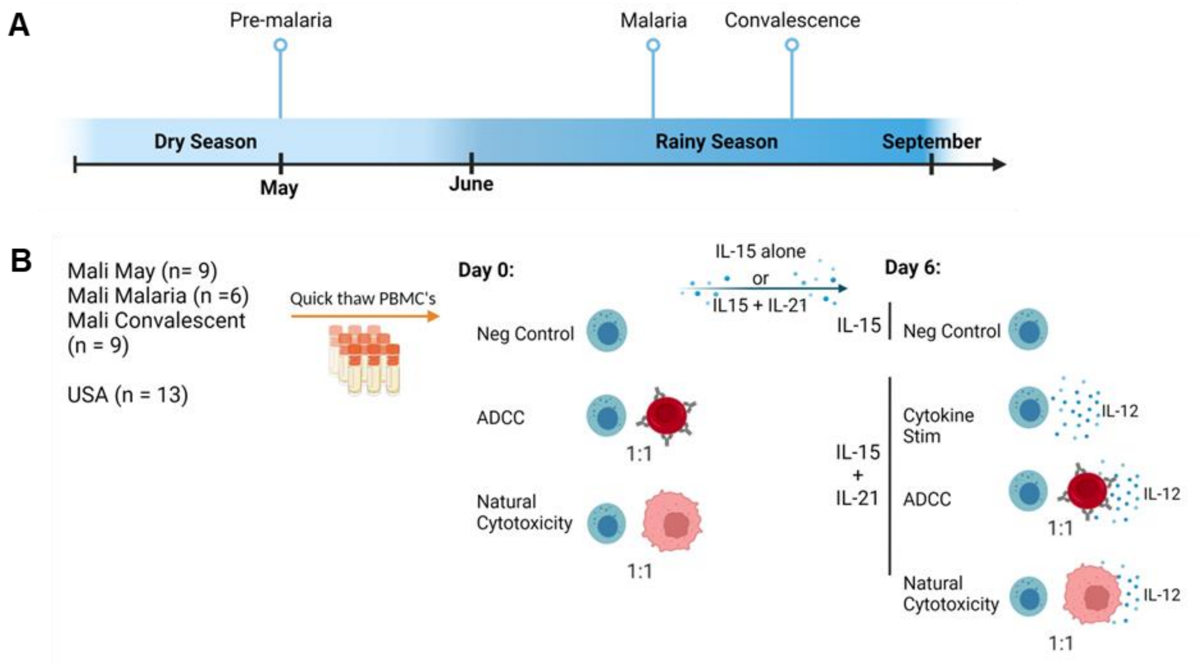


FIGURE 6: Experimental setup. (A) Timeline for sample collection at Malian study site. (B) Schematic overview of experimental design.

In accordance with previous studies showing that human NK cells secrete IL-10 in response to IL-15 stimulation only when IL-21 or IL-21 and IL-12 (for maximal production) are added, we observed minimal IL-10 release from NK cells incubated with IL-15 alone (for both US and Mali individuals, less than 1% of NK cells secreted IL-10). Incubation with IL-15, -21 and -12 however led to significantly greater IL-10 secretion from NK cells in both Malian and US individuals (Figure 7A-B). IL-10 release was significantly greater in the NK cells from Malian individuals compared to malaria naïve US controls following cytokine stimulation, ADCC and natural cytotoxicity assays (Figure 6C).

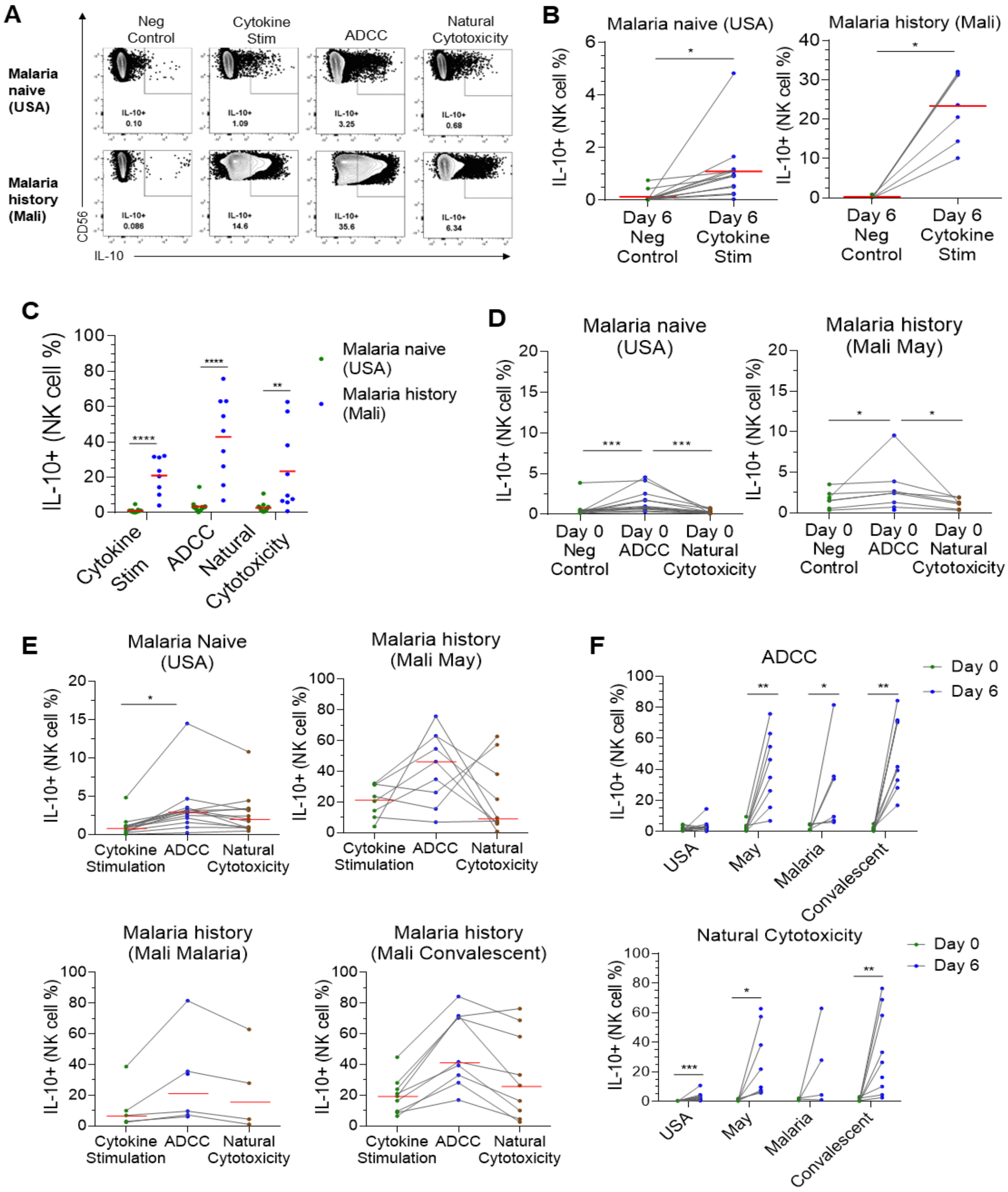


FIGURE 7: NK cells from Malian individuals secrete more IL-10 than NK cells from malaria naive (USA) controls.

(A-B) IL-10 expression on NK cells treated with IL-15 alone (neg control) or with a combination of IL-15, IL-21 and IL-12 (cytokine stim). (C) Comparison of IL-10 release derived from NK cells of malaria-exposed (Mali May group) vs malaria naïve (USA group) during cytokine stimulation alone, ADCC and natural cytotoxity. (D) Levels of IL-10 release were compared between the three assays (cytokine stimulation, ADCC and natural cytotoxity), for all 4 participant groups (USA, Mali May, Mali malaria and Mali convalescent

Figure 7 (cont'd)

timepoints) at day 0. (E) Same as D but for assays completed on day 6. (F) IL-10 released by NK cells on day 0 vs day 6 ADCC (top) and natural cytotoxity (bottom) assays. (B,C,D,E,F)*p<0.05, **p<0.01, ***p<0.001 as determined by Wilcoxon signed-rank test (B,C,F) or one-way ANOVA with Turkey's multiple comparison test (D,E); orange bars represent median values.

It was also observed that prior to the 6 day cytokine stimulation, NK cells from both US and Mali individuals released small but significantly higher than baseline levels of IL-10 following stimulation in ADCC, but not natural cytotoxicity assays (Figure 7D). This trend was also observed on day 6, where IL-10 release in ADCC assays was higher than cytokine stimulation alone; however only US individuals and Mali individuals at the convalescent timepoint showed statistically significant differences (Figure 7E). The expression of IL-10 was substantially greater at day 6 compared to day 0 in both ADCC and natural cytotoxicity assays (Figure 7F) indicating that prior exposure to IL-15, -21 and -12 may be needed for optimal IL-10 secretion. Taken together this indicates that the NK cells from Malian individuals more robustly secrete IL-10 compared to those from US donors, and that exposure to cytokines IL-15, -21 and -12 stimulates these cells for optimal IL-10 secretion which is greatest during ADCC events.

During ADCC, NK cell IL-10 secretion negatively correlates with CD107a expression in malaria timepoint samples.

From the observations noted above, we postulated that IL-10 may be secreted along with secretory granules during cytotoxic degranulation. Degranulation was assessed by monitoring the expression of lysosomal associated protein CD107a which is present on the surface of cells following the release of lysosomal contents. Pearson correlations were used to determine if IL-10 expression paralleled CD107a expression for any of the study groups (Figure 8).

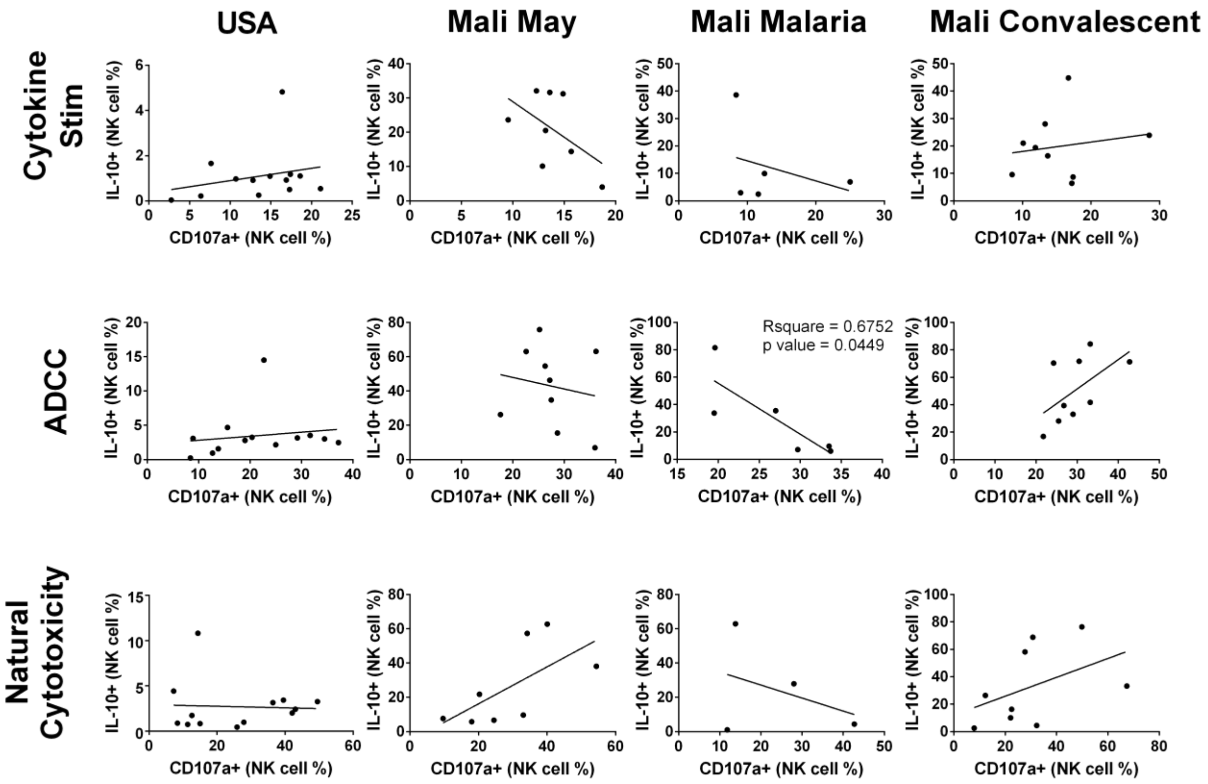


FIGURE 8: Pearson correlations of IL-10 release with CD107a show varying trends between groups. (A) NK Cell expression of IL-10 was plotted against NK cell CD107a expression for each group (US, and the 3 Mali timepoints) for each of the 3 stimulation assays. R square values are show for graphs with a significant (p<0.05) correlation coefficient.

Most graphs showed either no correlation or a trend of positive but non-significant correlation, with the exception of the malaria timepoint graphs. For this timepoint, IL-10 release following ADCC was significantly but inversely correlated with CD107a expression. Recent publications have shown a novel role of IL-10 as an autocrine signaling molecule with negative feedback capabilities [117]. In macrophages, IL-10 plays two distinct roles in autocrine signaling whereby it can act to raise the threshold of activation of the IL-10 secreting cell in an IFN-β mediated manner, or through a TNF-induced mechanism can suppress cells already activated during infection [117]. Given these observations, the inflammatory conditions (especially the cytokine milieu) that NK cells are exposed to during malaria infection may allow for a similar negative feedback role of IL-10, at least during the later sypmtomatic stages of malaria disease.

CD107a expression following ADCC and natural cytotoxicity is increased after the 6 day cytokine stimulation.

Another observation from our day 0 vs day 6 cytokine stimulation comparisons showed that CD107a expression in ADCC and natural cytotoxicity assays was greater following 6 days of IL-15 + IL-21 stimulation for all groups except for the Mali malaria timepoint (Figure 9A). One possible explanation may be the smaller sample size of the malaria timepoint group (n as low as 4 for the natural cytotoxicity assays) as the number of NK cells proportional to lymphocytes in this group was quite low and samples with fewer than 500 NK cells were excluded from analysis (all of those excluded were malaria timepoint samples). Within the other groups, the finding of upregulated CD107a following cytokine treatment with IL-15 and IL-21 indicates that these cytokines, either individually or cooperatively, enhance NK cell cytotoxicity.

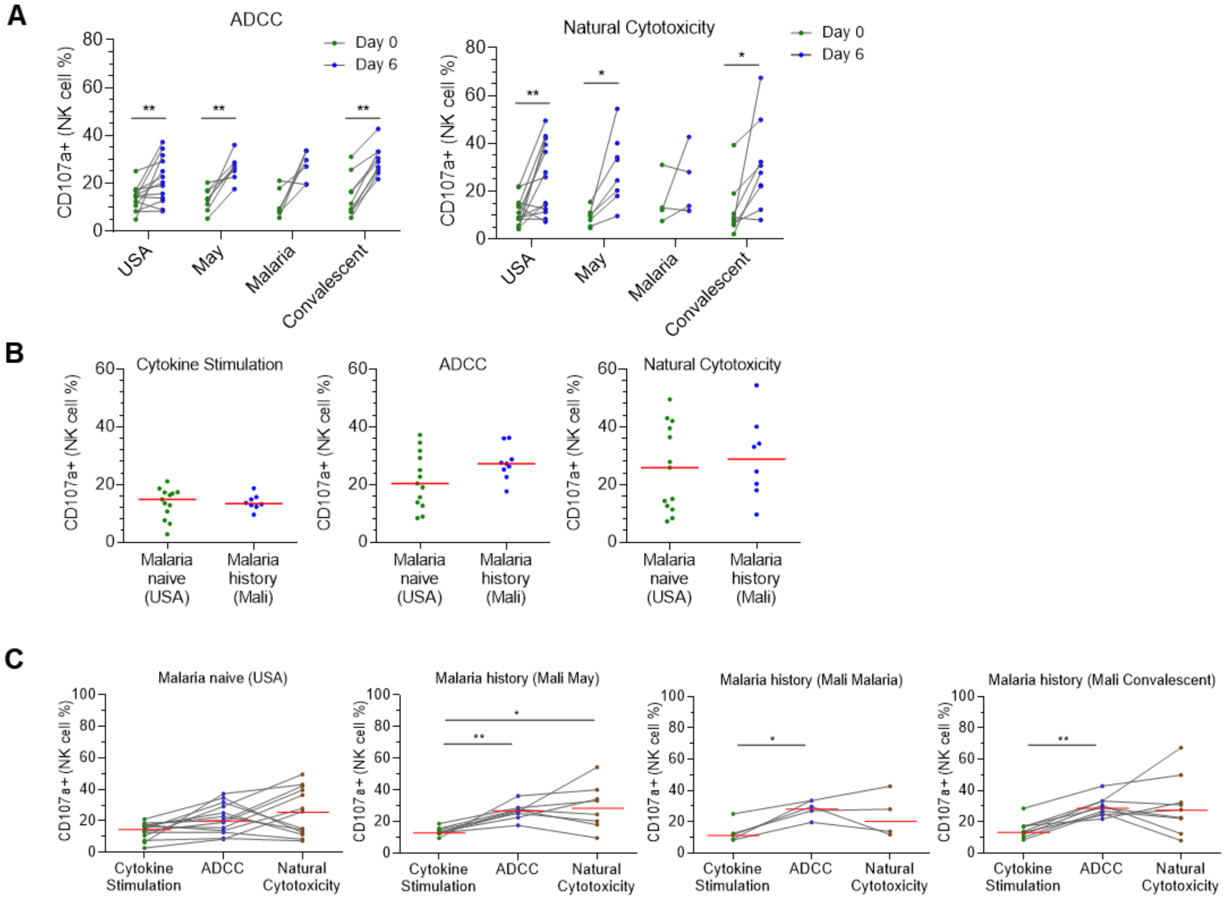


FIGURE 9: For US and non-malaria timepoint groups, CD107a expression during ADCC and natural cytotoxicity is increased following cytokine stimulation.
(A) Comparison of NK cell CD107a expression during ADCC and natural cytotoxicity prior to and following 6 day IL-15 plus IL-21 cytokine stimulation. (B) NK cell expression of CD107a during all three assays are compared between malaria naïve (USA) and Malian individuals at the May timepoint. (C) CD107a expression on NK cells at day 6 are compared between all 3 assays for each study group. (A,B,C) *p<0.05, **p<0.01, ***p<0.001 as determined by Wilcoxon signed-rank test (A,B) or one-way ANOVA with Turkey's multiple comparison test (C); orange bars represent median values.

We next compared CD107a expression between NK cells from US versus Mali individuals. There were no statistically significant differences in CD107a expression in NK cells from malaria naïve versus Malian individuals following cytokine stimulation alone, ADCC or natural cytotoxicity (Figure 9B).

For each study group we also compared NK cell CD107a expression between the three NK cell stimulation methods (cytokine stimulation, ADCC and natural cytotoxicity; Figure 9C). Statistically higher levels of CD107a were expressed during ADCC assays compared to cytokine stimulation alone in all Mali timepoint groups.

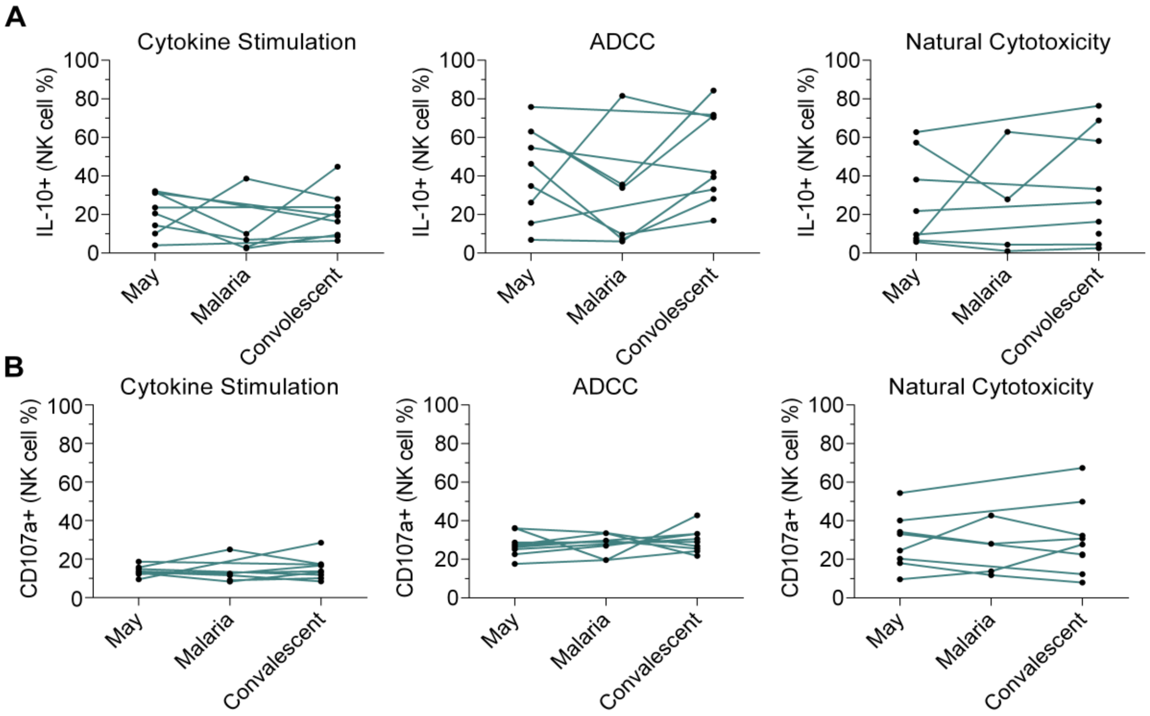


FIGURE 10: NK cell expression of IL-10 and CD107a did not change over the course of the malaria season.
(A) Day 6 expression of IL-10$^+$ NK cells in each stimulation condition (cytokine stimulation, ADCC and natural cytotoxicity). NK cells were analyzed from the same individuals at 3 different timepoints (May, malaria infection, convalescence). One-way ANOVA with post hoc Tukey multiple comparisons test was used for statistical analysis. (B) Same as (A) but assessing CD107a expression on NK cells. (A-B) Blue lines are drawn between paired samples.

We also evaluated both IL-10 secretion and CD107a expression on NK cells from individuals at the three timepoints (prior to, during and post-malaria infection; Figure 10).

No statistically significant changes were noted between any of the three timepoints over the course of the malaria season.

IL-10 secreting NK cells upregulate immune checkpoint molecules

The phenotype of IL-10 secreting NK cells has not yet been reported. To discover surface markers that correlate with IL-10 release, we utilized a 365 marker screening panel from Biolegend . Results from this initial screen revealed a number of markers that were preferentially expressed on IL-10^+ NK cells compared to IL-10^- NK cells (Figure 11A). Several of these markers included CTLA-4, 4-1BB, PD-1 and TIGIT (Figure 11B), which have been characterized as immune checkpoint molecules in NK cells [118,119,120]. To assess whether these markers are consistently expressed on IL-10 secreting NK cells from US and Mali individuals, a flow cytometry panel was designed to integrate these checkpoint molecules as well as markers for different NK cell subsets, for instance markers that characterize adaptive NK cells (Table 5).

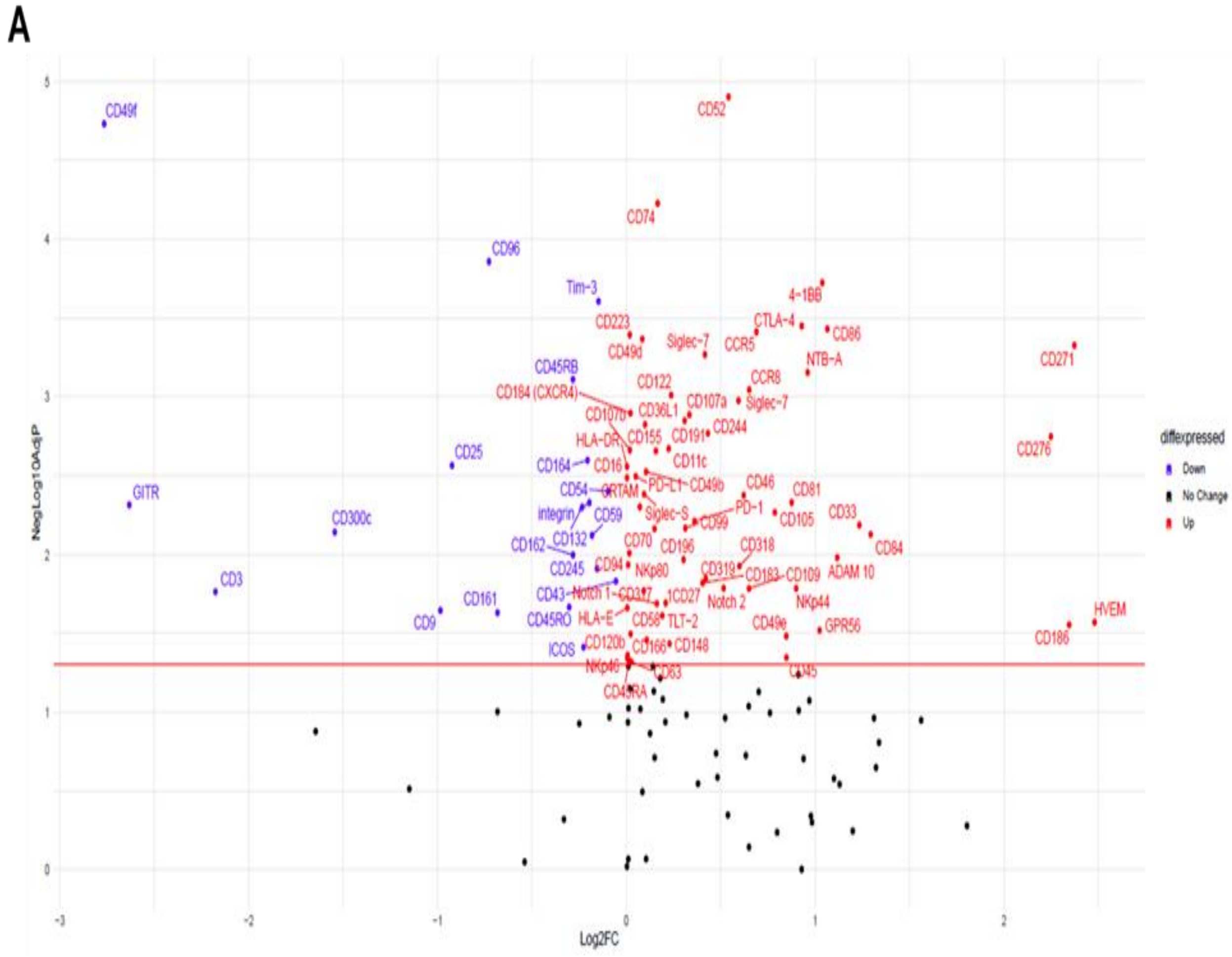
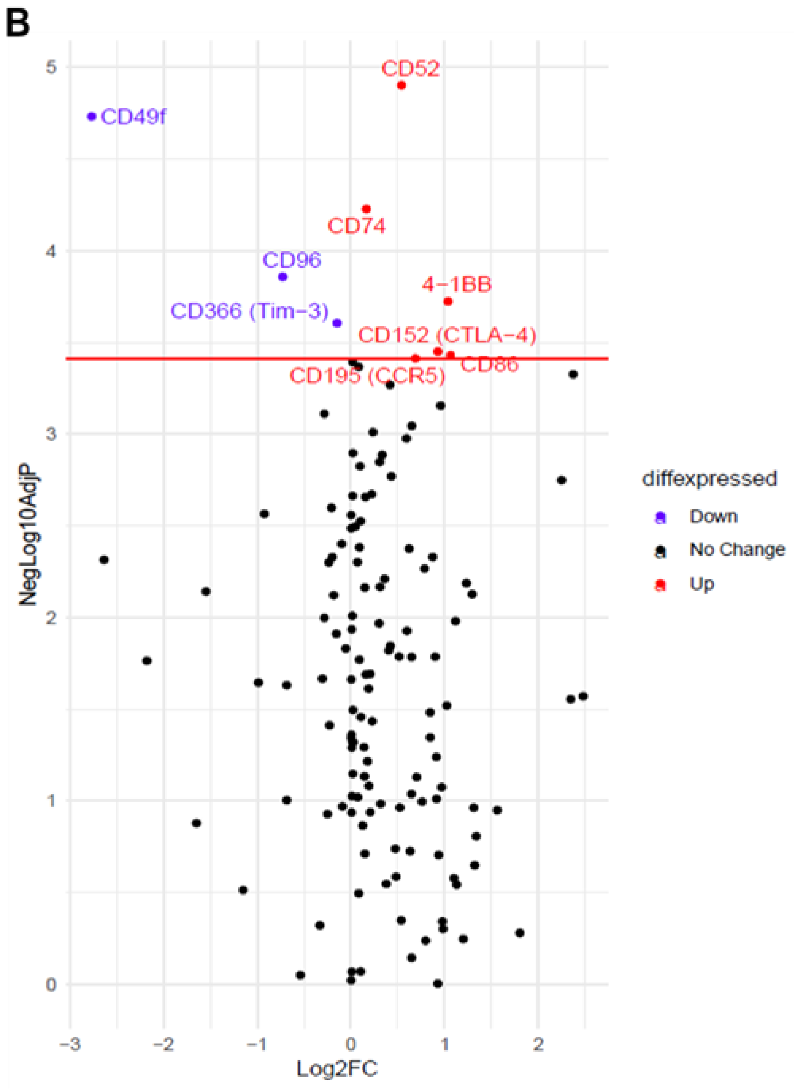


FIGURE 11: Volcano plots summarizing Biolegend screen results. (A) Volcano plot where the p value was set at 0.05 for significance (-log10 of around 1.30) and plotted on the y axis. The log2 fold changes of marker expression between IL-10+ versus IL-10- NK cells are plotted on the x axis. (B) Same as (A) except the p-value was corrected for multiple comparisons using a Bonferroni correction (adjusted p value was 0.000375). (A-B) Markers with less than 5% expression (% of NK cells) were excluded from analysis.

Figure 11 (cont'd)



Immunophenotyping of NK cells at baseline (day 0, negative control) showed similar levels of marker expression between US and Mali samples (Figure 12A). There was however statistically significantly lower basal levels of Siglec-7 expressed in the Malian samples. Siglec-7 is an inhibitory receptor expressed on NK cells. Unpublished data from our lab has indicated that adaptive NK cells show a deficit of Siglec-7, indicating that the NK cells from Malian individuals may have a higher percentage of NK cells that are adaptive compared to US controls.

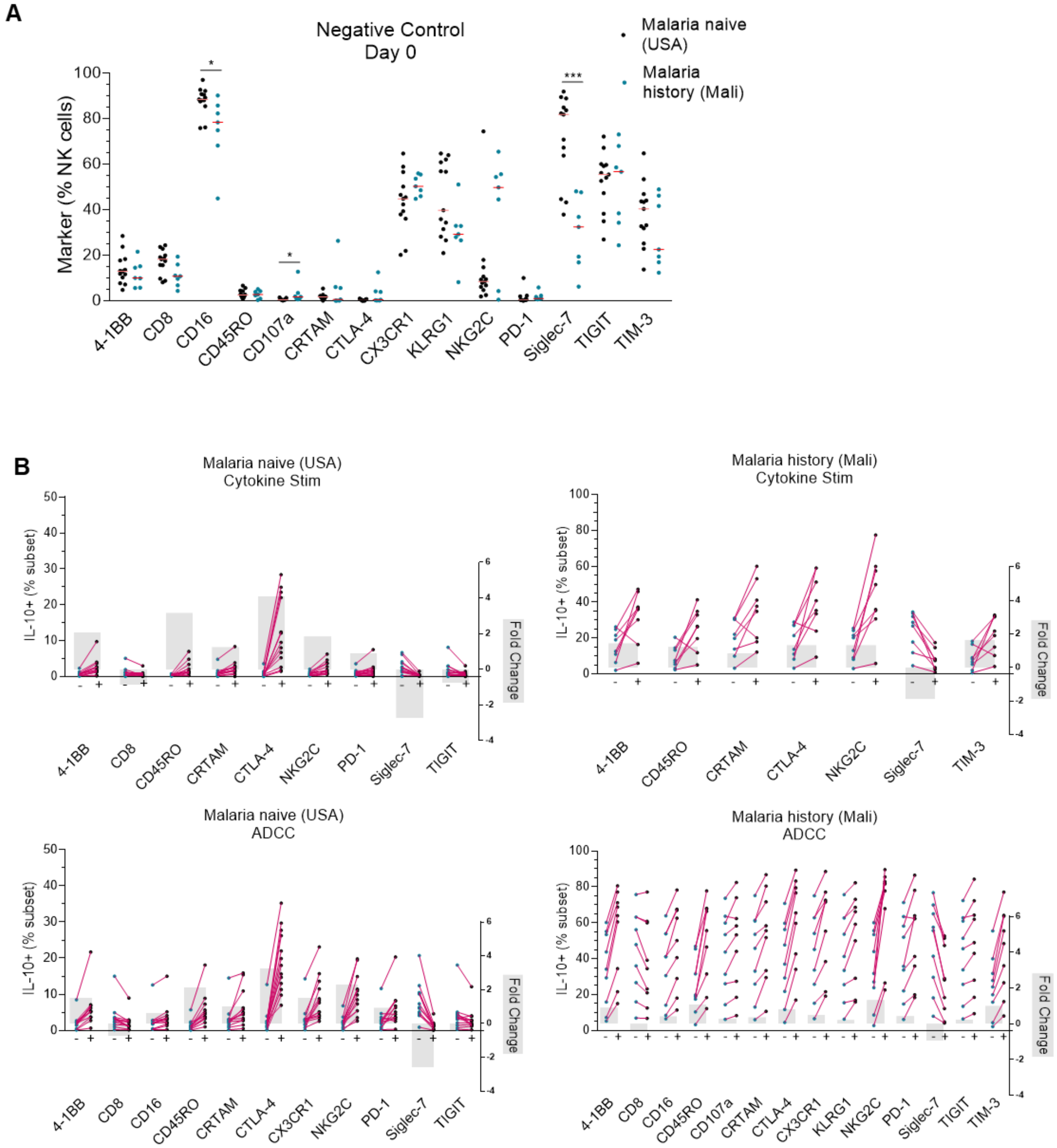
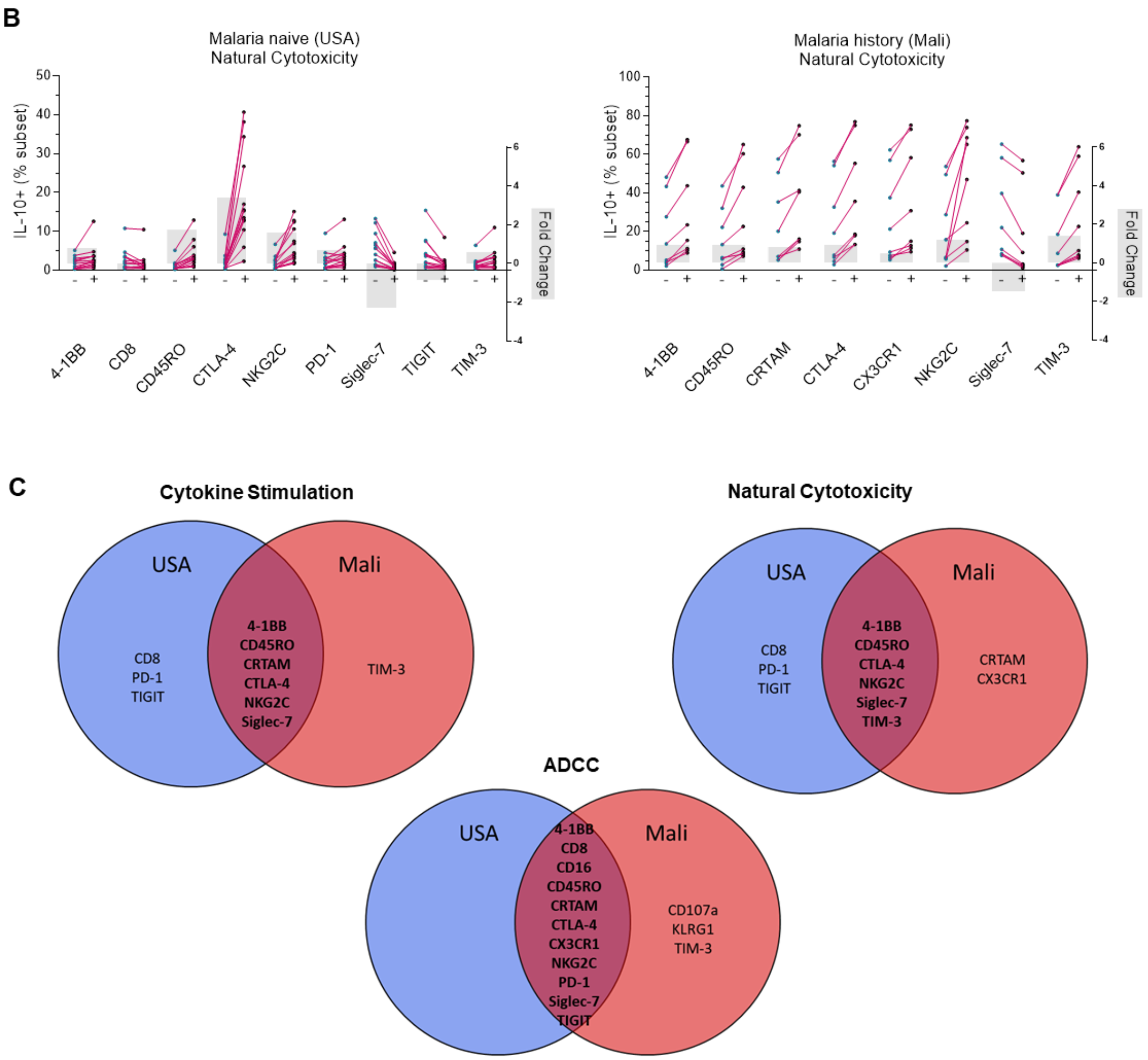


FIGURE 12: Secretion of IL-10 in NK cell subsets expressing several immune checkpoint molecules, or markers that are associated with adaptive or cytotoxic NK cells. (A) The baseline (day 0 negative control) expression of markers in general NK cell populations is shown for both malaria naïve (USA) and malaria history (Mali May timepoint) groups. (B) Markers that showed differential expression of IL-10 between marker(+) and marker(-) NK cells ($p < 0.05$ by Wilcoxon signed rank test) are shown for

Figure 12 (cont'd)

each study group for each assay. Magenta lines connect paired samples. The Log2 fold change for each marker is also shown (grey columns). (C) Visual summary of (B) whereby markers that are differentially expressed on IL-10⁺ vs IL-10⁻ NK cells only in the US control group are in blue, those different only in the Mali (May) group are in red, and markers that changed significantly in both USA and Mali samples are listed in the overlapping regions.



Comparisons of IL-10 secretion between marker⁺ and marker⁻ (for example 4-1BB⁺ versus 4-1BB⁻) NK cells revealed significant differences in proportions of IL-10⁺ NK cells in several subsets including ones upregulating immune checkpoint receptors (Figure 12B). Specifically, markers that showed significant differences in IL-10 secreting NK cells in both US and Mali groups in all 3 assays included 4-1BB, CTLA-4, CD45RO, NKG2C and Siglec-7 (Figure 12C). NK cells that expressed the immune checkpoint molecules 4-1BB and CTLA-4 had higher levels of IL-10 secretion compared to NK cells not expressing these markers. Agonism of 4-1BB has been shown to enhance NK cell ADCC and cytokine secretion, and stimulation of CTLA-4 via anti-CTLA-4 antibody therapies induce NK cell activation and degranulation within tumor microenvironments [121,122,123]. Similarly, another marker that showed differential IL-10 secretion between positive and negative subsets for all study groups across all 3 assays was CD45RO (Figure 12B-C). Typically this protein is considered a marker of T cell memory, but in NK cells CD45RO has been shown to distinguish degranulating NK cells and CD45RO$^+$ NK cells produced significantly more IFN-γ and were more cytotoxic compared to CD45RO$^-$ NK cells following IL-12 stimulation [124,125]. Taken together the findings of higher IL-10 secretion from the NK cells displaying these markers—4-1BB, CTLA-4 and CD45RO—indicate a cytotoxic phenotype associated with IL-10 release.

Immunophenotyping also revealed that NKG2C$^+$ NK cells had a higher level of IL-10 secretion compared to NKG2C$^-$ NK cells (Figure 12B-C). This activating receptor is associated with memory-like (adaptive) NK cell expansion in CMV infections [126,127]. Adaptive NK cells are highly adept at ADCC [128]. In the context of malaria, they have been shown to interact with and directly lyse iRBC's, and human studies have shown that adaptive NK cells correlate with protection from malaria [69,73]. Another marker in our panel, Siglec-7, has been shown by other's in our lab (data not yet published) to be downregulated on adaptive NK cells. For all study groups in all 3 assays, Siglec-7$^-$ NK cells expressed more IL-10 than Siglec-7$^+$ NK cells (Figure 12B-C). Taken together this implies that adaptive NK cells, which have enhanced cytotoxic potential compared to conventional NK cells, secrete higher levels of IL-10.

Of note, during cytokine stimulation and natural cytotoxicity assays, the US group had CD8$^-$, TIGIT$^+$ and PD-1$^+$ NK cell subsets that all had higher levels of IL-10 secretion compared to CD8$^+$, TIGIT$^-$ and PD-1$^-$ NK cell subsets, respectively (Figure 12B-C). This was only seen in the US group and not Mali groups, indicating that IL-10 secreting NK cells from US individuals may express a different phenotype or constitute a different subset of NK cells than the IL-10 secreting NK cells of Malian individuals. In the field of NK cell biology it is becoming increasingly evident that NK cell development and maturation are shaped by their cellular environment [129]. Pathogen and vaccine-driven signals have been shown to influence the differentiation of NK cell subsets with altered effector functions [130]. A well characterized example of this is human cytomegalovirus (HCMV) infection. Expanded subsets of NK cells expressing CD57 and NKG2C and lacking the transcription factor PLZF and adaptor protein FcεR1γ are elevated in HCMV seropositive compared to seronegative individuals [131]. In a similar way it is possible that chronic exposure to malaria or other pathogens common in sub-tropical regions (helminth infections for instance) have driven the differentiation of unique NK cell subsets.

In this study, while we did not know the CMV status of Malian participants, we did have serostatus information for all US participants. In the US samples, NK cell IL-10 secretion was not significantly different between CMV$^+$ vs CMV$^-$ groups (Figure 13).

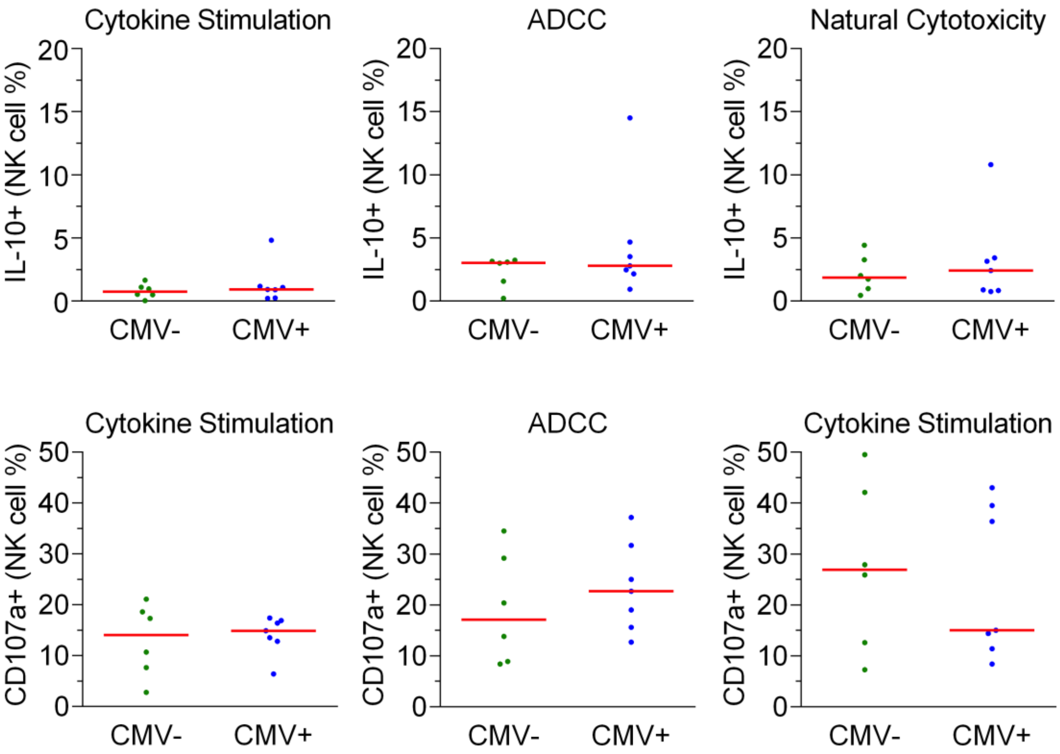


FIGURE 13: IL-10 (top row) and CD107a (bottom row) expression in CMV seropositive and seronegative individuals (USA). Groups were compared using Wilcoxon signed-rank tests (*p<0.05, **p<0.01, ***p<0.001); orange bars represent median values.

Following the 6 day cytokine stimulation, TIM-3 was dramatically upregulated and was expressed on nearly all NK cells in all groups (Figure 14). Such high expression on both IL-10^+ and IL-10^- NK cells renders this receptor rather ineffective as a marker for distinguishing the two populations and therefore it was excluded from further analyses. That said, it has been shown that TIM-3 stimulation catalyzes the activation of a complex transcription factor network that mediates the expression of NK cell maturation markers CD2, CD57 and NKG2C [132]. Even though TIM-3 may not be an ideal candidate for distinguishing IL-10 secreting NK cell subsets, it may still play a key role in mediating NK cell responses to IL-15.

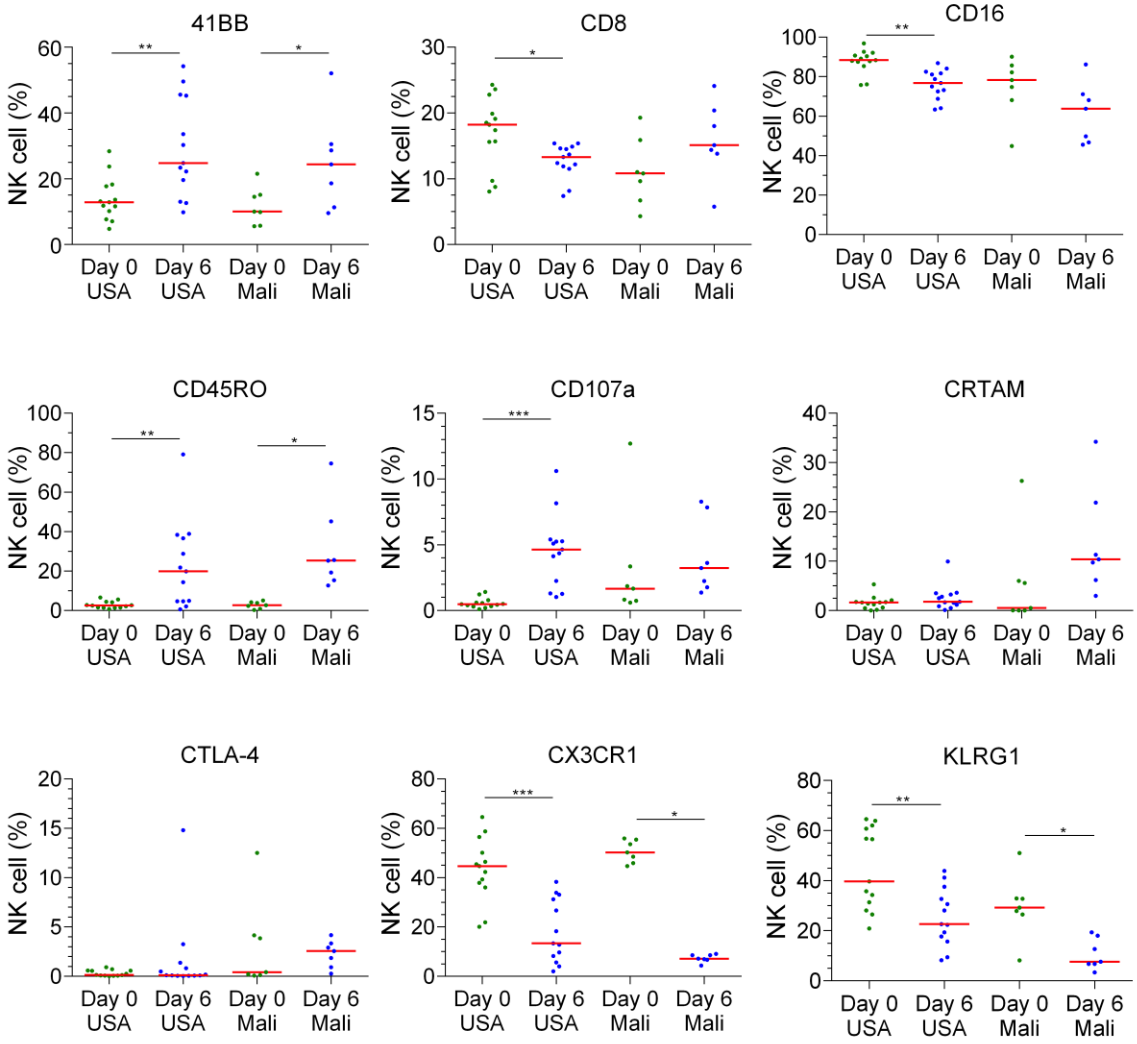
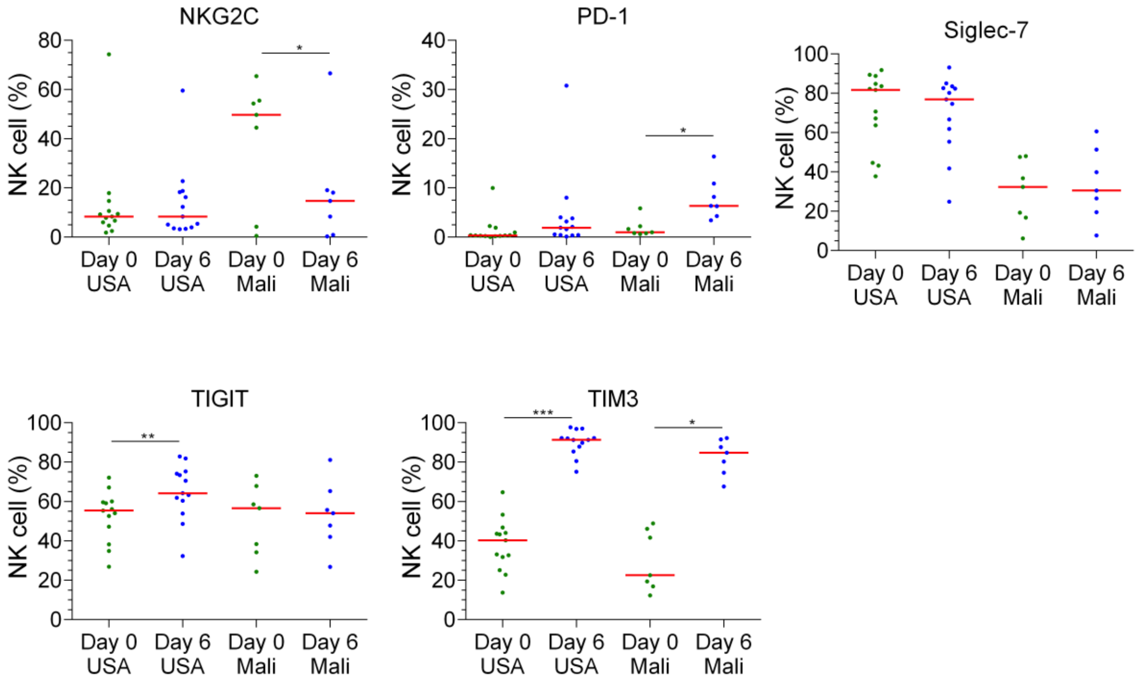


FIGURE 14: Day 0 and Day 6 expression (% NK cell) of phenotyping markers. Groups were compared using Wilcoxon signed-rank tests (*p<0.05, **p<0.01, ***p<0.001); orange bars represent median values. For day 0 and day 6 measurements for the Mali group, the samples used were from the pre-malaria timepoint.

Figure 14 (cont'd)



Discussion

The discovery that IL-15 complex treatment protects mice from ECM by an NK-cell derived IL-10 dependent mechanism suggests that IL-10 may play a role in ameliorating malaria pathology. Supporting this idea are reports that IL-10 mRNA levels are significantly higher in the spleen and brain of ECM resistant mice, and that neutralization of endogenous IL-10 significantly increases the development of ECM [65]. While most data from mouse models of malaria, especially ECM, indicate a protective role of IL-10, human studies have had more conflicting results [80]. Some studies have reported elevated levels of circulating IL-10 during severe cerebral malaria disease, as compared to non-severe uncomplicated malaria disease and healthy controls [59]. Other studies have shown that high ratios of IL-10 to TNF strongly correlated with protection against severe malaria anemia [133]. Our finding that NK cell secretion of IL-10 was higher in Malian individuals compared to malaria-naïve individuals may indicate that IL-10 from NK cells might play a protective role in malaria disease. Given the complex relationships between inflammatory cytokines and disease processes however, we cannot exclude the possibility that NK cell derived IL-10 and protection from severe disease may not be a direct straightforward correlation. While numerous studies have indicated and elucidated protective roles of NK cells during malaria disease, it has also been shown that NK cells can contribute to an overproduction of inflammatory cytokines, such as IFN-γ, that can contribute to immunopathology [55,61,134]. Similarly, it is possible that NK cell IL-10 secretion must strike a balance whereby anti-inflammatory effects are great enough to, for instance, dampen immunopathology, but are not so great as to hinder parasite control and elimination. To further investigate the full role of NK cell derived IL-10 in protecting against severe disease more studies are needed in the setting of severe malaria such as cerebral malaria or severe malaria anemia.

The finding that IL-10 secretion is only stimulated by IL-15 in the presence of other cytokines, namely IL-21 and IL-12, is consistent with previous studies [66]. Interestingly, comparing NK cell IL-10 release in response to 3 different stimulation techniques (cytokine stimulation, ADCC and natural cytotoxicity) showed that significantly higher levels of IL-10 were secreted during ADCC compared to cytokine stimulation alone in US and Mali convalescent timepoint groups. Cytotoxic degranulation leads to the lysis of target cells with subsequent spilling of intracellular components that can promote inflammation [135]. The lysis of iRBC's have been shown to release molecules, such as heme, that can act as damage-associated molecular patterns (DAMPs) which activate multiple inflammatory pathways including toll-like receptor signaling, neutrophil extracellular trap release and inflammasome formation [136,137]. Perhaps to dampen this effect, NK cells secrete the anti-inflammatory cytokine IL-10 during or after cytotoxic degranulation. Mechanistic studies into the potential secretion of IL-10 during NK cell cytotoxic degranulation have not yet been reported. Of note, we only observed higher IL-10 release from NK cells during ADCC, but not natural cytotoxicity. This may be due to differences in the target cells and what inflammation-mediating molecules are being released (for instance, heme release is unique to RBCs). Another potential explanation for the finding of higher IL-10 release during ADCC compared to natural cytotoxicity involves the different signaling pathways of these two mechanisms. Although there is significant overlap between downstream signaling events, K562 cells—which are used to elicit natural cytotoxicity—primarily express ligands for NKG2D and NCRs, while ADCC signals through CD16 [138]. The Fc receptor Fc γ RIIIa (CD16) associates with homo- or hetero-dimers of FcR γ and/or CD3 ζ chains which bear immunoreceptor Tyr-based activation motifs (ITAMs). NKG2D associates with the adaptor molecule DAP10, which bears the activating Tyr-based motif YxxM, which is distinct from the ITAMs [139]. While Src family kinases phosphorylate both ITAMs and the YxxM motif of DAP10, phosphorylated ITAMs recruit the Tyr kinases ZAP-70 and Syk whereas DAP10 signaling does not require these proteins [140]. Studies decreasing DAP10 expression in mice and human NK cells have shown that while signaling through other adapter proteins (like DAP12) can induce both cytotoxicity and cytokine secretion, signaling through DAP10 mostly activates cytotoxicity [141,142].

Others have shown that IL-10 induces metabolic changes in NK cells that enhance their cytotoxic functions [143]. From this, we postulated that NK cells secreting high levels of IL-10 might also exhibit high levels of degranulation during ADCC and natural cytotoxicity. However, we found that CD107a (a marker of degranulation) did not positively correlate with IL-10 secretion. In US donors there was no correlation, perhaps due to very low levels of IL-10 secreted in this group that may have been too low to induce enhanced cytotoxicity as mentioned above. In Mali donors the trend was mixed, with mostly non-significant positive correlations for the Mali May and convalescent timepoints, but a significantly negative correlation in the malaria group. Notably, our study design included only Malian individuals that became symptomatically infected with malaria. By selecting only individuals who developed malaria (and therefore had not yet reached the point of clinical immunity), we may have been selecting for individuals who had sub-optimal IL-10 responses during malaria infection, although these responses were still greater in magnitude compared to US controls. Future studies assessing IL-10 secretion from NK cells in older adults who have developed more complete clinical immunity to infections, or studies of age-matched asymptomatically infected individuals would provide insights into NK cell IL-10 responses during symptomless malaria infection.

Additionally, at a cellular level, the different correlation between CD107a expression and IL-10 secretion in NK cells during the malaria timepoint compared to the May and convalescent timepoints may indicate that NK cell responses to IL-10 are dependent on other environmental signals. Others have shown that NK cell-intrinsic STAT3 activation is vital for IL-10 production following IL-15C treatment for ECM. It was also demonstrated that this activation could be amplified in a positive feedback manner as IL-10 was shown to enhance STAT3 activation [144]. Some researchers have shown a beneficial role for STAT3 activation in NK cells, reporting upregulation of the activating receptor NKG2D and enhanced degranulation and cytotoxicity following STAT3 activation [145]. Other studies have shown that in hypoxic conditions and in tumor microenvironments NK cell STAT3 activation leads to reduced cytotoxic killing [146]. It is conceivable that differences between study conditions, whereby the first used healthy donor NK cells, and the second utilizied NK cells in a hypoxic environment, may contribute to conflicting results. In our study, perhaps exposure of NK cells that were taken during the malaria timepoint to factors such as inflammatory cytokines or iRBCs may have influenced their subsequent responses to IL-15 and/or IL-10. Investigations into the activation status of NK cell STAT3 during human malaria would lend insight into IL-10 signaling and feedback mechanisms.

Using paired samples that were analyzed at both day 0 and day 6, we observed that CD107a expression increased in both ADCC and natural cytotoxicity following the six day IL-15 and IL-21 treatment. This finding of enhanced degranulation following cytokine stimulation, especially with IL-15, is congruent with other findings [147,148,149]. This was significant for all groups except for the malaria timepoint samples. One possible explanation for this could be that the NK cells taken at the malaria timepoint are more exhausted than those from healthy US individuals or NK cells taken at the May or convalescent timepoints. NK cells that were extracted at the malaria timepoint had already been exposed to parasites and inflammatory conditions long enough for patients to develop symptoms. From that point they were then further stimulated during the 6 day cytokine incubation, followed by further activation in ADCC and natural cytotoxicity assays. It has been shown that NK cells switch from granzyme B-mediated cytotoxicity to death receptor-mediated cytotoxicity during serial killing [150]. In our malaria timepoint samples, it is possible that serial exposure to target cells may be causing NK cells to switch to a death receptor or FasL mediated killing.

Immunophenotyping revealed that cytokine checkpoint molecules were upregulated on IL-10$^+$ NK cells compared to IL-10$^-$ NK cells. Often studied as targets for cancer therapeutics, immune checkpoint molecules can positively or negatively regulate the activation of the immune response. While many of the NK cell immune checkpoint molecules discovered to date are inhibitory in nature, we found that the checkpoint molecules upregulated on IL-10$^+$ NK cells in Malian individuals are ones that have been characterized as being activating in nature. For instance, the marker 4-1BB (CD137) is co-stimulatory and is expressed on activated T and NK cells [119]. Its cognate ligand is 4-1BBL, which is present on professional antigen-presenting cells (APCs), such as dendritic cells and monocytes and macrophages [119]. We found that 4-1BB$^+$ subsets of NK cells had significantly higher expression of IL-10 compared to 4-1BB$^-$ NK cells. A recent study of 4-1BB expression in NK cells has demonstrated that anti-4-1BB stimulation combined with IL-27 and an NKG2D agonist increased ADCC activity of NK cells against prostate cancer cells [121]. Another immune checkpoint molecule that was expressed on NK cells with increased IL-10 secretion was CTLA-4. While CTLA-4 expression in T cells has been characterized by numerous studies, very few have investigated the role of CTLA-4 expression in NK cells. One study of human NK cells has reported that CTLA-4 haploinsufficiency in NK cells limits IFN-γ expression in response to cytokine stimulation as well as degranulation in response to target K562 cells [151]. This implies a stimulating role of CTLA-4 in NK cell effector functions. Lastly, the sialic acid-binding immunoglobulin-type lectin Siglec-7 has been identified as an inhibitory immune checkpoint receptor. In NK cells, anti-Siglec-7-blocking antibodies promote NK cell-mediated cytotoxicity against K562 cells [152]. Our finding that NK cells characterized by the absence of this inhibitory signal, that is Siglec-7$^-$ NK cells, expressed more IL-10 compared to Siglec-7$^+$ NK cells implies that IL-10$^+$ NK cells may be less inhibited than IL-10$^-$ NK cells. Taken together, the upregulation of 4-1BB and CTLA-4 and the downregulation of Siglec-7 on IL-10 secreting NK cells indicate that these cells may be more readily activated and have increased effector functions (especially cytotoxicity) compared to IL-10$^-$ NK cells.

In line with the above hypothesis, we also found that CD45RO, a marker that has been reported to strongly correlate with NK cell degranulation, defined a subset of NK cells that had more IL-10 expression than CD45RO$^-$ NK cells. In response to IL-12 stimulation CD45RO$^+$ NK cells have been reported to secrete significantly more IFN-γ and demonstrate enhanced cytotoxicity compared to CD45RO$^-$ NK cells [124,125]. Similarly, higher IL-10 secretion was seen on NK cells upregulating NKG2C, an established marker of adaptive NK cells with enhanced ADCC capabilities. The finding that IL-10 producing NK cells express markers associated with readily activatable subsets of NK cells with enhanced effector functions was surprising. Why would cells releasing anti-inflammatory mediators also be primed for enhanced killing? The dearth of studies regarding the role of stimulatory and co-stimulatory immune checkpoint molecules in NK cells makes it challenging to postulate. As stated previously, it is possible that signaling mechanisms not yet fully understood promote IL-10 secretion in some disease states, like malaria, as a way to curb the inflammation that results from target cell lysis. The observation that several activating immune checkpoints are upregulated on these cells indicates that they play an important role in mediating immune responses.

During cytokine stimulation and natural cytotoxicity assays, the NK cells from the US group demonstrated higher IL-10 release in $CD8^-$, $TIGIT^+$ and $PD\text{-}1^+$ NK cell subsets compared to $CD8^+$, $TIGIT^-$ and $PD\text{-}1^-$ NK cell subsets, respectively. NK cells from Mali groups did not show a significant difference in IL-10 secretion in these subsets during those same assays, indicating that the IL-10 secreting NK cells of US individuals may express a different phenotype or constitute or include a different subset of NK cells than the IL-10 secreting NK cells of Malian individuals. For this study, the age range of Malian participants was 5 – 15 years old whereas the US donor range was older, at 25 – 60 years old. NK cell subset phenotypes, including those expressing immune checkpoint molecules, have been shown to change with age. For instance the percentage of $CD56^{bright}$ NK cells decreases over time. We, therefore, cannot exclude age-related differences as a potential factor contributing to different phenotypes between these two groups [153,154]. However, it is worth noting that many of the studies evaluating NK cell changes with age evaluate a much older patient profile (usually > 60 years old), and other studies including age groups composed of young adults and adolescents have found that the majority of NK cell phenotypic differences existed between adults and the pediatric groups but not adults and adolescents [155,156,157].

Other explanations for the finding that the $IL\text{-}10^+$ NK cells of US donors may differ in phenotype from those of Malian individuals include the possibility that these differences are genetically inherited. Similar to the development of sickle cell trait, it could be that the NK cell subset upregulation of some receptors and downregulation of others (PD-1 and TIGIT in this instance) in individuals living in malaria endemic countries may represent one more way in which *Plasmodium* has placed selective pressure on the human genome.

Lastly, heterogeneity of NK cells is a result of both genetic and environmental diversity. It is possible that environmental factors—like increased exposure to helminths or another infectious agent yet to be implicated, or *Plasmodium* itself—may stimulate the development of a subset of IL-15 respondent IL-10 secreting NK cells that express a cytotoxic phenotype, much as CMV induces the development of a compartment of NKG2C$^+$ adaptive NK cells [158]. In this study, while we did not know the CMV status of Malian participants, we did have serostatus information for all US participants. NK cell IL-10 secretion was not significantly different between CMV$^+$ vs CMV$^-$ groups (Figure 13). This implies that CMV does not play a role in the differentiation of IL-10 secreting NK cell subsets, at least for malaria-naïve individuals. However, it is possible that the very low levels of IL-10 secretion from US participant NK cells obscured potential differences. Additional studies using NK cells that secrete higher levels of IL-10, such as those from Malian individuals, could lend more insight into any potential roles CMV might have in IL-10 secreting NK cell differentiation.

Our findings of more robust IL-10 secretion in the NK cells of malaria-exposed individuals along with a phenotype associated with enhanced cytotoxicity and activation imply a new dual role for these NK cells as being both immunoregulatory and inflammatory. This suggests that these cells may be key in mediating host immune responses to malaria infection in order to achieve a balance between a response strong enough to control and clear parasites, but not so abberant as to cause immunopathology. With further studies it is possible that the protective capabilities of NK cells may someday be harnessed to control malaria-induced pathology.

CHAPTER 4: Summary and future directions

Summary

Our work presented in this thesis has contributed to the fields of malaria genetics and immunology in several ways. Our comparisons of molecular tools for genotyping malaria infections have given evidence for the increased utility of a 24 SNP barcode for determining recrudescence from reinfection over traditional *msp* genotyping. Our R code for geographically optimizing the 24 SNP barcode may aid in the design of smaller SNP barcodes that yield the same genotyping results as the full barcode while lowering the costs associated with this method. This is an important consideration in resource-restricted settings.

We also helped broaden understanding of the role of NK cell IL-10 secretion in the setting of malaria infection. Our findings indirectly support what has been observed in ECM models. That is, individuals more protected from severe malaria disease had higher levels of NK cell IL-10 secretion in response to IL-15 stimulation compared to individuals more vulnerable to severe disease. From this it appears that NK cell derived IL-10 may contribute to protection against malaria. However, given the complex relationships between cytokine signaling and both protection and pathology, additional studies of NK cell IL-10 release in different malaria disease states (especially asymptomatic infection and cerebral malaria) are needed to provide further context for this role.

We also characterized for the first time NK cell IL-10 secretion during uncomplicated malaria disease. This provided insight into potential differences in NK cell biology during symptomatic infection. For instance, we found a significant inverse correlation between CD107a (a marker of degranulation) expression and IL-10 secretion during the malaria timepoint. This was not observed for the Mali May or Mali convalescent timepoints, indicating a potential switch in NK cell response to IL-15 and IL-21 stimulation during malaria. It will be interesting for future studies to investigate mechanisms that could explain this, including investigations into the roles that inflammatory mediators that are present during malaria infection might play in mediating this switch.

We also described, for the first time, NK cell phenotyping markers that correlated with IL-10 secretion. We found that several of these markers are associated with enhanced cytotoxicity. This points to a novel role for IL-10 secreting NK cells as both initiators of inflammatory processes through target cell lysis, and immunoregulators by way of anti-inflammatory IL-10 secretion.

Together these studies highlight new findings, for instance the phenotype associated with IL-10^+ NK cells. We have indirectly correlated some of the findings in ECM with human malaria disease, adding to the literature supporting ECM as a malaria model. We hope to have also contributed more broadly to the understanding of NK cell biology by proposing a potential dual-role for IL-10 secreting NK cells. With these contributions, perhaps the puzzle that is malaria pathogenesis becomes just a little bit more put together.

Future Directions

All of the work presented here could benefit from additional follow-up studies providing mechanistic evidence supporting and expanding upon our findings. Regarding our genotyping tools comparisons, in Malawi we found that a subset of 6 SNPs performed almost as well as the originally designed 24 SNP method. Because genotyping relies on high levels of genetic variation to identify distinct isolates, it may be that the genetic diversity of

Plasmodium isolates in the Malawi region are particularly high, and that extremely varied genotypes could easily be identified by a very low number of SNPs. It is possible that other malaria endemic regions have less genetic variation between isolates and so may require the full 24 SNP barcode for genotyping. If study groups in other countries utilize this R code for generating a shortened barcode, it will be interesting to see how the truncated version performs compared to the full-length barcode in other regions. Factors that influence parasite genetic diversity, for instance transmission intensities and host vaccination rates, may indirectly affect the ability of shorter barcodes to distinguish individual parasite isolates. Perhaps future studies might reveal geographic regions that are best suited for using truncated genotyping barcodes and identify factors that might influence their usefulness.

Another related use of the R program we generated that has not yet been discussed is SNP prioritization. Rather than truncate the whole barcode, researchers could instead use the R program to prioritize SNPs in the full barcode so that those most likely to reveal genotypic differences would be run first. For instance, the R program could be used to generate the top 6 SNPs most likely to reveal genotypic differences between isolates. Researchers could then run all samples using these 6 prioritized SNPs, which should identify the majority of genetically distinct samples. These samples could then be excluded from further analysis. Any samples that were identical at all 6 SNPs could then go on to the next round of genotyping which would use the 6 "next best" SNPs and so on. The exclusion of samples that are determined to be genetically distinct early in the genotyping process would save significant reagents, time, and money. Using comparison methods similar to those in Chapter 2, it would be relatively straightforward for our lab to test the efficacy of this "prioritization" approach.

Our finding that NK cell secretion of IL-10 was higher in Malian individuals compared to malaria-naïve individuals indicates that NK cell-derived IL-10 may play a protective role in malaria disease. However, due to the complexity of the relationships between cytokines, immune signaling and disease pathologies we cannot exclude the possibility that NK cell derived IL-10 and protection from severe disease may not be a straightforward correlation. Other studies have reported elevated levels of circulating IL-10 during severe cerebral malaria disease [59]. In a *P. chabaudi* mouse model of malaria, IL-10 has also been reported to promote hyper-parasitemia [159]. During malaria infections there are a number of cellular sources of IL-10 in addition to NK cells, including $CD4^+$ and $CD8^+$ T cells, Treg cells, B cells, dendritic cells and macrophages [80]. It is possible that the cellular source of IL-10 and the timing of its release can impact malaria infection outcomes. Studying this in the human system would be challenging due to variables like unknown inoculation doses, unknown time of initial infection, and genetic variability of the infecting parasites. Mouse models of malaria disease, especially ECM, would be more feasible for studying the sources and timing of IL-10 release along the course of a malaria infection, and clarify the effects these variables might have on infection outcomes. Ultimately, findings in human malaria disease will be needed to confirm findings from mouse models. Studies of NK cell IL-10 secretion during asymptomatic and severe malaria, such as cerebral malaria, will provide a more complete picture of the regulatory roles of this protein.

There are several mechanisms by which NK cell IL-10 secretion may be mediating inflammatory processes during malaria. For instance IL-10 acts on antigen-presenting cells (APCs) like macrophages to inhibit their production of proinflammatory cytokines, including IFN-γ and TNF-α, thereby dampening downstream inflammation [80]. It also plays an important role in mediating pathology within the cerebral microvasculature. It has been shown to downregulate ICAM-I on endothelial cells—an important mechanism in preventing the sequestration of iRBCs within the brain which in turn protects against ECM pathology [80,160].

Another mechanism by which NK cell-derived IL-10 may mediate malaria pathology has been alluded to by other studies but has not yet been proven. During ECM, mice develop a fatal cerebral malaria-like disease that is caused by CD8$^+$ T cell-mediated pathology [161]. Burrack *et al* found that, IL-15C treatment during ECM decreased CD8$^+$ T cell activation in the brain and prevented associated pathology (especially blood brain barrier breakdown) [66]. It is possible that NK cell-derived IL-10 secreted in response to IL-15C treatment directly suppresses CD8+ T cell activity, perhaps through mechanisms such as decreasing CD8$^+$ T cell antigen sensitivity [162].

Our findings regarding NK cell IL-10 secretion during malaria disease implied to us that this cell response may play an important role in protecting against severe disease. To better understand the NK cell subsets secreting IL-10 we screened for extracellular markers associated with IL-10 release. Immunophenotyping IL-10 secreting NK cells yielded surprising results. We found that NK cells expressing several activating immune checkpoint molecules had higher levels of IL-10 secretion. Markers for adaptive NK cells and proteins associated with enhanced cytotoxicity were also upregulated on IL-10$^+$ NK cells. These findings indicate a cytotoxic phenotype for IL-10 secreting NK cells. We have proposed a theory that NK cell IL-10 secretion may be advantageous for limiting immunopathology that is promoted by target cell lysis. Our functional studies also lend support for this theory. We found that significantly higher levels of IL-10 were secreted during ADCC compared to cytokine stimulation alone in US and Mali convalescent timepoint groups. Perhaps cellular components released during the lysis of red blood cells provide signals for NK cells to release IL-10. It has been demonstrated that during ECM, heme activates the signaling molecule STAT3 [163]. STAT3 has been shown to be a key modulator of two signaling processes: 1.) It is required for mediating IL-10R signaling that activates a protein complex, the mammalian target of rapamycin complex 1 (mTORC1). Activated mTORC1 initiates metabolic reprogramming within NK cells that directly lead to their enhanced effector functions, specifically ADCC and natural cytotoxicity [143]. And 2.) Activation of STAT3 is required for NK cell production of IL-10 following IL-15C treatment in ECM. The activation of STAT3 is initiated by IL-15 and amplified by IL-10 signaling [144]. These observations, along with our findings that IL-10 release is enhanced during ADCC, provide a potential mechanism for how the two paths of IL-10 production and effector functions in NK cells could be linked. Additional studies are needed to fill in gaps, such as whether heme from iRBC lysis, through the activation of STAT3, can contribute to IL-10 production and/or enhanced ADCC. Also, the activation of STAT3 during IL-15C treatment in ECM was shown to increase IL-10 production (shown by elevated levels of IL-10 mRNA), but further studies are needed to investigate whether additional signals are needed for IL-10 secretion.

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