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The role of innate(-like) lymphocytes and their inhibitory receptors in the immune response to malaria

By

Perri Claire Callaway

A dissertation submitted in the partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Infectious Diseases and Immunity

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Margaret Feeney, Co-chair
Professor Eva Harris, Co-chair
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Professor Ellen Robey

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Abstract

The role of innate(-like) lymphocytes and their inhibitory receptors in the immune response to malaria

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Professor Margaret Feeney, Co-chair
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P. falciparum (Pf) causes millions of cases of clinical malaria and hundreds of thousands of deaths each year, primarily in children under the age of five in sub-Saharan Africa. With increasing drug resistance, the need for an effective malaria vaccine is evident. In order to design an effective malaria vaccine, the immunological correlates of protection from malaria must be better understood. This is particularly important to understand in endemic populations given that many malaria vaccine candidates that were promising in naïve individuals were significantly less effective in endemic populations. Here, we examine the role that the innate and innate-like lymphocytes, NK cells and Vγ9Vδ2 T cells (Vδ2s), respectively, play in the immune response to malaria and how they are altered by chronic *Pf* exposure. We found that NK cells, like Vδ2s, are altered by chronic *Pf* exposure and shift towards a higher proportion of CD56negCD16+ NK cells but are able to recover after the chronic exposure is stopped. These CD56negCD16+ NKs are less likely to respond to cytokine stimulation but are effective at antibody dependent cellular cytotoxicity (ADCC). We next examined the role that inhibitory receptors on these innate(-like) lymphocytes might play in dampening the malarial immune response. First, we analyzed individuals' killer cell immunoglobulin-like receptors (KIRs) and their HLA ligand repertoires and found that two KIR-HLA ligand pairs were associated with an increase in parasite prevalence. Then, we studied the expression of KIRs in addition to two other inhibitory receptors with MHC-I ligands, LILRB1 and NKG2a, on Vδ2s. We observed that chronic *Pf* exposure was associated with an increase in KIRs and LILRB1 on Vδ2s and that Vδ2s with these inhibitory receptors were less likely to respond to TCR stimulation than their inhibitory receptor negative counterparts. Altogether, these data indicate that chronic *Pf* exposure leads to an immune repertoire that is less responsive to more generalized, soluble stimuli in part due to inhibitory receptors and instead takes advantage of the specificity of antibodies developed during chronic exposure to have a more narrow response. However, the importance of robust Vδ2 and NK cell responses during early infections is highlighted by the association of two inhibitory KIR-HLA ligand pairs with an increase in parasite prevalence suggesting impaired parasite clearance by the immune system. These findings have the potential to help guide future vaccine design targeted at

Pf endemic populations by directing the immune response to cells that have maintained their *Pf* responsiveness or by strongly stimulating cells that have become less responsive to *Pf* stimuli.

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Chapter 1

Introduction

Each year there are over two-hundred million cases of malaria resulting in over half of a million deaths. The vast majority of these deaths are in children under the age of five in sub-Saharan Africa (1). Malaria is caused by protozoan parasites of the *Plasmodium* species. While *Plasmodium falciparum* (*Pf*) is responsible for the vast majority of malaria morbidity and mortality, *Plasmodium vivax* accounts for a large portion of the malaria cases outside of sub-Saharan Africa (1). While there are effective anti-malarial drugs, drug resistant parasites are emerging and the need for an effective vaccine is apparent (2). The recent approval of the malaria vaccine RTS,S is promising; however, this vaccine has been shown to be less effective in malaria endemic populations and has a longer, complex regimen that can be hard to deliver in low resource settings (3,4).

P. falciparum does not induce sterilizing immunity

The high levels of malarial morbidity are, in part, caused by the failure to develop sterilizing immunity after an infection, resulting in individuals being infected multiple times throughout their life if not in a single year (5). While sterilizing immunity is not achieved, after many episodes of malaria some individuals develop what has been termed "clinical immunity". Clinical immunity is the development of immunity from symptoms, so even though an individual may still be infected with *Pf*, they will be protected from the clinical presentations of malaria (6,7). Recent data also suggests that these individuals are better at controlling parasite density, implying a more robust immune response against the parasite in addition to more tolerogenic mechanisms (7).

It is thought that sterilizing immunity is not developed in large part due to the high antigenic variation of the *Pf* (8,9). The genome of *Pf* contains over 5000 genes and is far more complex than other bacterial and viral pathogens (10). Of particular interest are the *var*, *rif* and *stevor* gene families that are variably expressed on the surface of infected red blood cells (iRBCs) and can be switched out for different genes in the same gene family (11–13). The *var* genes, more specifically, are expressed on the surface of iRBCs in order to facilitate the sequestration of the parasite in the vasculature by binding to different host proteins on endothelial cells. This sequestration enables the parasite to avoid clearance in the spleen. The parasite will vary which of the *var* genes it expresses and thus vary which host protein that the *var*-encoded protein Pfemp1 will bind to. This variation can make it hard for the immune system to make effective immune responses against relevant antigens (11). However, recent studies have illuminated how forming an immune response against specific variants of these gene families can protect from specific manifestations of clinical disease. Perhaps the most well studied example of this is protection from placental malaria after antibodies to the Pfemp1 variant VAR2CSA are developed. VAR2CSA is a variant of the *var* gene product that binds to the host protein chondroitin sulfate A (CSA), a protein that is only expressed in the placental vasculature so in most *Pf* infections VAR2CSA is unable to bind to its ligand and therefore unlikely to be immunogenic (14,15). However, in primigravid women, VAR2CSA is able to bind CSA in the placenta and cause an accumulation of parasites which can lead to placental inflammation and poor pregnancy outcomes (16). While primigravid women have no antibodies against VAR2CSA, women who have been pregnant before and have mounted an immune response against VAR2CSA are protected from placental malaria which is thought to be due to antibodies

against VAR2CSA neutralizing its ability to bind to CSA in the placenta (17,18). While immune responses against specific variants seems to abrogate the likelihood of specific severe disease manifestations, the vast variation in *Pf*'s antigen presentation ensures that individuals are still susceptible to infection.

High variation in *P. falciparum* transmission at different Ugandan study sites

As part of an effort to better understand malaria transmission worldwide, three study sites in Uganda were developed as one of the International Centers of Excellence for Malaria Research (ICEMR) and the PRISM (Program for Resistance, Immunology, Surveillance & Modeling of Malaria in Uganda) cohort was established (19). As part of this large collaboration an immunology lab was established to process immunological specimens (peripheral blood mononuclear cells, serum, whole blood preserved in RNAlater etc). Immunological specimens were processed from two of the study sites, Jinja and Tororo. Jinja is a peri-urban locality with a low incidence of malaria while Tororo, near the Eastern border with Kenya is more rural and has historically had a significantly higher incidence of malaria. Data from a third site, Kanungu, was also collected although no immunological samples were processed. This site had an intermediate level of malaria and is in southwest Uganda. The collaboration was able to accurately measure malaria incidence by calculating an annual entomological incidence rate (aEIR). In order to do this, they trapped all of the mosquitos within a single night at a specific location and tested them for presence of the malaria parasite. Thus, the aEIR estimates how likely it is that someone is bitten by a mosquito carrying the malaria parasite. The aEIR for Jinja is only 3.8 compared to Tororo's much higher rate at over 120 (20,21). We have been able to leverage this difference in *Pf* exposure between these two otherwise similar study sites in order to understand how clinical immunity develops and how *Pf* exposure modulates the immune system.

However, due to the well characterized high incidence of malaria in Tororo, the Ugandan government targeted it for indoor residual spraying (IRS) of insecticides. Beginning in December 2014, multiple rounds of IRS were conducted in Tororo. This led to a dramatic reduction in the aEIR in Tororo from over 120 to zero (22,23). Because the PRISM study continued to collect samples during this period of dramatic reduction, it served as a natural experiment of what happens to an individual's immune system which had previously been continuously exposed to *Pf* when it no longer has that exposure.

The role of Vγ9Vδ2 T cells in the immune response to *P. falciparum*

Vγ9Vδ2 T cells (Vδ2s) are a subset of γδ T cells that indirectly respond to non-peptidic small molecule ligands called phosphoantigens (pAgs). In humans, this subset is the predominant subset of γδ T cells in the blood and constitutes 3-5% of the total T cells in the blood. To activate Vδ2s, pAgs bind intracellularly to two host molecules, butyrophilin2a1 (BTN2a1) and butyrophilin3a (BTN3a1) leading to a conformational change in the BTN molecules that the Vδ2 TCR is then able to recognize (24–26). Mammals produce some pAgs, notably IPP, as a biproduct of the mevalonate pathway of isoprenoid biosynthesis which can accumulate intracellularly in cancer cells leading to the activation of Vδ2s (27). However, some microbes, including *Pf,* produce distinct pAgs as a byproduct of their non-mevalonate pathway of isoprenoid synthesis. One of these, HMBPP, is able to activate Vδ2s one thousand times more

potently than IPP (28). As a result, Vδ2s are intrinsically reactive to a *Pf* infection. In fact, during an acute episode of malaria in a naïve individual, Vδ2s can expand to be up to 50% of the T cells in the blood (29–31). Vδ2s have also been shown to be an important correlate of immunity in several malaria vaccine trials (32–35). Their strong response during acute *Pf* infections and their potential role in generating a protective immune response following vaccination with malaria vaccine candidates highlights the need to better understand these historically understudied cells.

Despite a strong initial increase in Vδ2s during an acute infection, the lab has previously shown that the overall frequency of Vδ2s declines after many episodes of clinical malaria. We have also shown that the frequency of Vδ2s is lower in individuals from the high *Pf* exposure study site Tororo compared to individuals from the low exposure study site Jinja. We have further shown that these Vδ2s are less responsive to *Pf* antigen (36–38). These changes have implications for the efficacy of malaria vaccines in endemic populations. By studying why these changes occur and if they can be reversed, this research will inform better vaccine design.

The role of Natural Killer cells in the immune response to *P. falciparum*

Natural Killer (NK) cells are a subset of innate lymphocytes that respond to a balance of activating and inhibitory receptors. Inhibitory receptors with constitutively expressed host ligands ensure that NK cells do not attack healthy host cells while activating receptors respond to ligands like pathogen associated molecular patterns (PAMPs) and cytokines (39). Generally, NK cells are further subset by their expression of the markers CD56 and the Fcγ receptor CD16. CD56brightCD16- NK cells are considered less mature whereas CD56dimCD16+ NKs are considered more mature (40,41). NK cells have been shown to play important roles in both anti-viral and anti-tumoral immune responses but their role in parasitic infections is less well defined.

While many have conjectured about putative *Pf* or iRBC ligands for different activating NK cell receptors in the context of malaria, no definitive interactions have been identified. However, data suggests that NK cells are activated during *Pf* infection, likely indirectly through myeloid and T cells. Evidence further suggests that they may be important in producing IFNγ early on in infection (42). More recent studies have focused on the ability of NK cells to recognize and kill opsonized iRBC through antibody dependent cellular cytotoxicity (ADCC) via CD16. One study found that a subset of NK cells called adaptive NK cells dominated the ADCC response to iRBCs and correlated with protection from parasitemia (43).

Immunopathology in malaria

It is thought that a large portion of the symptoms attributed to clinical malaria may actually be due to the host response to the parasite. These host responses are considered immunopathogenic. The large amount of pro-inflammatory cytokines produced by the immune system contribute to the cyclical fevers associated with acute malaria (44,45). It has been hypothesized that both Vδ2s and NK cells contribute to immunopathology in malaria given their more innate(-like), quicker responses, ability to produce high levels of pro-inflammatory cytokines and capacity for cytotoxicity (42,46). Their early and less specific production of pro-inflammatory cytokines can lead to damage to the host rather than, or in addition to, restriction of the parasite.

The development of clinical immunity, or immunity from symptoms, implies immune mechanisms which are able to control immunopathology. The immune system has a number of mechanisms by which to balance inflammation with tolerance, including Tregs, anti-inflammatory cytokines and inhibitory receptors. These cells and molecules act as the brakes on the immune system to keep it from attacking and damaging self. We became interested in the role that inhibitory receptors play broadly in the immune response to malaria, whether in stymieing an effective immune response or tamping down an out of control response.

Killer cell immunoglobulin-like receptors

Killer cell immunoglobulin-like receptors (KIRs) are a family of 14 transmembrane proteins, some of which are stimulatory and some of which are inhibitory. The inhibitory family members have a longer intracellular domain that associates with immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The longer intracellular domain of the inhibitory receptors is denoted as an L in the KIR name and the shorter intracellular domain of stimulatory KIRs is denoted as an S (e.g. KIR2DL1 is inhibitory while KIR2DS1 is stimulatory) (47,48). Both stimulatory and inhibitory KIRs share MHC-I as ligands, however different KIRs have different subsets of MHC-I as ligands. For example, KIR2DL1 recognizes the ligand group HLA-C2 which is a subset of HLA-C alleles. KIR3DL1 recognizes the HLA-Bw4 ligand group which is comprised of HLA-A and HLA-B ligands that share the Bw4 motif. There are two pairs of KIRs that are alleles of the same gene rather than distinct genes: one pair is KIR2DL2 and KIR2DL3 and the other is KIR3DL1 and KIR3DS1, despite the latter pair's contrasting functions (49,50). Generally, the inhibitory KIRs have been studied more in depth and have been better characterized.

KIRs whose genes are nearly universally expressed in all people are considered framework KIRs. Non-framework KIRs are found at varying levels within different human populations, but generally two different haplotypes have been defined in humans. Haplotype A, defined by the set presence of the KIRs: KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DS4 and a pseudogene, is considered to be the more inhibitory haplotype with only one stimulatory KIR. Haplotype B, in contrast, is almost any other combination of KIRs that includes a KIR not in Haplotype A and as a result can have a much higher proportion of stimulatory KIRs (51,52). In addition to inter-individual variation in KIR genes, there is even higher inter-individual variation in the presence of KIR ligands. As a result, a given individual may have a specific KIR but not its ligand or vice versa.

KIRs are primarily expressed on NK cells been but are also expressed on subsets of T cells, including Vδ2s (53). Within NK cells, KIRs are expressed at higher levels on the more mature CD56dimCD16+ subset of NK cells compared to the less mature CD56brightCD16- NK cells (54). KIRs are important in the licensing or education of NK cells that enables them to respond to MHC-deficient cells in the context of tumor cells or viral infections when MHC has been downregulated. In turn, KIRs also help to prevent NK cells from responding to normal, healthy host cells (47). The role of KIRs on T cells is less well defined.

Presence or absence of specific KIRs and their HLA ligands have been associated with a multitude of different infectious, autoimmune and other disease outcomes (55–60). Of particular

note is the association of KIR3DS1 and non-progressors in HIV infection (61). While previous studies have looked for associations between KIRs and malaria, most have focused on associations with severe malaria rather than susceptibility to infection (62).

LILRB1

LILRB1 is a transmembrane inhibitory receptor that has also been known as ILT-2, LIR-1 and CD85j. It is part of the LILR (leukocyte immunoglobulin-like receptor) family which, like KIRs, contains stimulatory and inhibitory receptors. A "B" in a LILR name denotes than it is an inhibitory receptor, which are differentiated from stimulatory LILRs by the presence of ITIMs on their intracellular domains, while an "A" denotes a stimulatory receptor. Some, but not all, of the LILR family have been shown to bind to classical MHC-I proteins. LILRB1 not only binds to classical MHC-I but also binds to non-classical HLA-G at an even higher affinity (but does not bind to HLA-E) (63). LILRB1 is broadly expressed on both myeloid and lymphoid cell lineages. LILRB1 is expressed at particularly high levels on monocytes and B cells and is expressed at lower levels on NK cells and T cell subsets, including Vδ2s (64,65). Further, different *LILRB1* polymorphisms have been shown to affect the surface expression levels of LILRB1 (66).

In the context of infection, LILRB1 has been shown to be the target of multiple immune evasion strategies by different pathogens. The HCMV protein UL-18 is able to bind LILRB1 and has been shown to inhibit NK cells, an important component of the HCMV immune response (67,68). There is also evidence that LILRB1 can bind to the dengue virus E protein, leading to a downregulation of interferon stimulated genes (ISGs) enabling monocytes to becomes infected with DENV (69). Of the most interest is the recent finding that LILRB1 can bind to a subset of *P. falciparum* proteins called RIFINs (repetitive interspersed families of polypeptides) (70).

RIFINs, encoded by the highly polymorphic *rif* gene family, are expressed on the surface of red blood cells infected with *Pf*. It is thought that these variant proteins contribute to the formation of rosettes, enabling binding of iRBCs to the microvasculature, and may therefore contribute to the development of severe malaria (71). In 2017, LILRB1 was identified as an inhibitory receptor capable of binding to a subset of iRBCs from malaria patients and RIFINs were identified as the LILRB1 ligand. However, not all RIFINs were able to bind to LILRB1 and the authors were unable to identify a specific sequence that conferred the ability to bind it. They were, however, able to show that some RIFINs can bind to LILRB1 on B cells leading to a decrease in the production of IgM by those B cells. The same study found that individuals with severe malaria had a higher number of iRBCs that were able to bind LILRB1 than individuals who had non-severe malaria (70). Together, these data show that LILRB1 plays an important role in regulating the immune response to malaria, but more studies are needed to determine which LILRB1+ cells are mediating the observed difference in malaria severity.

NKG2a

NKG2a is an inhibitory receptor primarily expressed on NK cells that recognizes HLA-E. In addition to expression on NK cells, NKG2a is also expressed on subsets of T cells including Vδ2s (72). Like KIR and LILRB1, it is part of a family (NKG2) of receptors that are both

inhibitory and stimulatory. Both the stimulatory and inhibitory family members dimerize with CD94 on the cell surface. The NKG2a ligand, HLA-E, is a non-classical MHC-I molecule that presents leader sequences from other classical MHC-I molecules. This enables NKG2a to serve as a monitor of classical MHC-I expression within a cell. In a tumor or infected cell, downregulation of classical MHC-I would lead to altered expression or presentation of HLA-E on the cell surface (73,74). Interestingly, in a similar manner to how it ligates LILRB1 to dampen the immune response, HCMV has also developed a peptide that can mimic the leader sequences of classical MHC-I that are loaded onto HLA-E thus leading to appropriate recognition by NKG2a despite infection (75).

On NK cells, NKG2a is expressed more frequently on less mature CD56brightCD16- NK cells and is downregulated on CD56dimCD16+ NK cells as they begin to express KIRs (54). NKG2a like KIRs is thought to be an important component of NK cell licensing or education with NK cells either relying predominantly on NKG2a or KIRs for licensing/education (76,77). NKG2a on Vδ2s has recently been described as a marker for Vδ2s that are hyper-responsive to cancerous cells (78).

Summary

In Chapter 2 of this dissertation, I take advantage of a unique natural experiment to show that while NK cells are altered in the context of chronic *Pf* exposure, cessation of this exposure leads to a return to baseline levels of both NK cells and Vδ2s. In Chapter 3, I leverage the extensive clinical data and genotyping of the PRISM cohort to find that KIR ligands HLA-C2 and HLA-Bw4 are associated with increases in parasite prevalence. Finally, in Chapter 4 I combine the study of the innate-like Vδ2 subset and inhibitory receptors to examine how LILRB1, KIR and NKG2a expression alters and is altered by the Vδ2 immune response to *Pf*.

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Chapter 2

Cessation of chronic *P. falciparum* exposure leads to rebounds in previously decreased Vγ9Vδ2 T cell and NK cells subsets

Introduction

While the overall incidence of malaria deaths has decreased over the past 20 years, progress has plateaued in the last several years with an observed increase in incidence and deaths in 2020 (1). One of the tools that has been effectively utilized to decrease malaria incidence in sub-Saharan Africa is indoor residual spraying (IRS) of insecticides. The application of insecticides on the indoor surfaces of residences leads to a drastic reduction in the amount of malaria in a community through the killing of *Anopheles* mosquitos (2).

Historically, Tororo, Uganda experiences high levels of malaria with an annual epidemiological incidence rate (aEIR) of over 120 in 2011-2012, indicating someone living in Tororo would be bitten by a mosquito carrying *P. falciparum* (*Pf*) 120 times a year (2). In 2014, the Ugandan Ministry of Health undertook a campaign of IRS targeting fourteen districts in Northern and Eastern Uganda, including Tororo (3). After multiple rounds of IRS the aEIR was dramatically reduced to zero (2). In contrast, Jinja, Uganda has an aEIR of just 3.8 and has not been targeted by the government to undergo any IRS (4). This reduction in aEIR over a relatively short period of time within the same population provided a unique opportunity to study the dynamics of the immune response to malaria and how they change with different levels of exposure.

Previously, we have shown that continuous, chronic *Pf* exposure, like that experienced by residents of Tororo, leads to an overall decline in the frequency of Vγ9Vδ2 T cells (Vδ2s) (5). Vδ2s are a subset of γδ T cells that are intrinsically reactive to *Pf*; their TCR indirectly recognizes a non-peptidic small molecule (HMBPP) that is a biproduct of *Pf*'s isoprenoid biosynthesis pathway. Further, we have shown that the remaining Vδ2s adopt some characteristics of NK cells; they are more likely to express the Fcγ receptor CD16 and NK receptors like killer cell immunoglobulin-like receptors (KIRs) (5,6). The rounds of IRS spraying enabled us to examine how the Vδ2 repertoire might change when no longer continually exposed to *Pf* antigens.

Given our observations with Vδ2s, we wondered whether NK cells might undergo parallel changes during continuous, chronic *Pf* exposure. NK cells are increasingly gaining recognition as an important part of the immune response to malaria (7). Of particular interest is their ability to perform antibody dependent cellular cytotoxicity (ADCC) of opsonized infected red blood cells and the correlation of different NK subsets with protection from malaria (8,9). Leveraging our low exposure study site, Jinja, and our high exposure study site, Tororo, we were able to examine differences in the NK cell repertoire likely due to high *Pf* exposure. We were then able to use longitudinal samples from before and after IRS in Tororo to further assess NK cell changes due to *Pf* exposure.

Here, we find that NK cells undergo parallel changes to Vδ2s in individuals that have experienced chronic *Pf* exposure, shifting to more antibody dependent responses. We further show that alterations in both the Vδ2 and NK cell compartments are reversed once environmental exposure to *Pf*-infected mosquitos is reduced by IRS. These findings highlight not only that *Pf* can drastically alter the immune repertoire but also that these changes may be transient.

Results

Repeated malaria episodes and chronic *Pf* exposure are associated with a decrease in CD56dimCD16+ NK cells and a parallel increase in CD56negCD16+ NK cells

By comparing samples from Tororo, Uganda with historically high *Pf* exposure and samples from Jinja with little *Pf* exposure, it is possible to examine perturbations in the NK cell compartment that may be due to chronic *Pf* exposure. We subset NK cells into 5 different subgroups based on their expression of CD56 and CD16 (Fig. 1a). CD56dimCD16+ are considered the more mature counterpart to the less mature CD56brightCD16- NK cells. In addition to these canonical subsets, two transitional subsets have been described, CD56dimCD16-and CD56brightCD16+ NK cells. CD56negCD16+ NK cells are increasingly being recognized as an additional subset of NK cells that are often elevated in chronic infections like HIV and herpesviral infections (10–14). While many studies have described these CD56negCD16+ NK cells as dysfunctional or functionally impaired, at least one recent studied suggests that they may instead rely on different antibody dependent activation pathways (15).

Intriguingly, we see a significantly lower proportion of CD56dimCD16+ NK cells in the high exposure site of Tororo (Fig. 1b). There is a correspondingly higher proportion of CD56negCD16+ NK cells in individuals from Tororo (Fig. 1c). This suggests that there is a shift from CD56dimCD16+ to CD56negCD16+ NK cells in individuals who are experiencing chronic *Pf* exposure. While this has not previously been observed in the context of malaria alone, it has been shown in individuals with Burkitt's lymphoma, a cancer caused by EBV but associated with malaria (15). While individuals from Tororo have a higher proportion of CD56negCD16+ NK cells, CD56dimCD16+ NK cells are still the highest prevalence subset for most individuals (Fig. 1d). There is also a significant difference in the proportion of CD56dimCD16- NK cells with individuals from Jinja, the low exposure site, displaying higher levels. Given that this is a more transitional subset of NK cells it might be indicative of NK cells from Tororo individuals leaving this compartment and becoming more mature CD56negCD16+ or CD56dimCD16+ NK cells.

In order to better determine if these observed differences between individuals from Jinja and Tororo were due to their varied *Pf* exposure and malaria incidence, we assessed whether the proportions of CD56negCD16+ and CD16dimCD16+ NK cells were associated with the number of clinical malaria episodes an individual had had in the previous year. We show that CD56dimCD16+ NK cells are negatively correlated with the number of malaria episodes in the previous year (Fig. 1e) but that CD56negCD16+ NK cells are positively correlated with the number of malaria episodes (Fig. 1f). This provides even stronger evidence that repeated episodes of clinical malaria contribute to a higher proportion of CD56negCD16+ NK cells, paralleling other chronic infections.

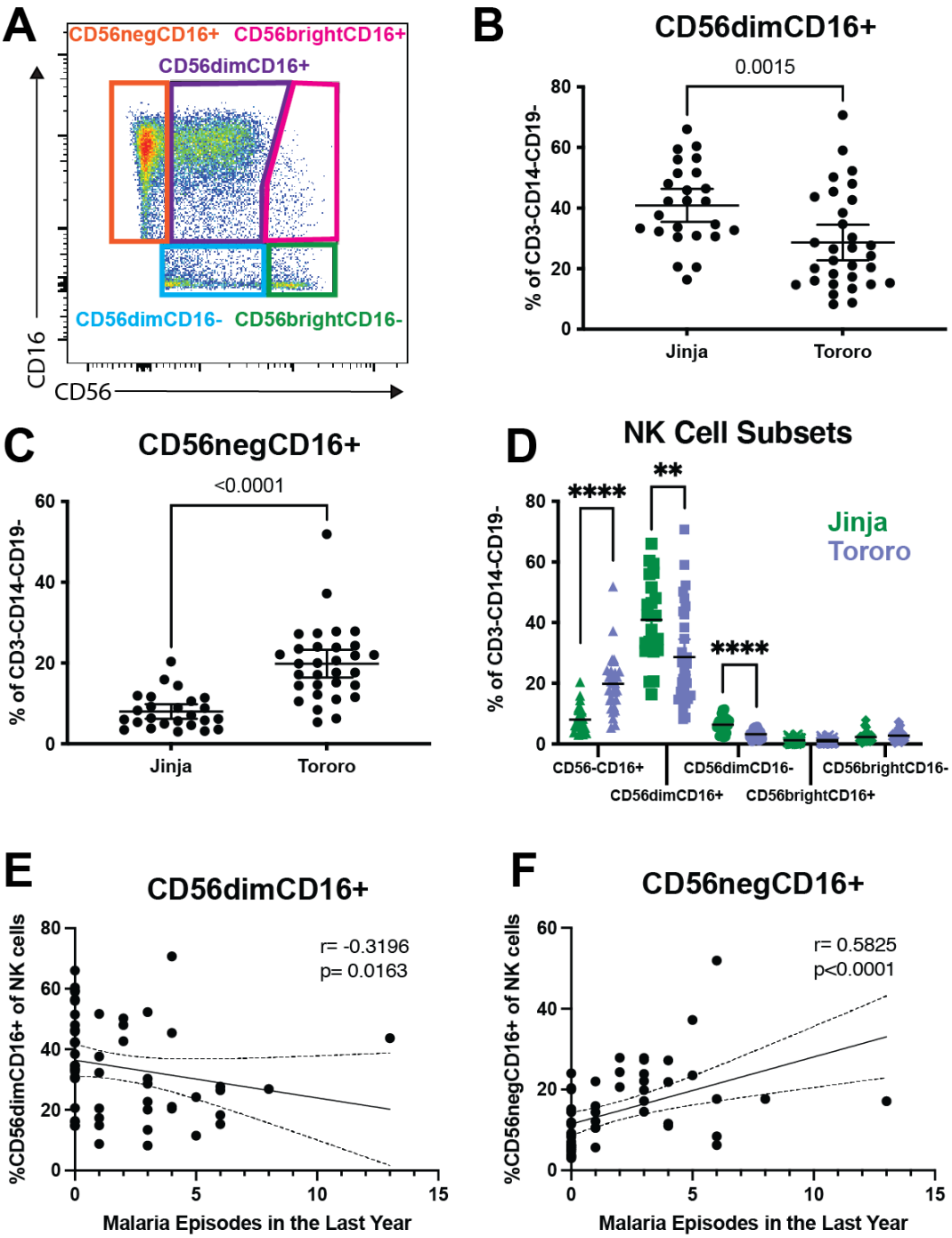

Figure 1: a) Gating schema for NK cell subsets. Gated on CD3-CD19-CD14- lymphocytes, excluding CD56-CD16- double negatives. b) Frequency of CD56dimCD16+ NK cells in individuals from either Jinja or Tororo. c) Frequency of CD56negCD16+ NK cells in individuals from either Jinja or Tororo. d) Frequency of all NK cell subsets in individuals from either Jinja or Tororo. e) Correlation of frequency of CD56dimCD16+ NK cells and number of malaria episodes in the last year. f) Correlation of frequency of CD56dnegCD16+ NK cells and number of malaria episodes in the last year.

Cessation of chronic *Pf* exposure by indoor residual spraying leads to a reversal of Vδ2 T cell and NK cell trends

Beginning in December 2014 the Ugandan government initiated an indoor residual spraying campaign of the Tororo study site. This application of pesticides to the interior walls of homes kills mosquito vectors and led to a dramatic reduction in the malaria incidence of the community (16,17). Given that we observed changes in both the Vδ2 and NK cell compartments with chronic *Pf* exposure, we utilized the unique opportunity provided by IRS to study how these compartments might change again with the cessation of *Pf* exposure.

We first examined Vδ2s, which our previous work has repeatedly shown decline with increased malaria incidence and chronic *Pf* exposure (5,6,18). We have further shown that while the frequency of Vδ2s declines, the overall proportion of Vδ2s expressing the Fcγ receptor, CD16, increases. We've found that these CD16+ Vδ2s have a more NK cell like phenotype and are less responsive to TCR stimulation while gaining the ability to be stimulated by opsonized infected red blood cells through CD16. Here we build on these results and observed that with the cessation of *Pf* exposure in Tororo, individuals' frequencies of Vδ2s rebounded within 6 months of the initiation of IRS and that this increase was maintained at the one-year post-IRS timepoint (Fig. 2a). We also see a corresponding decline in the number of CD16+ Vδ2s post-IRS (Fig. 2b). While this supports our previous findings that chronic malaria exposure affects Vδ2 frequency and the proportion of CD16+ cells, the mechanisms by which this occurs remain unclear. It does, however, suggest that the low frequency of Vδ2s in individuals who have had many episodes of malaria may be transient. Our observations align with a study in which Vδ2s recover functionality once individuals with HIV are virally suppressed and chronic antigenemia is reversed (19).

We next assessed if the changes we had observed in the NK cell compartment with chronic *Pf* exposure were also transient and reversible. After the initiation of IRS and the consequent dramatic reduction of *Pf* exposure, we observed a corresponding increase in CD56dimCD16+ NK cells (Fig. 3a). Paralleling the previously observed inverse association in the proportion of CD56negCD16+ and CD56dimCD16+ NK cells, we see a decrease in the proportion of CD56negCD16+ NK cells post-IRS (Fig. 3b). The maintenance of these inverse proportions before and after IRS highlights the possibility that CD56negCD16+ NK cells may be derived from CD56dim NK cells under conditions of chronic stimulation.

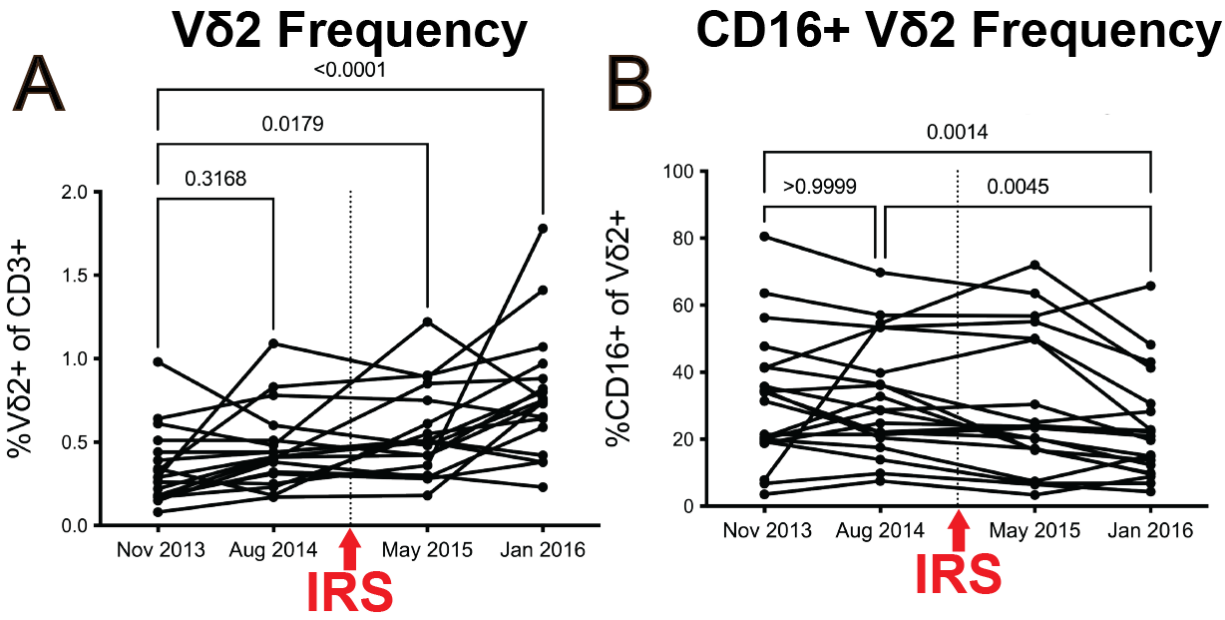

Figure 2: a) Frequency of Vδ2s at two time points before indoor residual spraying of pesticides (IRS) and at two time points after. b) Frequency of CD16+ Vδ2s at two time points pre-IRS and two time points post-IRS.

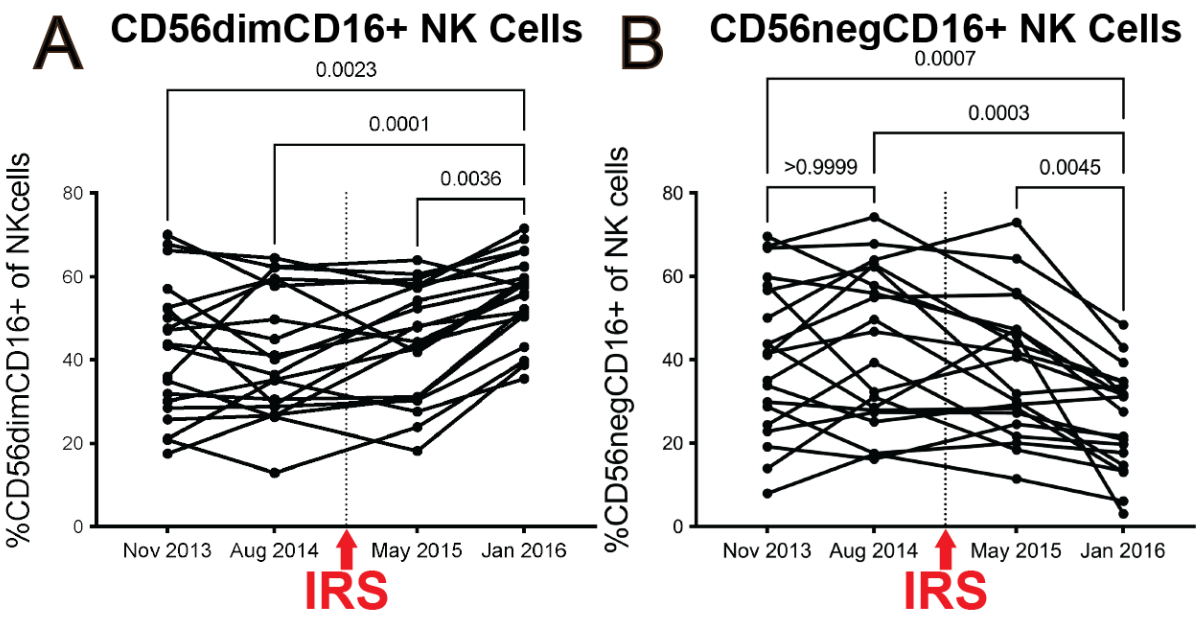

Figure 3: a) Proportion of CD56dimCD16+ NK cells at two time points before indoor residual spraying of pesticides (IRS) and at two time points after. b) Proportion of CD56negCD16+ NK cells at two time points pre-IRS and two time points post-IRS.

Discussion

Here we have shown that continuous exposure to *Pf* and high incidence of malaria episodes in childhood may modulate the NK cell compartment, leading to a higher proportion of the CD56negCD16+ NK cells and a lower proportion of the CD56dimCD16+ NK cells. We further show that when continuous *Pf* exposure rapidly and dramatically decreases, that not only do we see a reversal of the changes observed in the NK cell compartment, but we also see a reversal in changes we have previously observed in Vδ2s; they begin to increase again and the proportion of CD16+ Vδ2s declines.

CD56negCD16+ NK cells have previously been identified as an expanded subset in a number of chronic viral infections but to our knowledge have not been shown to expand in a parasitic infection. In initial studies of CD56negCD16+ NKs during HIV infection, these cells were thought to be dysfunctional due to their low responsiveness to cytokine stimulation. However, in recent years people have shown that they can be efficient mediators of ADCC (15). Intriguingly, we have shown in our previous work that high *Pf* exposure and repeated episodes of malaria lead to an increase in the proportion of CD16+ Vδ2s and that these CD16+ Vδ2s are capable of ADCC in response to opsonized infected red blood cells while being less responsive to TCR stimulation (5). This parallel shift to γδ T cells and NK cells that are more capable of ADCC while being less responsive to other less specific stimuli during chronic infections suggests a mechanism by which the immune system is able to minimize general inflammation while still responding to antibody bound pathogens in a narrower manner. This shift becomes particularly important in the greater context of the immune system during chronic *Pf* exposure; while these shifts in NK cells are occurring, the antibody repertoire against *Pf* antigens is growing. Shifting to NK and γδ T cells more focused on ADCC may enable the immune system to better take advantage of these *Pf* specific antibodies.

We had the opportunity to examine the durability and stability of these *Pf* induced changes in Vδ2s and NK cells when IRS led to an almost complete cessation of *Pf* transmission at the Tororo study site which prior to IRS had very high levels of *Pf* transmission. Post-IRS we observed both an increase in the frequency of Vδ2s and a decrease in the proportion of CD16+ Vδ2s. This suggests that continuous *Pf* exposure is necessary to maintain the depressed levels of Vδ2s and the increased proportion of CD16+ Vδ2s seen in individuals who have had repeated episodes of malaria. This may indicate these cells are being continuously culled in the peripheral immune system with little impact on thymic production. However, it should be noted that these studies all occurred in children who might be more capable of Vδ2 rebound than adults who have a lower capacity to produce new T cells.

In the NK cell compartment, we also observed a reversal of *Pf* induced alterations. The rebound of the CD56dimCD16+ population and the inverse decline of the CD56negCD16+ NK cells suggests that these cells might be either derived from the same progenitor population or that CD56negCD16+ NK cells may be derived from CD56dimCD16+ NK cells. A caveat to our study, however, is that we were only able to define NK cells by their absence of other lineage markers and the presence of CD16 or CD56. Thus, it is difficult to definitively state that the CD56negCD16+ population is NK cells. However, data from our lab and a collaborator show that the vast majority of these cells are CD7+ further defining them as NK cells. Additionally, in

data not shown here, we have observed that KIRs are expressed at the same or higher levels on CD56negCD16+ NK cells as CD56dimCD16+ NK cells, another indication these are NK cells. Overall these findings have interesting implications for NK cells during chronic infections, however, better characterization of marker expression and function are necessary. Combined with our data on Vδ2 T cells, our findings also have implications for the efficacy of malaria vaccines in endemic regions.

Methods

Study Approval

All study participants or the guardian of participants under 18 years of age gave written informed consent. The Uganda National Council of Science and Technology, the Makerere University School of Medicine Research and Ethics Committee, and the University of California, San Francisco Committee on Human Research approved all study protocols.

Study Site and Sample Processing

Samples were obtained from children enrolled in a large malaria surveillance study of two Ugandan districts: a suburban town in the Jinja district, with an annual entomological inoculation rate (aEIR) of 3.8, and the rural region of Tororo district, with an aEIR of 125 (4). Details from this study have been described elsewhere (2,4). In December 2014, the Ugandan government initiating an indoor residual spraying campaign in Tororo district which led to a drop in the aEIR to zero (2). PBMC were obtained at quarterly routine blood draws. 6 to 10 milliliters of blood were obtained in acid citrate dextrose tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Histopaque; GE Life Sciences) and cryopreserved in liquid nitrogen before analysis. For these analyses only timepoints where the individual was not parasitemic were included.

Flow Cytometry

Thawed PBMC were stained for surface markers following standard protocols (20,21) using the following antibodies and viability dye from Biolegend: CD56 (clone HCD56), CD16 (clone 3G8), CD19 (clone HIB19), CD14 (clone M5E2), Vδ2 (clone B6), CD3 (clone OKT3), L/D Aqua.

Flow Cytometry Data Analysis

Flow cytometry profiles were gated on single cells, Aqua Live/Dead negative, CD19-, CD14- lymphocytes. For NK cell gating cells were further gated on CD3- cells then gated on cells that were either CD56+ or CD16+. Without CD7 in the panel we could not establish if CD56-CD16- cells were NK cells. Cells that were positive for both CD3 and Vδ2 were defined as Vδ2 T cells. Samples were run on an LSRII flow cytometer (Becton Dickinson) with FACSDiva software. Data were then analyzed using FlowJo (Tree Star).

Statistical Analyses

All statistical analyses were performed using Prism 9.0 (GraphPad), and/or Stata (version 15, StataCorp). Comparisons of cell subsets between Jinja and Tororo were performed using Mann-Whitney tests. Spearman correlation was used to compare continuous variables. Scatter plots show best fit linear regression lines with 95% confidence intervals. Friedman tests with multiple comparisons were use to analyze cell subsets before and after IRS. In all analyses, a P value <0.05 was considered significant.

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Chapter 3

Inhibitory Killer Cell Immunoglobulin-like Receptor Ligands Are Associated With Higher *P. falciparum* Parasite Prevalence

This chapter is adapted from: Digitale JC*, Callaway PC* et al. Association of Inhibitory Killer Cell Immunoglobulin-like Receptor Ligands With Higher *Plasmodium falciparum* Parasite Prevalence. The Journal of Infectious Diseases, 2020, jiaa698, https://doi.org/10.1093/infdis/jiaa698 (*these authors contributed equally)

Introduction

Plasmodium falciparum malaria has caused hundreds of millions of deaths throughout history and continues to kill nearly half of a million people annually (1). As a result, *P. falciparum* has exerted strong selective pressure on human evolution (2,3). Most of the human genetic mutations known to alter malaria susceptibility lead to erythrocyte defects or cytoadhesion variants (2,3). However, a recent GWAS analysis of 17,000 individuals estimated that known variants account for only 11% of total host genetic influence on malaria susceptibility (4). Surprisingly, only a few gene variants implicated in the host immune response have been robustly associated with malaria susceptibility. Such associations could be particularly valuable in illuminating causal mechanisms of immune protection. Notably, most studies of host genetic susceptibility to malaria, including the GWAS study noted above, have compared severe malaria cases to controls with non-severe (but symptomatic) malaria(5,6). This analytic framework could overlook genes that influence protection at the earlier pre-erythrocytic stages of the *P. falciparum* life cycle, before blood stage infection is established.

Human killer cell immunoglobulin-like receptors (KIR) are a family of inhibitory and activating transmembrane receptors whose known ligands are primarily HLA class I molecules. The KIR locus is the second most polymorphic region in the human genome after HLA, and is evolving even more rapidly, likely in part due to selective pressure from pathogens (7). Human haplotypes vary both in the number of KIR genes and in the proportion that are inhibitory or activating. While some KIR genes are universal, others are expressed by only a subset of individuals (8). Moreover, within an individual, only a fraction of cells express a given KIR molecule (9). KIR are expressed on natural killer (NK) cells, some gamma delta T cells, and some CD8 T cells, all of which play a role in the antimalarial immune response (9–12). Interactions of KIR with their HLA ligands modulate the activation threshold of the cell and have been shown to influence the outcome of several, predominantly viral, infections (13–15). Because the KIR and HLA loci are on different chromosomes and segregate independently, associations of KIR-HLA compound genotypes with susceptibility to particular pathogens can shed light on mechanisms critical for immunity and immunopathogenesis.

To date, the association of KIR and their HLA ligands with susceptibility to malaria has been examined in very few studies and almost entirely in the context of case-control comparisons of severe vs. non-severe malaria (5,6). It has been hypothesized that KIR expression may influence activation of NK cells during malaria, and one study noted an association between KIR genotype and the *in vitro* NK cell response to *P. falciparum* infected red blood cells (iRBC) (16). Here, we examined the influence of well-defined KIR-HLA ligand pairs on the prevalence of parasitemia and malaria in a longitudinal cohort of 890 individuals in Uganda to determine the influence of KIR on susceptibility to *P. falciparum* infection. This analysis revealed that individuals with more inhibitory KIR-HLA pairs have higher odds of parasitemia, strongly supporting a role for cellular immunity in host restriction of the parasite.

Results

Clinical cohort and KIR-HLA genotyping

We analyzed data from 657 children and 233 adults recruited from 292 households at three study sites in Uganda (Table 1). KIR and HLA class I loci genotyping was performed (Table 2), and individuals were categorized based on the presence of well-characterized KIR ligand groups (i.e. HLA-C2 for KIR2DL1, HLA-C1 for KIR2DL2/3, and HLA-Bw4 for KIR3DL1, Supplemental Table 1). The prevalence of inhibitory KIR genes KIR2DL1 and KIR3DL1 was very high (both >98.5%), consistent with previous reports in East African populations (22). To control for KIR-HLA differences in ethnicity, participants were categorized as Bantu or non-Bantu based on the language of their consent form (Table 1). However, as ethnicity was largely collinear with site, including it in models did not improve model fit beyond site fixed effects; it was therefore omitted from final analyses.

Subjects were followed from August 2011-June 2016 with blood smears performed every 3 months to assess *P. falciparum* parasitemia. Reflecting the varying transmission intensity between sites, the prevalence of parasitemia was highest among children in Tororo (64%), followed by Kanungu (36%) and Jinja (13%). The incidence of malaria, defined as parasitemia accompanied by fever, also differed among the sites and was inversely associated with age (18).

To assess whether KIR and their HLA ligands influence the risk of *P. falciparum* infection or its clinical manifestations, we tested associations of individual KIR genes, HLA ligand groups and KIR-HLA compound genotypes with 5 malaria outcome measures: prevalence of parasitemia, clinical malaria incidence, parasite density (stratified by routine quarterly visits and malaria visits), conditional probability of clinical malaria given parasitemia, and temperature at parasitemic visits (conditional on parasite density). For each association, we report the p-value from a multi-level model (p), an empirical p-value after permutation tests (p*), and a q-value (q) calculated by applying the false discovery rate correction for multiple comparisons to the empirical p-values. There was no association between the presence of any individual KIR gene with any of these outcomes (Supplemental Tables 2a-f).

Inhibitory KIR2DL1 ligand HLA-C2 increases risk of P. falciparum parasitemia

Among the best characterized KIR-HLA ligand interactions are inhibitory KIR2DL1 with the HLA-C2 subgroup of HLA-C alleles (defined by lysine at amino acid position 80) and KIR2DL2/L3 with the HLA-C1 subgroup (asparagine at position 80) (23). Since KIR2DL1 (98.6%) and KIR2DL2/3 (99.3%) were both present in virtually all subjects, the presence of their HLA ligands determines which inhibitory KIR/HLA-C pair(s) an individual has. Further, within these inhibitory KIR/HLA-C ligand interactions, there is a hierarchy of inhibition. KIR2DL1/HLA-C2 provides the strongest inhibition, followed by KIR2DL2/HLA-C1, while KIR2DL3/HLA-C1 provides the weakest inhibition (24–26). We found that individuals with at least one copy of the KIR2DL1 ligand HLA-C2 had 1.31-fold higher odds of being parasitemic (p=0.017, p*=0.010, q=0.047). Conversely, having at least one HLA-C1 allele decreased the odds of being parasitemic (OR=0.79, p=0.016, p*=0.013, q=0.051).

Next, we assessed the zygosity of HLA-C ligand groups to determine whether the copy number of HLA-C1 or HLA-C2 influenced parasite prevalence. HLA-C1/C1 individuals are only able to receive inhibitory signals via KIR2DL2/3, whereas HLA-C2/C2 individuals receive inhibitory

signals predominantly via KIR2DL1; heterozygous individuals have the ligand for all three(23). Comparison of these groups revealed a stepwise increase in parasite prevalence, with HLA-C2/C2 individuals having 1.54-fold higher odds of parasitemia compared to HLA-C1/C1 individuals (Table 3). Heterozygous (HLA-C1/C2) individuals had intermediate odds. These results suggest that engagement of KIR2DL1 with its ligand HLA-C2, the most inhibitory of the KIR/HLA-C ligand pairs, increases parasite prevalence in comparison to engagement of KIR2DL2/3 with their ligand HLA-C1, which provide weaker inhibitory signals. KIR2DL1 expressing cells from HLA-C2/C2 homozygous individuals will receive stronger inhibitory signals, potentially dampening the effector cell response to *P. falciparum* infection.

KIR2DL2 and KIR2DL3 segregate as alleles of the same locus, but KIR2DL2 provides stronger inhibition when bound to HLA-C1 (24). Overall, no difference in parasitemia risk was observed between KIR2DL3/C1 and KIR2DL2/C1 individuals (Table 4). However, KIR2DL2 is in linkage disequilibrium with the homologous activating receptor KIR2DS2 (Wn*=0.85, Fisher's exact test $p<0.001$), which may be capable of binding HLA-C1 in a peptide-dependent manner (27,28). To test whether KIR2DS2 has an opposing effect that obscures the stronger inhibitory impact of KIR2DL2, we examined the impact of KIR2DL2 and KIR2DL3 among individuals lacking KIR2DS2. This revealed that presence of at least one copy of KIR2DL2 increased the odds of being parasitemic 1.6-fold compared to those with only KIR2DL3 (Table 4). While KIR2DL3/HLA-C1, the least inhibitory KIR-HLA combination in the hierarchy, was associated with lower parasite prevalence, previous data indicate that it markedly increases the risk of cerebral malaria, an inflammatory complication of malaria (5). Together these data further support a hierarchy of inhibition in which KIR-HLA combinations with stronger inhibitory potential increase parasite prevalence.

Inhibitory KIR3DL1 ligand HLA-Bw4 increases risk of P. falciparum parasitemia

Another inhibitory KIR molecule, KIR3DL1, recognizes a subset of HLA-A and HLA-B alleles that contain the HLA-Bw4 motif, defined by amino acid residues 77-83 (29). Like KIR2DL1, KIR3DL1 was nearly universal in our cohort (98.7%), hence its effect on parasite prevalence could not be estimated directly. However, only 74.8% of individuals had an HLA-Bw4 ligand, enabling us to estimate the effect of the KIR-HLA pair. Among individuals with HLA-Bw4, the odds of parasitemia were 1.31-fold higher than in individuals lacking HLA-Bw4 ($p=0.012$, $p^*=0.006$, $q=0.040$). This further suggests that increased KIR inhibition increases the likelihood of parasitemia, perhaps due to dampened cellular immunity.

Because both HLA-C2 and HLA-Bw4 were found to increase parasite prevalence and have previously been shown to be in linkage disequilibrium, we looked for an additive effect of the two ligand groups by analyzing the combined genotype. Having both HLA-C2 and HLA-Bw4 increased the odds of parasitemia by 1.46 times compared to HLA-C2 alone ($p=0.001$, $p^*<0.001$, $q=0.008$) and by 1.51 times compared to HLA-Bw4 alone ($p=0.002$, $p^*=0.001$, $q=0.012$), suggesting an additive inhibitory effect of the two KIR-HLA ligand pairs. Together, this supports a model wherein the more inhibitory signals that KIR expressing effector cells can receive, the greater the risk of parasitemia.

Higher surface expression of inhibitory KIR ligand HLA-C is associated with increased odds of parasitemia

To further elucidate how HLA-C influences *P. falciparum* infection, we examined the relationship of HLA-C surface expression levels to parasite prevalence. HLA-C molecules have been ascribed two distinct roles in cellular immunity—as ligands for inhibitory KIR, and as antigen presenting molecules for CD8 T cells—both of which can influence infectious disease outcomes (13,14,30). In contrast to HLA-A and -B molecules, surface expression of HLA-C is 10-15 fold lower and varies substantially amongst individual allotypes (31). During viral infections such as HIV infection, HLA-C alleles that are more highly expressed at the cell surface have been associated with a more potent CD8 T cell response that correlates with enhanced viral control (32,33). We hypothesized that if the protection associated with certain HLA-C ligand groups is attributable to differences in peptide presentation, then higher HLA-C expression would similarly correlate with a reduced likelihood of parasitemia. We assigned each HLA-C allele a value based on previously determined expression levels (21) and calculated a total HLA-C expression level for each individual. We found that higher HLA-C expression was associated with an *increase* in the odds of *P. falciparum* parasitemia (1.12-fold increase per one standard deviation of expression; p=0.014, p*=0.006, q=0.040). This contrasts with HIV infection, where highly expressed HLA-C allotypes are protective. These data suggest that the mechanism by which HLA-C molecules influence restriction of *P. falciparum* parasitemia is not antigen presentation to T cells, but rather a role in KIR-mediated inhibition of effector cells.

Relationship of KIR-HLA genotype to other parasitological and malaria outcomes
Finally, because immune mechanisms responsible for restricting parasitemia may differ from those that protect against symptomatic malaria, we also examined the association of KIR-HLA ligand pairs with the other parasitological and clinical outcomes noted above (Supplemental Table 2b-f). We did not observe an association of KIR-HLA ligand pairings with incidence of clinical malaria, probability of clinical malaria if parasitemic, body temperature at parasitemic visits, or parasite density. Together these results are most consistent with an influence of KIR-expressing effector cells on restriction of parasite replication, rather than on the downstream inflammatory consequences that eventuate in fever and other clinical symptoms.

In addition, we performed multiple sensitivity analyses to assess the robustness of our results. A more sensitive measure of parasitemia (LAMP) was performed on samples that were negative by microscopy at routine visits. For adults, these data were available for only a portion of follow-up time. When the definition of parasitemia was expanded to include *P. falciparum* infections detectable only by LAMP among children for the entire follow-up period and among children and adults for the period LAMP data were available for both, similar associations for all results stated above were observed. In addition, we analyzed models: 1) stratified by site to determine whether our results were robust across transmission intensities and ethnicities, 2) restricted to children as they suffer the largest burden of parasitemia, and 3) excluding individuals with hemoglobin S (sickle cell) mutations to ensure our results were not confounded by hemoglobin variants. These secondary analyses yielded similar results, further strengthening our findings.

Discussion

Using longitudinal data from individuals with varying malaria exposure, we found that the inhibitory KIR ligands HLA-C2 and HLA-Bw4 were both associated with a higher prevalence of *P. falciparum* parasitemia. Our data indicate that both the number of inhibitory KIR-HLA ligand pairs and the strength of their cellular inhibitory signaling influence the risk of *P. falciparum* parasitemia, but notably, do not impact clinical malaria. Our findings suggest that the cellular immune response plays a critical role either in the prevention or clearance of blood-stage *P. falciparum* infection. These associations represent a novel genetic determinant of *P. falciparum* parasitemia, and support an expanded role for KIR in modulating host immunity to parasitic infections.

This novel association of KIR-HLA compound genotypes with parasite prevalence is somewhat paradoxical given that *P. falciparum* spends most of its life cycle outside of HLA-expressing cells. Following intradermal inoculation by mosquitoes, *P. falciparum* sporozoites migrate via the dermis and lymphatics to infect hepatocytes, the only stage in the parasite life cycle within an HLA-expressing host cell (34). Within the hepatocyte, parasites expand 2,000-40,000 fold, then emerge synchronously as merozoites, which establish a self-propagating cycle of erythrocytic infection (35,36). Because erythrocyte membranes contain little to no HLA (37), we hypothesize that the influence of KIR on cell-mediated anti-parasite immunity may occur primarily during the liver stage when the multiplicity of infection is also lowest. It has previously been shown that increasing KIR mediated NK cell inhibition is associated with poorer clearance of HCV and HBV in the liver (13,14).

Alternatively, the observed impacts of KIR-HLA pairs on parasite prevalence may be due to enhanced spontaneous clearance of parasitemia rather than cessation of infection at the liver stage. Here, it is notable that no association of any individual KIR gene with clinical outcomes was observed, suggesting that the influence of KIR is dependent upon interactions with an HLA-bearing cell. Previous evidence suggests that NK cell activation by iRBCs is dependent on contact with monocytes and myeloid dendritic cells, creating a point at which KIR mediated inhibition of NK cells may occur, despite the absence of HLA on RBCs themselves (38). While NK-iRBC conjugates have been observed, direct activation of NK cells by iRBCs has not been conclusively demonstrated (16). While conventional αβ T cells express KIR only infrequently, KIR are often expressed on γδ T cells. Further, we have shown KIR to be highly expressed on the CD16+ Vγ9Vδ2 T cell subset, which is increased with frequent malaria exposure and can be activated by opsonized iRBCs in a TCR-independent manner (39). Thus KIR may not only modulate antibody dependent cellular cytotoxicity of NK cells but also of γδ T cells (40,41).

It is also notable that we did not observe an influence of KIR or their HLA ligands on symptomatic malaria. In endemic regions, "clinical immunity"—defined as protection from symptomatic malaria—develops gradually with increasing age and exposure to *P. falciparum*, but sterilizing immunity (i.e. protection against parasitemia) does not (42). Hence, it is likely that the immune mechanisms underlying sterile protection and restriction of parasite replication differ from those that mediate clinical immunity, with the latter involving immunoregulatory processes that dampen the inflammation responsible for fever and other symptoms, while fostering successful long-term parasitism. By definition, sterile immunity is directed at the pre-

erythrocytic stages of parasite development. The pre-erythrocytic immune response can prevent or greatly limit the establishment of blood-stage parasitemia and is believed to be mediated by CD8 T cells (12,43), although recent data suggest that NK cells (44–46) and γδ T cells (47–49), which both frequently express KIR, may also play important roles. It remains possible that KIR-HLA interactions exert an impact on the symptomatic or inflammatory manifestations of malaria that we were underpowered to detect, given that all study subjects had access to free clinical care which afforded prompt diagnosis and treatment of malaria. Indeed, Hirayasu et. al. reported that the risk of cerebral malaria, an inflammatory complication, is markedly elevated among individuals with KIR2DL3/HLA-C1, which is the least inhibitory pairing amongst KIR2DL1/2/3 ligands (5). Immunopathology contributes substantially to the neurological damage caused by cerebral malaria (50) and NK cells have been found to accumulate in the brain microvasculature (51). Thus, lower KIR inhibition could allow unrestrained NK cell activation resulting in immune-mediated pathology. This would be consistent with the hypothesis that mechanisms that prevent initial *P. falciparum* infection and those that prevent severe disease are distinct and may have a balancing effect on the maintenance of different KIR and HLA ligands in malaria endemic populations.

Our results also underscore that case-control studies of severe vs. non-severe malaria have important limitations as a framework to identify immune susceptibility genes. Because such comparisons are restricted to individuals in whom parasitemia is already established, they cannot identify genetic factors influencing host restriction at the pre-erythrocytic stages of infection. Moreover, the development of severe malaria is likely to be influenced by genetic factors governing inflammation, as well as non-genetic factors such as socio-economic status and access to care. We believe that additional genetic determinants of malaria may be uncovered through analytic methods that incorporate rigorous longitudinal assessments of both parasite infection and symptoms, enabling evaluation of immune protection at varying stages of malaria, from initial infection and establishment of parasitemia, to development of symptomatic or even severe malaria.

While it is well established that KIRs influence the immune response to and ultimate outcome of numerous viral infections, their impact on the control of non-viral infections has been less certain. Our finding that inhibitory KIR ligands increase the risk of *P. falciparum* parasitemia indicates that the influence of KIR on host immunity extends to parasitic infections. Moreover, our findings suggest an important role for the cellular immune response in restricting *P. falciparum* infection, perhaps during the earliest stages of infection, before blood-stage parasitemia is established. These findings represent a novel genetic determinant of malaria susceptibility and further our understanding of antimalarial immunity with implications for vaccine design.

Methods

Study Approval

Ethical approval was granted from Makerere University School of Medicine Research and Ethics Committee, London School of Hygiene and Tropical Medicine Ethics Committee, UCSF Committee on Human Research, and Uganda National Council for Science and Technology. All participants and/or their parents/guardians gave written informed consent.

Study Sites

Nagongera, in Tororo district, is a high-transmission, rural area in southeastern Uganda. Kihihi, in Kanungu district, is a rural area in southwestern Uganda with moderate transmission. Walukuba, in Jinja district, is a relatively low-transmission, peri-urban area near Lake Victoria. Households in the three sites were enumerated and approximately 100 households per site were randomly selected. All children aged 6 months to 10 years and one adult caregiver per household who met eligibility criteria (usually a female) were invited to participate between August and October 2011. Children aged out of the cohort when they reached 11 years of age. Participants were followed through June 2016; visits after December 31, 2014 from Tororo were excluded from this analysis because transmission changed dramatically at this site after intensive indoor residual spraying campaigns were started. Analytic follow-up time was a median of 55 months in Jinja, 57 months in Kanungu, and 39 months in Tororo.

Clinical and parasitological outcomes

Subjects were enrolled into East Africa International Center of Excellence for Malaria Research longitudinal cohorts at three sites in Uganda with differing malaria transmission intensity(17). Households were randomly selected and all children aged 6 months to 10 years and one caregiver were offered enrollment.

Participants visited the clinic quarterly for thick blood smears. Caregivers were instructed to bring children to study clinics for free care for any illness. Thick blood smears were performed on children presenting febrile (>38.0°C) or reporting fever in the previous 24 hours. Parasitemia was determined by microscopy and parasite density was calculated as previously described(18). LAMP (loop-mediated isothermal amplification) was performed on samples negative by microscopy at routine visits throughout follow-up for children and for part of follow-up for adults.

At each household, mosquitoes were collected monthly using CDC light traps, tested for sporozoites using ELISA(19) and an annual entomological inoculation rate (EIR) was calculated(18).

HLA and KIR Genotyping

HLA genotyping was performed using a targeted next generation sequencing (NGS) method. Briefly, locus-specific primers were used to amplify a total of 23 polymorphic exons of HLA-A, B, C (exons 1 to 4), DPA1 (exon 2), DPB1 (exons 2, 3), DQA1 (exon 2), DQB1 (exons 2, 3), DRB1 (exons 2, 3), and DRB3, 4, 5 (exon 2) genes with Fluidigm Access Array (Fluidigm Singapore PTE Ltd, Singapore). The 23 Fluidigm PCR amplicons were pooled and subjected to sequencing on an illumina MiSeq sequencer (Illumina, San Diego, CA). HLA alleles and

genotypes were called using the Omixon HLA Explore (beta version) software (Omixon, Budapest, Hungary).
KIR genotyping for the presence or absence of each KIR gene was conducted by PCR with sequence-specific priming (PCR-SSP) as described previously(20), with some modifications. Each PCR was conducted in a volume of 5 μl using 5 ng genomic DNA and SYBR Green PCR Master Mix with Platinum Taq (Invitrogen). Presence and absence of specific PCR products was detected by melting curve analysis on the 7900 Real-Time PCR System (Applied Biosystems).

HLA-C Expression Levels
HLA-C expression levels were imputed using estimates based on previously published measurements of HLA-C allotypes(21).

Outcomes
We analyzed the association of KIR and their HLA ligands with five different outcomes related to malaria immunity:

1. Parasite prevalence: Prevalence of at least one symptomatic or asymptomatic parasite positive visit per quarter (by microscopy).
2. Annual malaria incidence rate: Number of symptomatic malaria episodes per person-year.
3. Parasite density: Log parasite density measured during visit (in analyses, stratified by two visit types: routine quarterly parasite-positive visits and symptomatic malaria visits).
4. Probability of symptoms if infected: Whether a participant was febrile if they were parasitemic. (Episodes of asymptomatic parasitemia where fever developed within 7 days and repeat episodes of febrile parasitemia within 7 days of initial episode were excluded so that each malarial episode was only counted once.)
5. Temperature at parasitemic visits: Objective temperature at parasitemic visits, conditional on parasite density.

Statistical Analyses
First, we assessed the effect of each KIR individually on these outcomes. Next, we explored KIR-HLA compound genotypes as follows. HLA ligand group definitions are in Supplemental Table 1. KIR2DL1, which binds HLA-C2, was present in nearly all subjects (98.6%). KIR2DL2 and KIR2DL3 segregate as alleles of the same locus and have the same HLA ligand, HLA-C1; virtually everyone in this sample has KIR2DL2 and/or KIR2DL3 (99.3%). We examined the effect of having at least one copy of HLA-C2 among those with KIR2DL1 and of having at least one copy of HLA-C1 among those with KIR2DL2 and/or KIR2DL3. Building on this, we investigated HLA-C1/C2 zygosity in the entire sample to determine the effect of multiple copies of each ligand. We then assessed the effect of a joint variable: no HLA-C1 ligand (i.e. HLA-C2 homozygous), HLA-C1 with KIR2DL2 only, HLA-C1 with KIR2DL2 and KIR2DL3, or HLA-C1 with KIR2DL3 only. We repeated this analysis among those with no KIR2DS2 (for the outcome of parasite prevalence only). Another inhibitory KIR, KIR3DL1, was also nearly ubiquitous in the sample (98.7%). We again considered the effect of its ligand, HLA-Bw4, among those with the receptor. Given that both HLA-C2 and Bw4 affected parasite prevalence in the same direction, we assessed the combined genotype of HLA-C2 and HLA-Bw4 (neither HLA-C2 nor HLA-Bw4, only HLA-C2, only HLA-Bw4, or both HLA-C2 and HLA-Bw4). Finally, we looked at the level of HLA-C expression (normalized as a z-score).

We used logistic regression for parasite prevalence and probability of symptoms if infected, Poisson models for malaria incidence, and linear models for log parasite density and temperature. In summary, the models followed this general form (an example model for parasite prevalence shown):

$$logit(Parasite\ prevalence_{ijk}) = KIR\text{-}HLA\ compound\ genotype_{ij} + Site_j + Log(aEIR_j) + Sex_{ij} + Age_{ijk} + Child_{ij} + Age*Child_{ijk} + u_i + y_j$$

where i indicates individuals, j indicates households, and k indicates specific visits. For example, age_{ijk} denotes the age of child i from household j during visit k. As $aEIR_j$ represents the average annual EIR recorded for household j (time-invariant), we assume relatively stable transmission intensity over the course of study follow-up.

We used Monte Carlo permutation tests to calculate exact p-values. We permuted exposure variables 10,000 times and calculated the two-sided p-value as the number of permutations that yielded coefficients greater than or equal to the absolute value of the observed coefficient divided by the number of models that converged (>99%) using the Stata ritest package version 1.1.4 (52). For each outcome, we controlled for multiple testing separately using the false discovery rate (FDR) approach with the Stata qqvalue package (53).

Sensitivity Analyses

We performed the following sensitivity analyses for all models with the outcome of parasite prevalence: stratified by site, among children only, and among sickle cell wild type. We also estimated models using a measure of parasitemia defined as positive if a subject were positive either by microscopy or by LAMP among children for the entire follow-up period and among children and adults for the period LAMP data were available for both. Adult LAMP data was available in Jinja and Kanungu only from August 2011 to September 2013 and for the full duration of follow-up time for Tororo. Controlling for ethnicity (Bantu/non-Bantu) based on the language of their consent form did not improve model fit as compared to controlling for site because these two variables were collinear.

Tables

Table 1. Characteristics of study population

	Jinja	Kanungu	Tororo
Households (N)	**97**	**100**	**95**
Median household EIR (range)	2.1 (1.5-22.0)	6.3 (3.0-44.5)	67.6 (11.5-588.4)
Adults (N)	**89**	**78**	**66**
Median incidence of symptomatic malaria episodes per person year (range)	0.0 (0.0-2.0)	0.2 (0.0-3.7)	0.0 (0.0-3.0)
Parasite prevalence (% quarters parasite+)	4.9%	12.8%	12.0%
Children (N)	**187**	**267**	**203**
Median age at enrollment, years (range)	4.4 (0.5-10.0)	4.7 (0.5-9.9)	5.1 (0.6-10.0)
Median incidence of symptomatic malaria episodes per person year (range)	0.2 (0.0-5.1)	1.2 (0.0-8.1)	2.1 (0.0-8.8)
Parasite prevalence (% quarters parasite +, range)	12.8%	36.4%	64.2%
Median follow up time, months (range)*	55.1 (2.3-58.6)	56.7 (0.0-58.3)	39.0 (1.8-40.3)
Sickle cell, n(%)			
AA	201 (72.8%)	323 (93.6%)	195 (72.5%)
AS	72 (26.1%)	22 (6.4%)	70 (26.0%)
SS	0 (0.0%)	0 (0.0%)	2 (0.7%)
No result	3 (1.1%)	0 (0.0%)	2 (0.7%)
Bantu ethnicity, N (%)	180 (65.5%)	341 (98.8%)	2 (0.7%)

*Note: 3 people in the sample had only one visit; Tororo visits after December 31, 2014 were excluded because transmission changed dramatically at this site after intensive indoor residual spraying campaigns were started.

Table 2. KIR and Ligand Prevalence

KIR and HLA prevalence	N=890
KIR	%
KIR2DL1†	98.6
KIR2DL2	54.7
KIR2DL3‡	84.8
KIR2DL4	99.7
KIR2DL5	57.9
KIR2DP1	98.1
KIR2DS1	23.5
KIR2DS2	49.0
KIR2DS3	23.0
KIR2DS4	68.3
KIR2DS5	42.6
KIR3DL1	98.7
KIR3DL2	99.6
KIR3DS1	13.0
HLA	%
C1/C2 ligand group zygosity*	
C1/C1	19.5
C1/C2	53.1
C2/C2	27.4
Bw4 ligand group (at least one copy)	74.8

Note: Reduced Ns due to failed amplification or lack of DNA.

†N=888

‡N=883

*N=889

Table 3. Effect of HLA-C zygosity on parasite prevalence

C1/C2 zygosity	OR	P-value	Empirical p- value	Empirical FDR q-value	N
C1/C1	Ref				173
C1/C2	1.26	0.045	0.036	0.111	472
C2/C2	1.54	0.001	0.001	0.008	244

Table 4. Effect of KIR2DL2/3 on parasite prevalence

	OR	P-value	Empirical p-value	Empirical FDR q-value	N
2DL2/3					
no C1	1.24	0.061	0.045	0.115	244
C1 + 2DL2 only	1.03	0.845	0.837	0.837	87
C1 + 2DL2/2DL3	0.93	0.543	0.510	0.640	273
C1 + 2DL3 only	Ref				278
2DL2/3 among those without 2DS2					
no C1	1.19	0.199	0.171	0.354	136
C1 + 2DL2 only or 2DL2/2DL3	1.60	0.024	0.016	0.056	45
C1 + 2DL3 only	Ref				272

Supplemental Table 1: KIR Ligand Groups

Ligand Group	KIR	Ligands	
		Allele group	*Allele*
C1	KIR2DL2/3	C 1, 3, 7, 8, 12, 13, 14, 16 (except 16:02)	
C2	KIR2DL1	C 2, 4, 5, 6, 15, 17, 18	C 16:02
BW4	KIR3DL1	B 13, 27, 37, 38, 44, 47, 49, 51, 52, 53, 57, 58, 59 A 23, 24, 32	

Supplemental Table 2a: Parasite Prevalence

Exposure	Comparison group	Odds ratio	P-value	Empirical p-value	Empirical FDR q-value	N
KIR2DL2	Absent	0.97	0.702	0.666	0.764	890
KIR2DL3	Absent	0.92	0.466	0.444	0.640	883
KIR2DL5	Absent	1.06	0.524	0.475	0.640	890
KIR2DS1	Absent	0.94	0.559	0.504	0.640	890
KIR2DS2	Absent	0.90	0.279	0.227	0.439	890
KIR2DS3	Absent	0.98	0.819	0.807	0.837	890
KIR2DS4	Absent	1.09	0.365	0.331	0.540	890
KIR2DS5	Absent	1.09	0.366	0.310	0.534	890
KIR3DS1	Absent	1.08	0.567	0.516	0.640	890
At least one HLA C2 (among those KIR2DL1+)	No C2	1.31	0.017	0.010	0.047	878
At least one HLA C1 (among those KIR2DL2+/2DL3+)	No C1	0.79	0.016	0.013	0.051	883
HLA C1/C2 zygosity						889
C1/C2 heterozygote	C1 homozygote	1.26	0.045	0.036	0.111	
C2 homozygote	C1 homozygote	1.54	0.001	0.001	0.008	
C2 homozygote	C1/C2 heterozygote	1.22	0.045	0.043	0.115	
KIR2DL2/3 + HLA C1/C2						882
No C1 (C2 homozygote)	C1 + 2DL3 only	1.24	0.061	0.045	0.115	
C1 + 2DL2 only	C1 + 2DL3 only	1.03	0.845	0.837	0.837	
C1 + 2DL2/2DL3	C1 + 2DL3 only	0.93	0.543	0.510	0.640	
C1 + 2DL2 only	No C1 (C2 homozygote)	0.83	0.254	0.241	0.439	
C1 + 2DL2/2DL3	No C1 (C2 homozygote)	0.75	0.012	0.009	0.044	
C1 + 2DL2 only	C1 + 2DL2/2DL3	1.11	0.521	0.508	0.640	
KIR2DL2/3 + HLA C1/C2 (among those without KIR2DS2)						453
No C1 (C2 homozygote)	C1 + 2DL3 only	1.19	0.199	0.171	0.354	

C1 + 2DL2 only or 2DL2/2DL3	C1 + 2DL3 only	1.60	0.024	0.016	0.056	
C1 + 2DL2 only or 2DL2/2DL3	No C1 (C2 homozygote)	1.34	0.177	0.157	0.347	
At least one HLA Bw4 (among those with KIR3DL1)	No Bw4	1.31	0.012	0.006	0.040	880
HLA C2 + HLA Bw4						890
Only C2	Neither C2 nor Bw4	0.93	0.718	0.698	0.773	
Only Bw4	Neither C2 nor Bw4	0.90	0.625	0.588	0.702	
Both C2 and Bw4	Neither C2 nor Bw4	1.35	0.109	0.078	0.185	
Only Bw4	Only C2	0.97	0.837	0.822	0.837	
Both C2 and Bw4	Only C2	1.46	0.001	0.000	0.008	
Both C2 and Bw4	Only Bw4	1.51	0.002	0.001	0.012	
HLA C Expression (z-score)		1.12	0.014	0.006	0.040	889

Supplemental Table 2b: Incident malaria

Exposure	Comparison group	Incident rate ratio	P-value	Empirical p-value	Empirical FDR q-value	N
KIR2DL2	Absent	1.01	0.91	0.91	0.94	887
KIR2DL3	Absent	1.03	0.75	0.73	0.94	880
KIR2DL5	Absent	1.10	0.21	0.16	0.45	887
KIR2DS1	Absent	0.91	0.28	0.22	0.46	887
KIR2DS2	Absent	0.99	0.90	0.89	0.94	887
KIR2DS3	Absent	0.98	0.81	0.80	0.94	887
KIR2DS4	Absent	1.01	0.90	0.89	0.94	887
KIR2DS5	Absent	1.05	0.48	0.44	0.72	887
KIR3DS1	Absent	0.96	0.75	0.71	0.94	887
At least one HLA C2 (among those KIR2DL1+)	No C2	1.14	0.15	0.11	0.45	875
At least one HLA C1 (among those KIR2DL2+/2DL3+)	No C1	0.89	0.14	0.12	0.45	880
HLA C1/C2 zygosity						886
C1/C2 heterozygote	C1 homozygote	1.12	0.22	0.19	0.45	
C2 homozygote	C1 homozygote	1.23	0.06	0.03	0.26	
C2 homozygote	C1/C2 heterozygote	1.09	0.25	0.23	0.46	
KIR2DL2/3 + HLA C1/C2						879
No C1 (C2 homozygote)	C1 + 2DL3 only	1.12	0.21	0.19	0.45	
C1 + 2DL2 only	C1 + 2DL3 only	0.97	0.82	0.80	0.94	
C1 + 2DL2/2DL3	C1 + 2DL3 only	1.00	0.99	0.99	0.99	
C1 + 2DL2 only	No C1 (C2 homozygote)	0.87	0.27	0.25	0.47	
C1 + 2DL2/2DL3	No C1 (C2 homozygote)	0.89	0.20	0.18	0.45	
C1 + 2DL2 only	C1 + 2DL2/2DL3	0.97	0.82	0.81	0.94	
At least one HLA Bw4 (among those with KIR3DL1)	No Bw4	1.20	0.03	0.02	0.26	877
HLA C2 + HLA Bw4						887
Only C2	Neither C2 nor Bw4	1.09	0.62	0.59	0.92	

Only Bw4	Neither C2 nor Bw4	1.15	0.45	0.38	0.67	
Both C2 and Bw4	Neither C2 nor Bw4	1.32	0.08	0.04	0.26	
Only Bw4	Only C2	1.06	0.67	0.64	0.94	
Both C2 and Bw4	Only C2	1.22	0.04	0.02	0.26	
Both C2 and Bw4	Only Bw4	1.15	0.19	0.15	0.45	
HLA C Expression (z-score)		1.07	0.07	0.05	0.26	886

Supplemental Table 2c: Parasite density: Routine quarterly parasite-positive visits

Exposure	Comparison group	Fold increase in parasite density	P-value	Empirical p-value	Empirical FDR q-value	N
KIR2DL2	Absent	1.06	0.58	0.56	0.91	540
KIR2DL3	Absent	0.86	0.29	0.29	0.73	537
KIR2DL5	Absent	0.98	0.83	0.82	0.93	540
KIR2DS1	Absent	0.97	0.81	0.80	0.93	540
KIR2DS2	Absent	1.00	0.99	0.99	0.99	540
KIR2DS3	Absent	1.14	0.27	0.27	0.73	540
KIR2DS4	Absent	0.90	0.37	0.35	0.74	540
KIR2DS5	Absent	0.93	0.47	0.46	0.80	540
KIR3DS1	Absent	0.93	0.65	0.62	0.91	540
At least one HLA C2 (among those KIR2DL1+)	No C2	1.20	0.20	0.17	0.73	532
At least one HLA C1 (among those KIR2DL2+/2DL3+)	No C1	0.89	0.31	0.31	0.73	534
HLA C1/C2 zygosity						540
C1/C2 heterozygote	C1 homozygote	1.18	0.27	0.25	0.73	
C2 homozygote	C1 homozygote	1.24	0.20	0.18	0.73	
C2 homozygote	C1/C2 heterozygote	1.05	0.68	0.69	0.93	
KIR2DL2/3 + HLA C1/C2						535
No C1 (C2 homozygote)	C1 + 2DL3 only	1.11	0.44	0.42	0.79	
C1 + 2DL2 only	C1 + 2DL3 only	1.04	0.84	0.83	0.93	
C1 + 2DL2/2DL3	C1 + 2DL3 only	1.03	0.82	0.82	0.93	
C1 + 2DL2 only	No C1 (C2 homozygote)	0.94	0.73	0.73	0.93	
C1 + 2DL2/2DL3	No C1 (C2 homozygote)	0.93	0.59	0.59	0.91	
C1 + 2DL2 only	C1 + 2DL2/2DL3	1.01	0.97	0.97	0.99	
At least one HLA Bw4 (among those with KIR3DL1)	No Bw4	0.83	0.14	0.12	0.73	535
HLA C2 + HLA Bw4						540

Only C2	Neither C2 nor Bw4	1.83	0.02	0.01	0.28	
Only Bw4	Neither C2 nor Bw4	1.33	0.30	0.25	0.73	
Both C2 and Bw4	Neither C2 nor Bw4	1.34	0.21	0.16	0.73	
Only Bw4	Only C2	0.73	0.12	0.11	0.73	
Both C2 and Bw4	Only C2	0.73	0.02	0.02	0.28	
Both C2 and Bw4	Only Bw4	1.01	0.97	0.97	0.99	
HLA C Expression (z-score)		1.05	0.39	0.37	0.74	540

Supplemental Table 2d: Parasite density: Malaria visits

Exposure	Comparison group	Fold increase in parasite density	P-value	Empirical p-value	Empirical FDR q-value	N
KIR2DL2	Absent	0.97	0.73	0.73	0.98	590
KIR2DL3	Absent	1.33	0.03	0.02	0.22	586
KIR2DL5	Absent	0.95	0.60	0.59	0.98	590
KIR2DS1	Absent	0.91	0.42	0.38	0.98	590
KIR2DS2	Absent	0.98	0.82	0.81	0.98	590
KIR2DS3	Absent	0.92	0.49	0.48	0.98	590
KIR2DS4	Absent	1.03	0.82	0.81	0.98	590
KIR2DS5	Absent	0.92	0.38	0.37	0.98	590
KIR3DS1	Absent	0.83	0.23	0.21	0.98	590
At least one HLA C2 (among those KIR2DL1+)	No C2	1.00	0.98	0.98	0.98	580
At least one HLA C1 (among those KIR2DL2+/2DL3+)	No C1	1.03	0.79	0.78	0.98	584
HLA C1/C2 zygosity						590
C1/C2 heterozygote	C1 homozygote	1.02	0.90	0.90	0.98	
C2 homozygote	C1 homozygote	0.98	0.91	0.90	0.98	
C2 homozygote	C1/C2 heterozygote	0.97	0.75	0.74	0.98	
KIR2DL2/3 + HLA C1/C2						584
No C1 (C2 homozygote)	C1 + 2DL3 only	0.91	0.46	0.45	0.98	
C1 + 2DL2 only	C1 + 2DL3 only	0.66	0.02	0.02	0.22	
C1 + 2DL2/2DL3	C1 + 2DL3 only	0.99	0.95	0.95	0.98	
C1 + 2DL2 only	No C1 (C2 homozygote)	0.73	0.08	0.07	0.48	
C1 + 2DL2/2DL3	No C1 (C2 homozygote)	1.09	0.50	0.50	0.98	
C1 + 2DL2 only	C1 + 2DL2/2DL3	0.67	0.03	0.02	0.22	
At least one HLA Bw4 (among those with KIR3DL1)	No Bw4	0.95	0.70	0.69	0.98	582
HLA C2 + HLA Bw4						590
Only C2	Neither C2 nor Bw4	0.86	0.56	0.53	0.98	

Only Bw4	Neither C2 nor Bw4	0.81	0.44	0.40	0.98	
Both C2 and Bw4	Neither C2 nor Bw4	0.87	0.55	0.51	0.98	
Only Bw4	Only C2	0.94	0.75	0.75	0.98	
Both C2 and Bw4	Only C2	1.01	0.96	0.95	0.98	
Both C2 and Bw4	Only Bw4	1.07	0.67	0.66	0.98	
HLA C Expression (z-score)		0.99	0.79	0.78	0.98	590

Supplemental Table 2e: Probability of symptoms if infected

Exposure	Comparison group	Odds ratio	P-value	Empirical p-value	Empirical FDR q-value	N
KIR2DL2	Absent	1.12	0.44	0.40	0.92	698
KIR2DL3	Absent	1.09	0.65	0.64	0.98	694
KIR2DL5	Absent	1.18	0.26	0.22	0.92	698
KIR2DS1	Absent	0.96	0.79	0.77	0.98	698
KIR2DS2	Absent	1.14	0.37	0.33	0.92	698
KIR2DS3	Absent	1.00	0.99	0.99	0.99	698
KIR2DS4	Absent	0.86	0.32	0.29	0.92	698
KIR2DS5	Absent	1.12	0.45	0.43	0.92	698
KIR3DS1	Absent	1.02	0.92	0.92	0.98	698
At least one HLA C2 (among those KIR2DL1+)	No C2	1.01	0.95	0.94	0.98	687
At least one HLA C1 (among those KIR2DL2+/2DL3+)	No C1	0.92	0.57	0.56	0.98	692
HLA C1/C2 zygosity						698
C1/C2 heterozygote	C1 homozygote	0.99	0.95	0.95	0.98	
C2 homozygote	C1 homozygote	1.05	0.83	0.82	0.98	
C2 homozygote	C1/C2 heterozygote	1.06	0.72	0.71	0.98	
KIR2DL2/3 + HLA C1/C2						692
No C1 (C2 homozygote)	C1 + 2DL3 only	1.18	0.37	0.35	0.92	
C1 + 2DL2 only	C1 + 2DL3 only	1.07	0.80	0.79	0.98	
C1 + 2DL2/2DL3	C1 + 2DL3 only	1.21	0.28	0.28	0.92	
C1 + 2DL2 only	No C1 (C2 homozygote)	0.91	0.69	0.68	0.98	
C1 + 2DL2/2DL3	No C1 (C2 homozygote)	1.03	0.86	0.86	0.98	
C1 + 2DL2 only	C1 + 2DL2/2DL3	0.88	0.60	0.60	0.98	
At least one HLA Bw4 (among those with KIR3DL1)	No Bw4	1.22	0.22	0.20	0.92	690
HLA C2 + HLA Bw4						698
Only C2	Neither C2 nor Bw4	1.45	0.26	0.23	0.92	

Only Bw4	Neither C2 nor Bw4	1.90	0.07	0.05	0.92	
Both C2 and Bw4	Neither C2 nor Bw4	1.59	0.13	0.10	0.92	
Only Bw4	Only C2	1.32	0.29	0.28	0.92	
Both C2 and Bw4	Only C2	1.10	0.61	0.60	0.98	
Both C2 and Bw4	Only Bw4	0.84	0.42	0.39	0.92	
HLA C Expression (z-score)		1.04	0.54	0.51	0.98	698

Supplemental Table 2f: Temperature at parasitemic visits

Exposure	Comparison group	Coefficient	P-value	Empirical p-value	Empirical FDR q-value	N
KIR2DL2	Absent	0.04	0.24	0.22	0.66	698
KIR2DL3	Absent	0.09	0.07	0.06	0.58	694
KIR2DL5	Absent	0.03	0.40	0.39	0.66	698
KIR2DS1	Absent	-0.03	0.54	0.51	0.72	698
KIR2DS2	Absent	0.09	0.01	0.01	0.24	698
KIR2DS3	Absent	-0.04	0.29	0.28	0.66	698
KIR2DS4	Absent	-0.04	0.29	0.27	0.66	698
KIR2DS5	Absent	0.03	0.46	0.45	0.66	698
KIR3DS1	Absent	-0.04	0.45	0.43	0.66	698
At least one HLA C2 (among those KIR2DL1+)	No C2	0.00	0.95	0.95	0.95	687
At least one HLA C1 (among those KIR2DL2+/2DL3+)	No C1	-0.03	0.40	0.41	0.66	692
HLA C1/C2 zygosity						698
C1/C2 heterozygote	C1 homozygote	-0.01	0.80	0.79	0.85	
C2 homozygote	C1 homozygote	0.02	0.73	0.71	0.83	
C2 homozygote	C1/C2 heterozygote	0.03	0.43	0.43	0.66	
KIR2DL2/3 + HLA C1/C2						692
No C1 (C2 homozygote)	C1 + 2DL3 only	0.07	0.11	0.10	0.66	
C1 + 2DL2 only	C1 + 2DL3 only	0.02	0.79	0.78	0.85	
C1 + 2DL2/2DL3	C1 + 2DL3 only	0.10	0.04	0.03	0.45	
C1 + 2DL2 only	No C1 (C2 homozygote)	-0.05	0.40	0.38	0.66	
C1 + 2DL2/2DL3	No C1 (C2 homozygote)	0.02	0.61	0.62	0.75	
C1 + 2DL2 only	C1 + 2DL2/2DL3	-0.08	0.23	0.21	0.66	
At least one HLA Bw4 (among those with KIR3DL1)	No Bw4	0.05	0.26	0.23	0.66	690
HLA C2 + HLA Bw4						698
Only C2	Neither C2 nor Bw4	0.04	0.63	0.61	0.75	

Only Bw4	Neither C2 nor Bw4	0.10	0.27	0.22	0.66	
Both C2 and Bw4	Neither C2 nor Bw4	0.08	0.34	0.29	0.66	
Only Bw4	Only C2	0.06	0.36	0.33	0.66	
Both C2 and Bw4	Only C2	0.04	0.47	0.45	0.66	
Both C2 and Bw4	Only Bw4	-0.03	0.63	0.61	0.75	
HLA C Expression (z-score)		0.00	0.83	0.83	0.86	698

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Chapter 4

The role of inhibitory receptors on Vγ9Vδ2 T cells in the immune response to chronic *P. falciparum* exposure

Introduction

Despite a decline in the number of malaria cases and deaths during the last decade, progress has recently stalled and even reversed, with 627,000 malaria deaths in 2020 (1). The recent increases in malaria incidence and mortality in addition to the increasing prevalence of drug resistant parasites only underscore the need for an effective malaria vaccine (2). In order to do this, we must have a better understanding of the immune correlates of protection from malaria. Several vaccine studies have now highlighted the frequency of Vγ9Vδ2 T cells (Vδ2s) and their response to a number of malaria vaccine candidates as an important correlate of protection (3–6).

Vδ2s are the predominant subset of γδ T cells in the peripheral blood, accounting for 3-5% of the T cells in the blood overall. Vδ2s are intrinsically reactive to *Plasmodium falciparum (Pf)* infection in addition to a number of other microbial infections through recognition of non-peptidic small molecules called phosphoantigens (pAgs). pAgs are produced as a byproduct of the non-mevalonate isoprenoid biosynthesis pathway used by some bacteria and parasites. HMBPP, produced by *Pf* and other pathogens, is the strongest activator of Vδ2s. Interestingly, pAgs do not bind directly to the Vδ2 TCR, but are instead taken up by other host cells, bind intracellularly to butyrophilin2a1 (BTN2a1) and butryophilin3a1 (BTN3a1) which in turn leads to a conformational shift in these host proteins which the Vδ2 TCR is able to recognize (7–9). In the context of an acute malaria infection, this intrinsic reactivity can lead Vδ2s to expand to up to 50% of the T cells in the blood. In addition to high levels of proliferation, Vδ2s produce a number of pro-inflammatory cytokines and are able to degranulate upon activation (10–12).

Previous work from our group has shown, however, that as someone's cumulative malaria incidence or *Pf* exposure increases, the overall frequency of their Vδ2s declines. Not only do their total Vδ2s decline, but the remaining Vδ2s become less responsive to parasite antigen stimulation via their TCR (13,14). Simultaneously, we find an increase in percentage of Vδ2s expressing the Fcγ receptor CD16 and have shown that these Vδ2s can robustly respond to opsonized infected red blood cells (iRBCs) via CD16 (14,15). We have hypothesized that the reason Vδ2s may be less responsive to TCR stimulation while maintaining the ability to be activated through CD16 is due to inhibitory receptors whose host ligands are present in the context of the Vδ2 TCR recognizing BTN2a1/BTN3a1 on other host cells but are absent in the context of opsonized iRBCs.

To explore this, we focused on KIRs, LILRB1 and NKG2a because of their shared recognition of MHC-I. MHC-I is expressed universally on almost all host cells, with the notable exception of red blood cells. Due to these differences in MHC-I expression, we are able to test how these inhibitory receptors might modulate Vδ2 activation pathways in different contexts. KIRs are a family of 14 transmembrane receptors, some of which are inhibitory and some of which are stimulatory. The inhibitory family members have been better characterized and have more tools with which to study them. Here, we examine the three best-characterized inhibitory KIRs, KIR2DL1, KIR2DL2/L3 and KIR3DL1. Despite having distinct names, KIR2DL2 and KIR2DL3 segregate as alleles of the same gene and as a result share the same ligand. All HLA-C alleles are ligands of either KIR2DL1 or KIR2DL2/L3; alleles in the HLA-C1 ligand group are ligands of KIR2DL2/L3 and all alleles in the HLA-C2 group are ligands for KIR2DL1. In contrast, KIR3DL1 recognizes the Bw4 motif found on a subset of HLA-A and HLA-B alleles

(see Chapter 3 for additional ligand details). Earlier in this dissertation, we showed that individuals with HLA-C2 and HLA-Bw4 ligands are at an increased risk for *Pf* parasitemia. By examining the expression of KIRs on Vδ2s we hope to be able to uncover a functional explanation that might account for part of this association.

While MHC-I molecules are ligands for LILRB1, the non-classical HLA-G binds LILRB1 more strongly than the classical MHC-I molecules (16). LILRB1 is of particular interest in the context of malaria because it has recently been shown that some proteins in the RIFIN family of *Pf* can bind to LILRB1. Not only can these *Pf* proteins bind to LILRB1 but this interaction can inhibit at least B cells which suggests that RIFIN-LILRB1 binding might also lead to inhibition of other immune cells expressing the inhibitory receptor (17). We were interested in investigating if, given this direct interaction between LILRB1 and *Pf*, expression of LILRB1 might be altered with heavy *Pf* exposure and how LILRB1 might alter the Vδ2 response.

Finally, NKG2a recognizes the non-classical MHC-I molecule HLA-E. The role of NKG2a on NK cells is relatively well characterized and its expression is often contrasted with that of KIRs. Both NKG2a and KIRs play an important role in the development of NK cell licensing or education which enables them to respond to "missing self". However, NKG2a is more often expressed on less mature CD56brightCD16- NK cells whereas KIRs are more often expressed on more mature CD56dimCD16+ NK cells (18). On Vδ2s, a recent paper has suggested that NKG2a marks a subset of Vδ2s as having the highest antitumor effector functions (19). We examined if NKG2a expression patterns on Vδ2s mirrored the expression patterns of NK cells and if these NKG2a+ cells have higher anti-malarial functions.

In this study, we find heterologous expression levels and patterns amongst these different MHC-I receptors. Broadly we see increases in KIR and LILRB1 on Vδ2s from individuals with high *Pf* exposure and find that these inhibitory receptors are more likely to be expressed on CD16+ Vδ2s which we have previously shown to be elevated in individuals with high *Pf* exposure. LILRB1+ and some KIR+ Vδ2s also appear less likely to respond to parasite antigen through their TCR. By contrast NKG2a expression does not seem to be modulated by *Pf* exposure and NKG2a+ Vδ2s are more likely to respond to parasite antigen than not.

Results

Chronic *P. falciparum* exposure is associated with an increase in surface expression of LILRB1 and RNA expression of KIRs

Previous data from our lab has suggested that repeated episodes of malaria and continuous exposure to *Pf* modulate the Vδ2 repertoire (13,14). We investigated whether chronic exposure would more specifically modulate expression of the inhibitory receptors of interest on Vδ2s. In order to explore this, we leveraged samples from two study sites in Uganda, Jinja and Tororo. Jinja has a low incidence of malaria with most individuals exposed to *Pf* only a couple times per year whereas Tororo has a very high incidence of malaria and individuals are nearly continuously exposed to *Pf* (see Chapter 2 for more details on malaria incidence at the study sites). Using samples from these sites, we found that expression of LILRB1 on Vδ2s is increased in individuals from the high exposure study site Tororo while frequency of LILRB1+ Vδ2s does not increase, although trends in that direction (Fig. 1a). We further found an increase in the frequency of KIR2DL3+ Vδ2s and a trending increase in the frequency of KIR2DL2/L3+ Vδ2s, when an antibody was used that did not distinguish between the two alleles of KIR2DL2/3. We did not observe a difference in the frequency of expression with other KIRs (Fig. 1b). Single-cell RNA sequencing analysis of enriched Vδ2s from six individuals from Jinja and six individuals from Tororo reveal that at the transcriptomic level, Vδ2s segregate by study site. This indicates that the continuous *Pf* exposure experienced by individuals from Tororo is dramatically altering their Vδ2s so as to be fundamentally different from those of individuals from Jinja (Fig. 1c). When examining differences at the RNA level, the most well studied inhibitory KIRs are expressed at a significantly higher level on Vδ2s from Tororo than Jinja (Fig. 1c). In contrast to its surface expression, we do not see a difference in LILRB1 at the mRNA expression level suggesting that post-translational modifications may lead to differences in surface expression. There is some data to suggest that LILRB1 might be retained in the cytoplasm by most T cells with only a small percentage exhibiting surface staining for LILRB1 (20). Alternatively, we may not have observed differences in LILRB1 due to the overall lower number of cells in the RNA sequencing analysis (hundreds of Vδ2s) compared to the higher number of cells in the flow cytometry experiments (thousands of Vδ2s). We did not observe a difference in expression of NKG2a/*KLRC1* at either the protein or RNA levels between the two study sites (Fig 1c, 1d). Together these data indicate that chronic exposure to *Pf* leads to either an upregulation of KIRs and LILRB1 on Vδ2s or that KIR+ and LILRB1+ Vδ2s are more likely to be maintained while KIR- and LILRB1- Vδ2s populations decline. In contrast, NKG2a is neither selected for nor upregulated in the context of chronic *Pf* exposure. Of note, LILRB1 and the KIRs share classical MHC-I ligands whereas NKG2a recognizes the non-classical MHC-I, HLA-E.

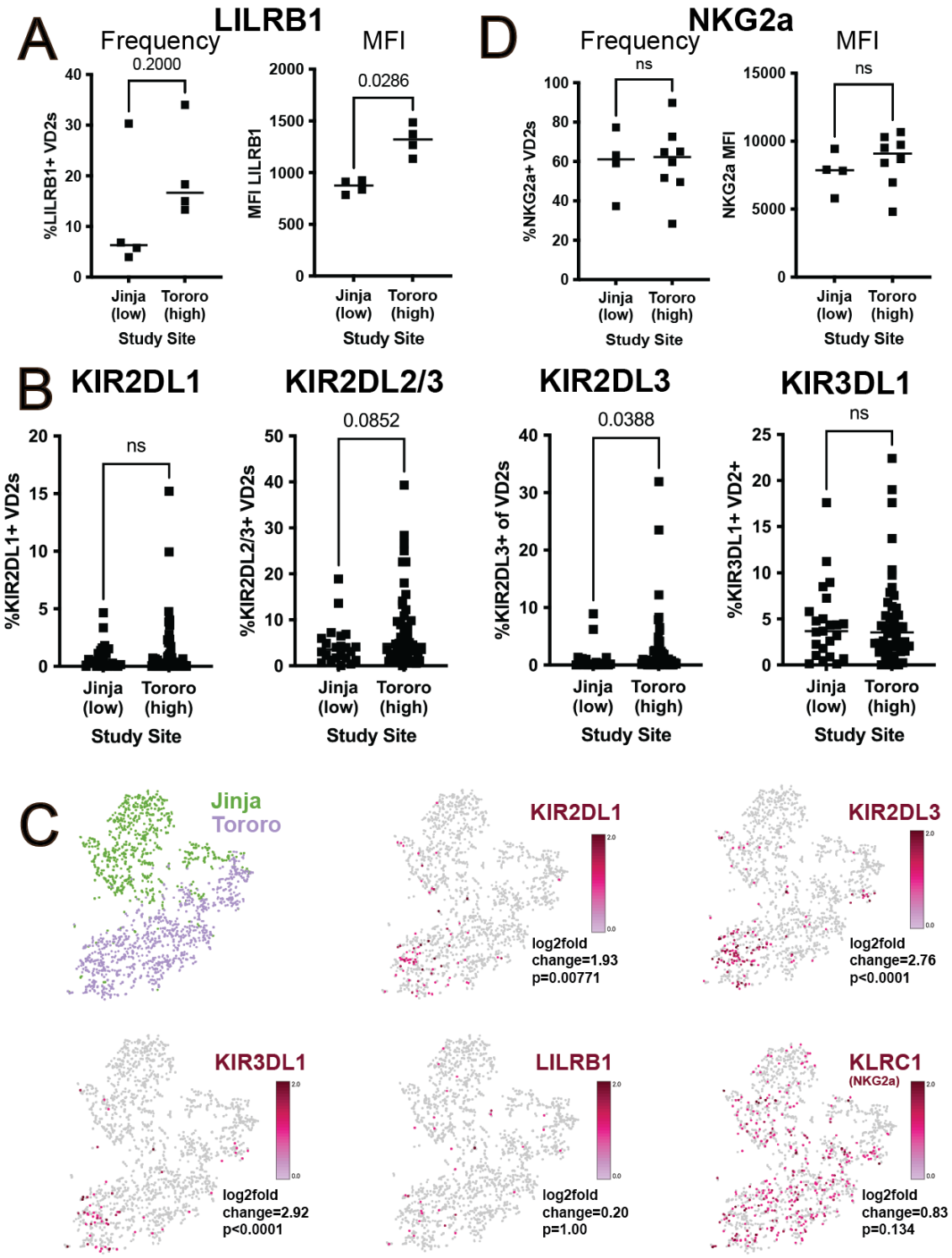

Figure 1: a) Left, frequency of LILRB1+ Vδ2s; right, mean fluorescence intensity (MFI) of LILRB1 on Vδ2s. b) Frequency of KIR+ Vδ2s, KIR2DL2/L3 are shown grouped because they are alleles of the same gene. c) Upper left, clustering of Jinja and Tororo VD2s, expression of different inhibitory receptors on Vδ2s. Log2fold change is the fold change of Tororo samples over Jinja samples with corresponding p-value. d) Left, frequency of NKG2a+ Vδ2s; right, MFI of NKG2a on Vδ2s.

Inhibitory receptors are differentially expressed between CD16+ and CD16- Vδ2s

Previously, we have shown that the Fcγ receptor CD16 defines two distinct subsets of Vδ2s. CD16+ Vδ2s are less likely to respond to iRBCs, HMBPP and *Pf* schizont extract but are capable of activation via CD16 by opsonized iRBCs independent of their TCRs (15). Given this decrease in responsiveness to many parasite antigens, we examined if CD16+ Vδ2s might express inhibitory receptors at a higher rate than CD16- Vδ2s and thus have lower responsiveness. We found that not only were CD16+ Vδ2s more likely to be LILRB1+ but that LILRB1+ Vδ2s were also more likely to be CD16+ (Fig. 2a). We also see a consistent pattern of KIRs being more frequently expressed on CD16+ Vδ2s, but unlike LILRB1, not the inverse (Fig. 2b). When we examine CD16 at the RNA level we see CD16 primarily expressed on individuals from Tororo, aligning with previous flow cytometry data (Fig. 2c). Further, mirroring the discrepancies observed between LILRB1 surface protein expression and RNA expression levels, we did not see a difference in LILRB1 RNA expression between CD16+ and CD16- Vδ2s (Fig. 2c). However, KIR2DL1, KIR2DL3 and KIR3DL1 were three of the most differentially expressed genes between CD16+ Vδ2s and CD16- Vδ2s (Fig. 2c). Once again despite high overall expression of NKG2a/*KLRC1* we see no difference between CD16+ VD2s and CD16- VD2s (Figs. 2d, data not shown). This was unexpected given CD56brightCD16- NK cells are more likely to express NKG2a than CD56dimCD16+ NK cells.

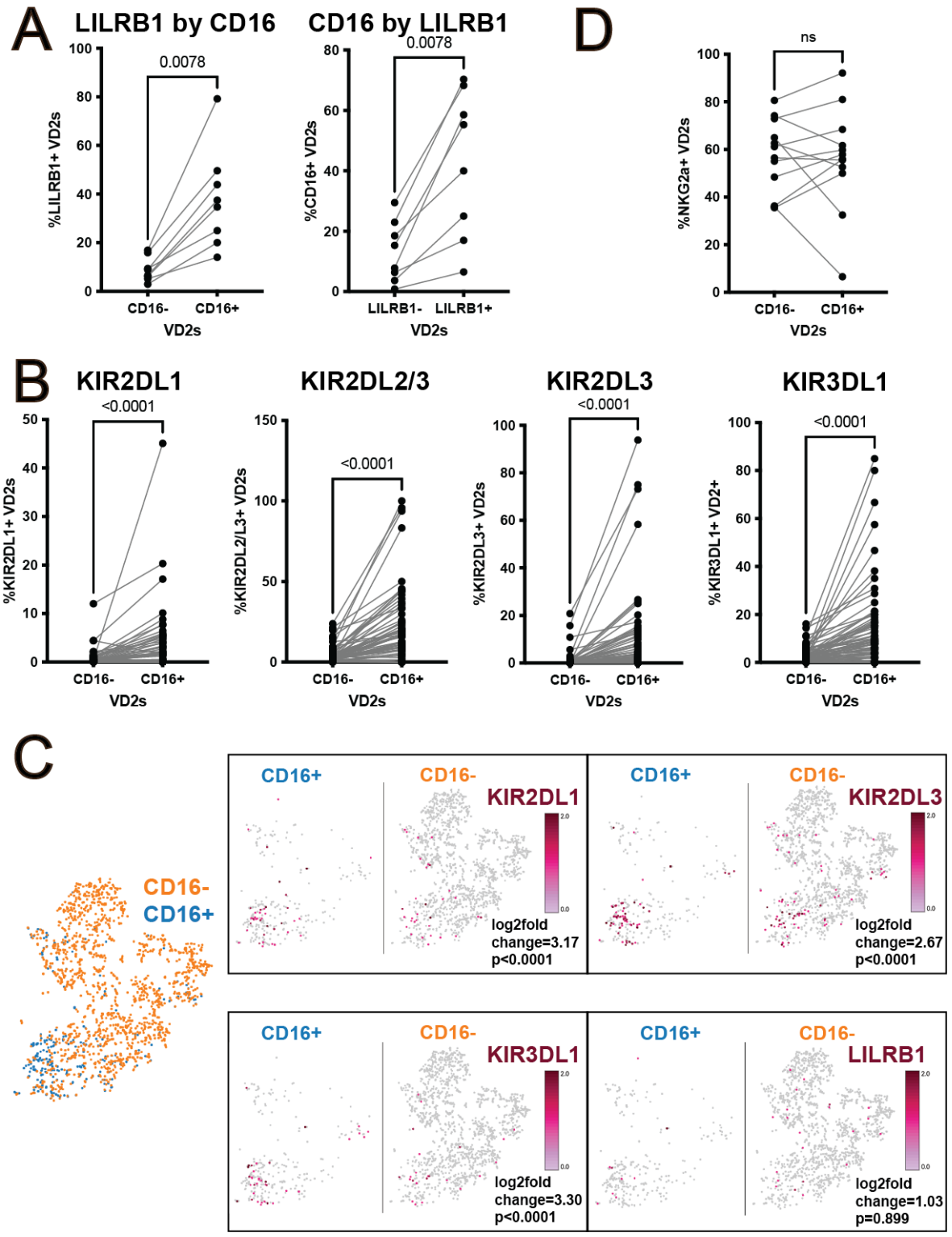

Figure 2: a) Left: LILRB1 is expressed more frequently on CD16+ Vδ2s than on CD16- Vδ2s; Right: CD16 is expressed more frequently on LILRB1+ Vδ2s than LILRB1- Vδ2s. b) KIRs are more frequently expressed on CD16+ Vδ2s than CD16- Vδ2s. c) KIR mRNA levels, but not LILRB1 mRNA levels, are higher on CD16+ than CD16- Vδ2s. d) There was no difference in frequency of NKG2a between CD16+ and CD16- Vδ2s.

LILRB1+ and KIR2DL1+ Vδ2s are less likely to respond to TCR stimulation while KIR3DL1+ and NKG2a+ Vδ2s are more likely to respond

Previous work from our group has shown that CD16+ Vδ2s can be activated not only through their TCR but also through CD16 (15). *In vitro*, we are able to able to stimulate Vδ2s through their TCR by adding HMBPP to PBMCs and through CD16 by coating a plate with an anti-CD16 antibody which leads to crosslinking of CD16 on the cell surface. We stimulated Vδ2s through either their TCR or CD16 and assessed if Vδ2s with inhibitory receptors were more or less likely to respond, using the degranulation marker CD107a as a readout.

LILRB1+ Vδ2s only trended towards being less likely to respond than LILRB1- Vδ2s during TCR stimulation, however, we noticed a bimodal distribution of the response among the LILRB1+ Vδ2s. Upon further investigation, the two populations of LILRB1+ Vδ2s were separated by study site and therefore by level of *Pf* exposure (Fig. 3a). LILRB1+ Vδ2s from high-exposure Tororo were less likely to respond than LILRB1+ Vδ2s from low-exposure Jinja. This only held true for the LILRB1+ Vδ2s—there was no difference among LILRB1- Vδ2s (Fig. 3b). This is interesting in light of the higher MFI of LILRB1 on Vδ2s from Tororo individuals (Fig. 1a). We hypothesize that there may be a threshold of LILRB1 expression at which it can exert a noticeable level of inhibition on Vδ2 activation.

The three primary KIRs that we examined have different HLA ligands resulting in combinatorial variation in whether individuals have the corresponding ligands for whichever KIRs they express. The HLA-C1 ligand group is the ligand for KIR2DL2/L3 while the rest of the HLA-C alleles fall into the HLA-C2 ligand group and are the ligand for KIR2DL1. The ligand for KIR3DL1 is a motif on some HLA-A and HLA-B molecules called Bw4 (see Chapter 3 for more details on KIR ligands). Using HLA-typing data (see Chapter 3 for more details), we were able to distinguish between individuals who had the relevant KIR ligand and those who did not, and thus were or were not able to receive an inhibitory signal through that KIR.

When stimulating Vδ2s with HMBPP, we saw that KIR2DL1+ Vδ2s were less likely to respond but that this was only true in individuals who had the matching KIR2DL1 ligand, HLA-C2 (Fig. 3c). However, when the same individuals' Vδ2s were stimulated with plate bound anti-CD16, we did not see this difference and if anything, the KIR2DL1+ Vδ2s trended towards being more likely to respond rather than less (Fig. 3d). This difference is notable because during HMBPP stimulation, KIRs' HLA ligands should be present at the immune synapse with butyrophilin2a1 and butyrophilin3a1, but should not be present when stimulating with plate-bound anti-CD16 because there is no "presenting" cell displaying HLA ligands. While similar trends were observed for KIR2DL3 (data not shown), we found that KIR3DL1+ Vδ2s were more likely to respond to HMBPP stimulation than KIR3DL1- Vδ2 but only in individuals with the HLA-Bw4 ligand (Fig. 3e). Further, when stimulated with anti-CD16, KIR3DL1+ Vδ2s were also more likely to respond but only in individuals who have the HLA-Bw4 ligand (Fig. 3f). These contradictory findings are surprising but might be due in part to the variation in inhibitory potential of different alleles of KIR3DL1 with some providing significantly less inhibition than others and additional variation in HLA-Bw4 isoforms which have been found to be associated with different clinical outcomes (21–23). An alternate explanation for why KIR3DL1+ Vδ2s

were more likely to respond to HMBPP stimulation might be that they are less likely to express the inhibitory KIR2DL1. Data from CD8+ T cells indicates that T cells are more likely to express only one inhibitory KIR at a time and the KIR3DL1 ligand HLA-Bw4 is in linkage disequilibrium with the KIR2DL1 ligand HLA-C2 (24). Therefore, if individuals have both HLA-Bw4 and HLA-C2 ligands, the KIR3DL1+ Vδ2s might be more likely to respond than the KIR2DL1+ Vδ2s. However, because KIR2DL1 and KIR3DL1 were in different flow cytometry panels, we were not able to directly assess this. Further, one study found that KIR2DL2 only inhibited late TCR signaling events and not early events on CD4+ T cells and that their cytotoxicity was maintained (25). This suggests that our five-hour stimulation may be too early to see definitive effects of some of the KIRs on Vδ2 activation.

In contrast to the patterns expected from its role on NK cells and function as an inhibitory receptor, we found that NKG2a+ Vδ2s were more likely to respond to HMBPP stimulation than NKG2a- Vδ2s (Fig. 3g). One reason that these NKG2a+ Vδ2s may be more likely to respond than their NKG2a- counterparts is because they express higher levels of the Vδ2 TCR (Fig. 3h). This suggests that these NKG2a+ Vδ2s have a higher baseline ability to respond to the TCR stimulus HMBPP despite having an inhibitory receptor that can bind to HLA-E on host cells presenting HMBPP through butyrophilin2a1/3a1. There were no differences in Vδ2 TCR levels between KIR+ and KIR- Vδ2s (data not shown). LILRB1+ Vδ2s expressed lower levels of the Vδ2 TCR than LILRB1- Vδ2s (Fig. 3h). Of note, individuals from Jinja and Tororo had similar levels of Vδ2 TCR expression on their LILRB1+ and LILRB1- Vδ2s, suggesting that the differences we observe in LILBR1+ Vδ2 responsiveness between study site is not driven by differences in Vδ2 TCR expression.

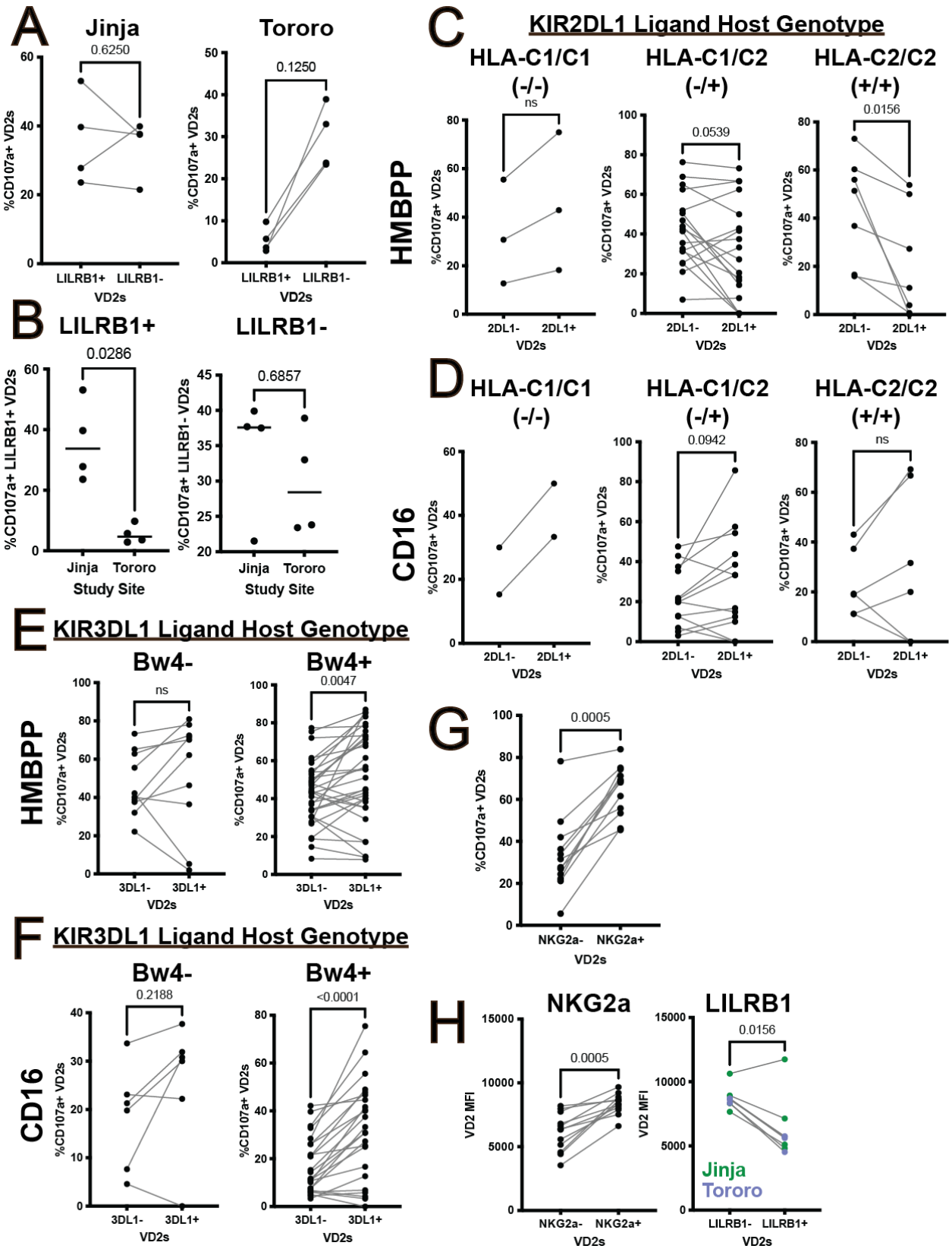

Figure 3: a) LILRB1+ Vδ2s from Jinja (left) do not respond more or less than LILRB1- Vδ2s in

response to HMBPP stimulation while LILRB1+ Vδ2s from Tororo (right) trend towards being less likely to respond to HMBPP stimulation. b) LILRB1+ Vδ2s from Tororo are less likely to respond to HMBPP stimulation than LILRB1+ Vδ2s from Jinja (left), but LILRB1- Vδ2s are not (right). c) KIR2DL1+ Vδ2s are less likely to respond to HMBPP stimulation but only when the KIR2DL1 ligand HLA-C2 is present. d) KIR2DL1+ Vδ2s are more likely to respond to CD16 stimulation when HLA-C2 is not at the immune synapse. (In c, d only individuals with >5 KIR2DL1+ Vδ2s were included in the analysis). e) KIR3DL1+ Vδ2s are more likely to respond to HMBPP than KIR3DL1- Vδ2s in individuals with the HLA-Bw4 ligand. f) KIR3DL1+ Vδ2s are only more likely to respond to CD16 when its ligand HLA-Bw4 is present. g) NKG2a+ Vδ2s are more likely to respond to HMBPP than NKG2a- Vδ2s. h) Left: NKG2a+ Vδ2s express a higher level of the Vδ2 TCR; Right: LILRB1+ Vδ2s express a lower level of the Vδ2 TCR.

Blocking the KIR and LILRB1 ligands HLA-B and HLA-C leads to an increase in Vδ2 stimulation

Next, we assessed if we could increase the Vδ2 response by blocking the ligand(s) for both LILRB1 and KIRs. We used the B.1.23.2 antibody clone which specifically recognizes HLA-B and HLA-C to block interactions between these ligands on the cells "presenting" HMBPP via butyrophilin2a1/3a1 and the inhibitory receptors LILRB1 and KIRs. We stimulated cells both with HMBPP and with *Pf*-infected red blood cells (iRBCs) and blocked MHC by preincubating the PBMC with the B.1.23.2 antibody for 30 min before adding the stimulus. We found that by blocking with this antibody we were able to significantly increase the stimulation of Vδ2s by HMBPP and increase the stimulation by iRBC but not significantly as measured by the degranulation marker CD107a (Fig 4a-b). The lower overall stimulation by iRBC might make it more difficult to observe a significant difference with the MHC blocking antibody.

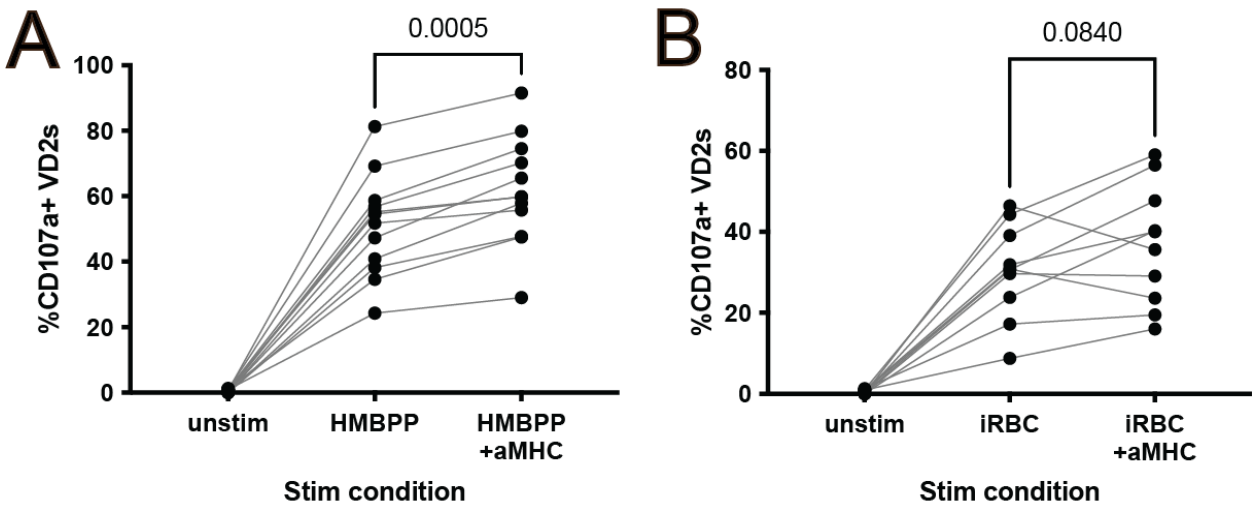

Figure 4: a) Pre-incubation with MHC antibody B.1.23.2 leads to an increase in Vδ2 stimulation by HMBPP. b) Pre-incubation with MHC antibody B.1.23.2 leads to a non-significant increase in Vδ2 stimulation by iRBC. Paired Wilcoxon test was used.

Discussion

We have shown that KIR and LILRB1 surface expression on Vδ2s seems to be modulated by *Pf* exposure but that NKG2a expression remains unaltered. We further saw that LILRB1 and KIR were differentially expressed by CD16+ and CD16- Vδ2s while NKG2a was not. We confirmed the differential KIR expression between Jinja and Tororo and CD16+ and CD16- Vδ2s with RNAseq data while intriguingly LILRB1 was not differentially expressed at the RNA level. We have further shown that these inhibitory receptors seem to impair the Vδ2 response to the *Pf* antigen HMBPP and that by blocking their shared ligand we are able to increase Vδ2 stimulation.

While our work has demonstrated that LILRB1 is expressed on Vδ2s and modulated by chronic *Pf* exposure, more studies are necessary to demonstrate if *Pf* RIFINs can directly bind to LILRB1 on Vδ2s and lead to inhibition as it does in B cells. If *Pf* RIFINs can lead to the direct inhibition of Vδ2s, it is intriguing that LILRB1 is upregulated on Vδ2s from individuals in high exposure Tororo suggesting that this upregulation might be a host mechanism of tolerance to the parasite. However, a class of antibodies has recently been described that contain receptor domains of LILRB1 within the antibody and that RIFINs can bind to these domains within the antibodies (26). These antibodies are thus able to block RIFINs from binding LILRB1 on immune cells and negatively regulating them, which in turn would suggest that the host is trying to prevent RIFINs from downregulating the immune response.

Our previous work on associations of KIR and their HLA ligands with an increased parasite prevalence highlights the important role that inhibitory receptors can play in the immune response to malaria (Chapter 3). In particular, we found that the KIR2DL1 ligand was associated with an increase in parasite prevalence. Combined with our data showing that KIR2DL1+ Vδ2s are less likely to respond to parasite antigen, it is possible that inhibition of Vδ2s by KIR2DL1 may contribute to the associated increase in parasite prevalence in individuals with HLA-C2. However, given the discrepancies with KIR3DL1 whose ligand HLA-Bw4 was also associated with an increase in parasite prevalence, it is likely that Vδ2s are not the primary cell type contributing to this association.

In contrast to LILRB1 and KIR, NKG2a has not been previously associated with any different malaria outcomes. We also did not find any association with NKG2a and *Pf* exposure or response, however, we did find that NKG2a+ Vδ2s were more responsive to TCR stimulation via HMBPP. This mirrors a recent paper that found NKG2a marked a subset of "hyper-responsive" Vδ2s in anti-tumor responses (19). Our data suggest that this might be due to higher expression of the Vδ2 TCR rather than due to functions of NKG2a. Together, the data indicate that NKG2a might serve as a biomarker for Vδ2s that are more poised to respond either due to higher TCR expression or co-expression with another unidentified molecule. Further investigation is needed to understand why NKG2a does not appear to inhibit Vδ2s in a context where its HLA-E ligand should be present.

The massive expansion of Vδ2s observed during early acute infection, while Vδ2s are still highly sensitive to HMBPP, likely contributes to the immunopathology associated with acute

malaria episodes. HMBPP is broadly produced by all iRBCs and is released into the blood stream each time a red blood cell is lysed to release more parasites, leading to the proliferation of Vδ2s and the production of many pro-inflammatory cytokines by Vδ2s (27). Our findings, which suggest that with chronic *Pf* exposure Vδ2s become less responsive to TCR stimulation via HMBPP, may be a mechanism to prevent immunopathology. Upregulating inhibitory receptors on these Vδ2s would further prevent a very generalized and over-inflammatory response by Vδ2s. By having a higher frequency of Vδ2s expressing CD16, it enables Vδ2s to still respond to iRBCs but in a more direct way that can take advantage of the specificity of antibodies for a more targeted response. Further, because opsonized iRBCs do not express the ligands for LILRB1 and KIR, Vδ2s are still able to be robustly activated through CD16. While a broader, more inflammatory Vδ2 response might be important to jump start the immune system during an individual's first few malaria episodes, it is less necessary later on when the individual has a more developed adaptive immune response.

Previous malaria vaccine studies have shown that the frequency and function of Vδ2s is an important correlate of protection (3–5). Thus, the Vδ2s of chronically exposed individuals being less responsive to parasite antigen like HMBPP may impair the immune response to malaria vaccines in endemic regions. More studies are needed to understand if the poor responses to malaria vaccines observed in malaria endemic regions compared to naïve populations is due, in part, to the lower frequency and inhibition of Vδ2s. Beyond malaria, our finding that blocking interactions between these inhibitory receptors and their MHC-I ligands is able to increase Vδ2 stimulation has implications for cancer immunotherapies wherein Vδ2s are being investigated as CAR-T therapies and LILRB1, KIR and NKG2a are being investigated as checkpoint inhibitors.

Methods

Study Approval

All study participants or the guardian of participants under 18 years of age gave written informed consent. The Uganda National Council of Science and Technology, the Makerere University School of Medicine Research and Ethics Committee, and the University of California, San Francisco Committee on Human Research approved all study protocols.

Study Site and Sample Processing

Samples were obtained from children enrolled in a large malaria surveillance study of two Ugandan districts: a suburban town in the Jinja district, with an annual entomological inoculation rate (aEIR) of 3.8, and the rural region of Tororo district, with an aEIR of 125 (28). Details from this study have been described elsewhere (28,29). In December 2014, the Ugandan government initiating an indoor residual spraying campaign in Tororo district which led to a drop in the aEIR to zero (29). PBMC were obtained at quarterly routine blood draws. 6 to 10 milliliters of blood were obtained in acid citrate dextrose tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Histopaque; GE Life Sciences) and cryopreserved in liquid nitrogen before analysis. For these analyses only timepoints where the individual was not parasitemic were included.

CD16 and HMBPP Stimulations and MHC blocking

Cells were rested overnight in RPMI supplemented with 10% FBS. Cells were then incubated with either HMBPP, iRBC or CD16 crosslinking antibody in addition to Monensin (Invitrogen/Caltag, 10 μg/ml), Brefeldin-A (Invitrogen/Caltag, 10 μg/ml) and CD107a antibody for 5 hours at 37°C. HMBPP was diluted in water and added at a final concentration of 15nM. iRBC were added at an E:T ration of 1:1 PBMC:iRBC. iRBC preparation is described in (15). Briefly, for CD16 crosslinking, ELISA plates were coated with anti-CD16 antibody (clone 3G8) overnight and described in detail in (15). For MHC blocking, clone B.1.23.2 (Thermo Fisher) was added at a concentration of 20mg/mL to the PBMC for 30min at 37°C then washed off before adding the stimulus.

Flow Cytometry

Thawed PBMC were stained directly after thawing for surface markers or after stimulation for surface markers and cytokines following standard protocols (30,31) using the following antibodies and Viability Dye from Biolegend (except when noted): CD56 (clone HCD56), CD16 (clone 3G8), CD19 (clone HIB19), CD14 (clone M5E2), Vδ2 (clone B6), CD3 (clone OKT3), L/D Aqua, KIR2DL1 (HP-MA4), KIR2DL3(R&D, clone 180701), KIR2DL/L3 (clone DX27), KIR3DL1 (clone DX9), LILRB1 (clone GHI-75), NKG2a, IFNg (clone 4S.B3), TNF (MAb11) and CD107a (H4A3).

Flow Cytometry Data Analysis

Flow cytometry profiles were gated on single cells, Aqua Live/Dead negative, CD19-, CD14- lymphocytes. Cells that were positive for both CD3 and Vδ2 were defined as Vδ2 T cells. Samples were run on an LSRII flow cytometer (Becton Dickinson) with FACSDiva software. Data were then analyzed using FlowJo (Tree Star).

Single Cell RNA Sequencing

Vδ2s were enriched from PBMC using the Stemcell EasySep™ Human PE Positive Selection Kit II, using a Vδ2 antibody conjugated to magnetic particles. Cells were counted and loaded on a 10X Next GEM Chip at 30,000 cells/lane total. Each multiplexed lane incorporated 5,000 cells from three different individuals. Cells were encapsulated into droplets and processed using the Chromium Single Cell 5' Library Gel Bead and Construction kit according to manufacturer's procedures. Libraries were sequenced on an Illumina NovaSeq (UCSF Center for Advanced Technology), using a read depth of 35,000 reads/cell. Count matrices were generated using Cell Ranger v6.0.2 (10X Genomics) and exported as a .cloupe file. All UMAPs were constructed in and differential gene expression analysis was calculated in the Loupe Browser (Version 6, 10X Genomics).

Statistical Analyses

All statistical analyses were performed using Prism 9.0 (GraphPad), and/or Stata (version 15, StataCorp). Comparisons of cell subsets between Jinja and Tororo were performed using Mann-Whitney tests. Wilcoxon tests were used for all paired comparisons between different cell subsets. Friedman tests with multiple comparisons were used when more than two paired groups were compared. In all analyses, a P value <0.05 was considered significant.

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Chapter 5

Conclusion

Summary

In this thesis, I set out to understand not only how NK cells, Vδ2s, and their inhibitory receptors modulate the immune response to *Pf*, but also how chronic exposure to *Pf* modulates these cells and their inhibitory receptor expression. Building on prior work from our lab that demonstrated Vδ2 frequency declines but the proportion of Vδ2s expressing CD16 increases with chronic *Pf* exposure, I show in Chapter 2 that with the cessation of *Pf* exposure that these observations are reversed. More specifically, spraying of pesticides by the Ugandan government led to a dramatic reduction in malaria incidence and created a natural experiment in which we could examine individuals who had previously experienced high levels of *Pf* exposure and now did not. After this dramatic reduction, we observed that Vδ2s rebounded and the proportion expressing CD16 declined. Given our previous observations that Vδ2s became more NK cell like after continuous exposure to *Pf* antigens, I next explored whether NK cells might also undergo changes in the context of chronic *Pf* exposure. I found that individuals experiencing chronic *Pf* exposure had significantly higher levels of CD56negCD16+ NK cells and lower levels of CD56dimCD16+ NK cells. This shift towards CD56negCD16+ NK cells, which are less responsive to other stimuli but are efficient mediators of ADCC, parallels the shift to CD16+ Vδ2s, which we have previously shown are capable of ADCC. We further show that like Vδ2s, the changes observed in the NK cell compartment are reversible once chronic *Pf* exposure stops. Data from Chapter 2 emphasize the strong influence that chronic *Pf* exposure has on the immune system and suggests that the immune system may rely more on antibody dependent responses than other activating stimuli in the context of chronic antigenemia.

In Chapter 3, we used clinical data from the Ugandan cohort study, PRISM, to assess if there were any associations between KIR and their HLA ligands with different malaria clinical outcomes. While we found that the presence of any KIR gene was not associated with any clinical outcomes, we did find that two HLA ligands were associated with increases in parasite prevalence. Presence of HLA-C2 and HLA-Bw4, the ligands for KIR2DL1 and KIR3DL1 respectively, were associated with an increase in parasite prevalence. Further we found that having two copies of the ligand HLA-C2 was associated with an even higher risk of increased parasite prevalence than individuals with only one copy. In addition, the effects of HLA-C2 and HLA-Bw4 were combinatorial; individuals who had both ligands were at an increased risk for parasitemia compared to individuals who only had one of these two ligands. Finally, we show that increases in HLA-C expression are also associated with increased parasite prevalence. This finding solidified that the observed associations with HLA ligands are due to their role as KIR ligands rather than their role as presentation molecules for CD8 T cells. Increased HLA-C expression should lead to increased presentation to CD8 T cells and therefore one would expect this to be associated with a decrease in parasite prevalence if this was the HLA ligand's primary function, however, we observe the opposite. These findings led us to hypothesize that KIR bearing cells might be inhibited by the KIR ligands HLA-C2 and HLA-Bw4 in the liver where *Pf* infection is initiated largely because it is the only stage of the parasite's lifecycles within an HLA-bearing cell.

Next, in Chapter 4, we examined not only how KIRs might modulate the Vδ2 response to malaria, but also how LILRB1 and NKG2a, which are also inhibitory receptors with MHC-I ligands, might influence the Vδ2 response as well. Following up on findings from Chapter 1 that

chronic *Pf* exposure modulated the Vδ2 repertoire, we examined if there might be differences in the expression of these inhibitory receptors on Vδ2s from individuals residing in high exposure Tororo. Indeed, we found that LILRB1 was upregulated and that several KIRs were more frequently expressed on Vδ2s from Tororo individuals. When we examined the CD16+ subset of Vδ2s, which we have previously shown to be at an increased frequency in individuals from Tororo, we found that they were more likely to express KIRs and LILRB1 than their CD16- counterparts. NKG2a, however, was not differentially expressed between study sites or between CD16+ and CD16- Vδ2s. In order to assess how these inhibitory receptors affected the function of Vδ2s, we then stimulated the cells either through their TCR with HMBPP or through CD16 with cross-linking antibodies. While KIR2DL1+ and LILRB1+ Vδ2 appeared less likely to respond to stimulation, KIR3DL1+ and NKG2a+ Vδ2s appeared more likely to respond to stimulation. These findings were surprising given that one would expect that all four receptors would provide an inhibitory signal to the Vδ2, making them less likely to respond. However, when we blocked HLA-B and HLA-C, ligands for KIRs and LILRB1, we saw an increase in stimulation, implying that the interaction with these receptors had previously been inhibiting these cells. These data demonstrate that inhibitory receptors previously associated with NK cells and other immune cells may play an important role in modulating the Vδ2 cell response in order to prevent immunopathology during chronic infections.

Future Directions

While my findings elucidate some of the changes in the Vδ2 and NK cell repertoires due to high *Pf* exposure, the mechanism by which these changes occur remain unclear. Long term *in vitro* culture with PBMCs and iRBCs might enable elucidation of some of these mechanisms. For example, it may be possible to determine if CD56negCD16+ NK cells are derived from CD56dimCD16+ NK cells by sorting the CD56dimCD16+ NK cells, labeling them, re-combining them with the rest of the PBMC and co-culturing the PBMC with iRBC. However, it is possible that other relevant molecules like antibodies may be excluded in this experimental setup. Similarly, in order to determine if CD16+ Vδ2s were CD16- Vδ2s that later began to express CD16 or if some express it early on and then proliferate, we can look at the Vδ2 TCR repertoire. For example, if we see an enrichment of a given TCR with CD16+ Vδ2s, it would suggest that CD16+ Vδ2 express CD16 early on and then proliferate. Further, while we have characterized the function of CD16+ Vδ2s in response to malaria antigens, we have not characterized the function of CD56negCD16+ NK cells and how their functions might be different to other NK cell subsets' functions in the malaria immune response. For example, the capability of CD56negCD16+ cells to respond to opsonized iRBC and perform ADCC could be assessed to confirm that this NK cell subset is capable of robust antibody dependent functions. Finally, the parallel changes we observed in the Vδ2 and NK cell subsets suggest that other cell types may undergo similar alterations in response to chronic *Pf* exposure that make them less likely to respond to more general stimuli like cytokines and more likely to respond to antibody bound antigens. Future studies could assess whether other cell types upregulate CD16 and other Fc receptors in addition to testing their capacity for antibody dependent responses.

While our findings from Chapter 3 highlight a potentially important role for NK cells in the immune response to malaria, what that role is remains unclear. In order to determine a potential mechanism underlying the associations we observed, it would be necessary to compare the NK

cell response to different *Pf* derived stimuli between individuals with and without the KIR ligands HLA-C2 and HLA-Bw4. However, given that we hypothesize that the effects of KIRs/KIR ligands on parasite prevalence might occur in the liver, it is difficult to experimentally assess. In order to assess the NK cell response to the liver stage of the parasite *in vitro*, it would be necessary to culture sporozoites in hepatocytes which is not regularly done. Further, the hepatocytes would need to be HLA-typed in order to understand if they express various KIR ligands. As an additional follow up to our experiments, it would be possible to also examine if there were any associations between the different alleles of KIR3DL1 and different malaria outcomes using the clinical data in Chapter 3. Previous studies have shown that different KIR3DL1 alleles are associated with different levels of cellular inhibition and clinical outcomes so may also influence parasite prevalence. Like KIR3DL1, LILRB1 polymorphisms have been shown to result in different clinical outcomes for some diseases, so similarly it would be possible to assess if LILRB1 polymorphisms were associated with different malaria outcomes if LILRB1 was allele typed in our cohorts.

In Chapter 4, we analyzed the expression and function of five different receptors on Vδ2s, however, due to limitations of conventional flow cytometry, we were unable to look at these receptors at the same time on the same cells. A logical next step to our analyses is to utilize the advances of mass cytometry or spectral flow cytometry to combine all of the inhibitory receptors into a single panel to look at co-expression on cells. Further, by incorporating all of the receptors into a single panel it is possible to tease apart any combinatorial effects that these receptors might have. For example, in the context of the MHC-I blocking experiment, it would be possible to look at the contribution of individual KIRs and LILRB1 at the same time to see if they add up to the increase in stimulation associated with blocking MHC-I. A larger panel would also enable the inclusion of all relevant NK cell markers into the same panel as the Vδ2 markers. This would enable comparison of expression of different inhibitory receptors between NK cells and Vδ2s. Further it would be possible to assess if malaria incidence or *Pf* exposure alters (co)-expression of inhibitory receptors on different NK subsets.