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The role of monocytes in the development of natural immunity to Plasmodium infection

Author
Baccarella, Alyssa M

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The role of monocytes in the development of natural immunity to *Plasmodium* infection

By

Alyssa Marie Baccarella

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Committee in Charge:

John Balmes, Chair
Charlie Kim
Kristine Madsen
John Schwartzberg

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Part I: Literature Review
Immunity to Malaria and the Role of Monocytes

Malaria as a global health problem

Malaria, caused by parasites of the genus *Plasmodium,* is a leading cause of infectious morbidity and mortality worldwide, resulting in an estimated 154-289 million infections and 660,000 deaths in 2010 (WHO Global Malaria Programme, 2012). Of those infected by *Plasmodium* parasites, children and pregnant women face the highest risks of severe complications and death (Murphy and Breman, 2001). Additionally, of those who experience cerebral malaria, a severe version of malaria characterized by coma and seizures, approximately 15% will experience neurological sequelae, with risks of permanent neurological impairment (van Hensbroek et al., 1997; Mung'ala-Odera et al., 2004). Unsurprisingly considering the incidence and high disease burden, malaria exacts a tremendous economic toll on already economically vulnerable regions (Sachs and Malaney, 2002).

Plasmodium falciparum, the major causative agent in human malaria, is estimated to have emerged as a species 100,000 years ago, at the same time as humans, and has been one of the greatest evolutionary pressures on the human immune system (Crompton et al., 2014; Kwiatkowski, 2005). Perhaps due to this coevolution, *Plasmodium sp.* have been particularly skilled at evading human immunity. Natural immunity against malarial disease is slow to develop and short lived once exposure to *Plasmodium* is removed (Schofield and Mueller, 2006). As a result, current clinical management relies heavily on pharmacological treatment of malarial disease; however, drug resistant strains of *Plasmodium falciparum* are on the rise, with drug development struggling to keep up (Kim and Schneider, 2013). An efficacious, long lasting vaccine is highly desirable, but its development has proven difficult (Wykes, 2013). The most clinically advanced vaccine candidate, RTS,S, provides less than 50% protection from malaria disease in children ages 5-17 months old, and has only a 27% efficacy in young infants, the vaccine's initial target population (Agnandji et al., 2014). Furthermore, this protection was greatly diminished by 18 months following vaccination (Agnandji et al., 2014). A better understanding of the mechanisms that promote and hinder the development of natural immunity to malaria could provide vital clues to guide the development of novel therapies and long lasting vaccines.

Malaria infection – *Plasmodium sp.* parasites life cycle

Plasmodium falciparum parasites have a complex life cycle that requires both human and *Anopholes* mosquito hosts. Initial human infection occurs when an infected female mosquito takes a blood meal from a human. Her saliva contains the sporozoite form of *P. falciparum*, and during the blood meal approximately 10-100 of these sporozoites are injected into the skin. After remaining in the skin for hours to days, the sporozoites migrate through the bloodstream

into the liver and invade the hepatocytes. Once in the hepatocyte, the infection remains free of clinical symptoms for roughly one week, during which the sporozoites asexually replicate, increasing in number by approximately 40,000 fold and differentiating into erythrocyte-infectious merozoites [reviewed in (Crompton et al., 2014)].

These merozoites egress from the hepatocytes in budding vesicles, initiating blood-stage infection. In the bloodstream, these merozoites begin a 48-hour cycle of red blood cell invasion, replication and red blood cell rupture. During blood-stage infection, the parasites employ multiple mechanisms to evade immune system detection. For the majority of the cycle, the most inflammatory molecular patterns of the parasite are hidden from the immune system within the immunologically quiescent red blood cell, which lacks machinery for responding to, or signaling the presence of, infection. Additionally, *P. falciparum* remodels the red blood cell surface to avoid entrance into the spleen, and thus recognition by innate immune effector cells and the resulting immune activation. To achieve this, the parasites express various antigenically distinct *P. falciparum* erythrocyte membrane protein 1s (PfEMP1s), which allow the parasites to bind to a variety of proteins on the vessel endothelial cells and sequester in the vessels. However, upon parasite egress from the red blood cell, cytokine-producing immune cells are exposed to pathogen-associated molecular patterns (PAMPs) on the parasites, and produce inflammatory cytokines that underlie the clinical manifestations of bouts of fever, lethargy and headaches at approximately 48 hour intervals. Some of the most severe malaria pathology may result from an unchecked cytokine response (Hunt and Grau, 2003; Perkins et al., 2011). These cytokine responses are somewhat correlated to the antigenic load of parasites in the blood, and human *Plasmodium* infection often can reach densities of 50,000 infected red blood cells per microliter of blood (Rodrigues-da-Silva et al., 2014). Finally, to complete the malaria life cycle, during a blood meal of an infected human, *P. falciparum* infected erythrocytes are taken up and infect the mosquito. In the mosquito, the parasites differentiate into male and female gametocytes, which then ultimately undergo a form of sexual reproduction and eventually once again differentiate into sporozoites that invade the mosquito's salivary glands (Crompton et al., 2014). In many endemic areas, transmission is seasonal, with the greatest number of infective bites occurring during the peak of rainy season, and very few infective bites occurring during the dry season (Dery et al., 2010).

Clinical Immunity, the shifting dogma

As alluded to above, immunity against malaria disease develops slowly in humans living in malaria-endemic regions. However, immunity manifests in at least two broad forms. One form known as "clinical immunity," is characterized by a lack of symptoms despite the presence of *Plasmodium* parasites in the bloodstream. Clinical immunity appears to be the predominant form of naturally acquired immunity. Sterile immunity, on the other hand, is characterized by protection from blood stage *Plasmodium* infection; however, it is suspected that this form of immunity is likely never naturally achieved.

In malaria-endemic areas, children develop immunity to malaria disease over the course of years. Although these children can be exposed to hundreds of infective mosquito bites a year, they only become less susceptible to severe malaria by around age five, and remain susceptible to clinical disease and episodes of febrile malaria until early adolescence (Marsh and Kinyanjui). Clinical immunity does eventually develop, however, and by adulthood, febrile malaria episodes are very rare.

The development of clinical immunity was initially attributed primarily to the development of malaria specific plasma cells, producing immunoglobulins against *Plasmodium* proteins. A 1961 study made the observation that transfer of immunoglobulins from malaria-immune adults to less immune, symptomatically *Plasmodium* infected children led to a reduction in *Plasmodium* parasitemia, which fit with what was known about the development of sterilizing immunity to many other pathogens (Cohen et al., 1961). However, while antibodies undoubtedly play a role in parasite control, more recent studies have revealed that naturally acquired *Plasmodium*-specific antibodies and B cells are short lived without constant parasite exposure, returning to only slightly higher than baseline after just 6 months of decreased malaria exposure (Crompton et al., 2010; Weiss et al., 2010). Additionally, vaccines focused on the promotion of *Plasmodium*-specific B cell responses have failed to meet expectations. It was recently shown that infection causes the deletion of *Plasmodium*-specific memory B cell responses, offering one potential explanation for the lackluster performance of these vaccines [reviewed in (Scholzen and Sauerwein, 2013)]. Given these poor antibody responses, it is clear that development of humoral immunity to malaria is impaired, and it is likely that clinical immunity is not completely explained by antibody responses.

Surprisingly, a recently published longitudinal study of children and adults in malaria-endemic Mali reported that over half of a large cohort of children and adults were asymptomatically infected with *Plasmodium* parasites at the beginning of the rainy season, often thought to be a time when infection rates are low (Tran et al., 2013a). Additionally, among those not infected at the start of the rainy season, there was no protection from blood-stage infection associated with age. Instead, protection from clinical disease (*i.e.* clinical immunity), and to a lesser extent decreased parasite load during blood stage infection, increased with age (Tran et al., 2013a).

These results all support the notion that immunity to malaria exists in the two forms described above, sterile immunity and clinical immunity. Importantly, these states must necessarily be defined by different underlying immunological mechanisms, either in character, magnitude, or both.

There is evidence that sterilizing immunity can be produced in humans under specific experimental conditions. Thus far, sterilizing immunity can only be produced in humans through exposure to irradiated sporozoites that are unable to establish productive infection (Clyde, 1975, 1990), modified sporozoites which can invade the liver but not replicate within the hepatocytes (Hoffman et al., 2002; Vaughan et al., 2010) or the exposure to sporozoites in the presence of the anti-malarial drug, chloroquine, which allows progression of the liver

infection but prevents development of blood-stage infection (Roestenberg et al., 2009). The immunity to sporozoites delivered with chloroquine is long lived and lasts at least 28 months (Roestenberg et al., 2009). However, the doses of sporozoites necessary to confer this immunity are 1000 times those that would be naturally encountered in an infective mosquito bite, and these sporozoites are antigenically homologous. While these systems are unlike those that would be encountered in nature, and questions remain about how such immunization approaches would fare in an endemic setting, they do provide insight into sterilizing immune responses against the parasites (Crompton et al., 2014).

Clinical immunity, on the other hand, is thought to be a combination of restriction (*i.e.*., parasite clearance) and tolerance (*i.e.*, the dampening of inflammatory immune responses in response) to *Plasmodium* parasites (Schneider and Ayres, 2008a). *Plasmodium* parasites contain many PAMPs that stimulate innate immune pattern recognition receptors (PRRs) [reviewed in (Gazzinelli et al., 2014)]. Given that these PAMPs are present in the blood stream during asymptomatic infection, it is likely that the innate immune system becomes less responsive to stimulation, as occurs in endotoxin tolerance (Boutlis et al., 2006a). Additionally, given that the parasite load is decreased during asymptomatic infection (Tran et al., 2013a), mechanisms of restriction must be increasing. It is thought that as age and incidence of infection increases, the ability to spontaneously clear parasites from the blood without pharmacological treatment increases; however, there have been documented cases of malaria transmission via blood transfusion from an asymptomatic carrier tens of years following possible malaria exposure (Djimdé et al., 2003; Hassanpour et al., 2011). A recent study (Portugal et al., 2014) reported that children acquire exposure-dependent, *P. falciparum*-specific immunoregulatory responses that dampen inflammatory responses but increase anti-parasite effector mechanisms in malaria endemic areas. However, the cell-specific contributions to these responses, and interplay between the adaptive and innate immune system have not yet been elucidated.

It is currently controversial whether clinical immunity, rather than sterilizing immunity, should be a goal in the development of malaria vaccination. The shift to thinking of malaria as a chronic disease is still in its infancy, and few studies have examined the consequences of long-term, asymptomatic infection. One recent study reported attention deficits in asymptomatically infected children performing cognitive tasks (Nankabirwa et al., 2013). Additionally, the presence of asymptomatic parasitemia in a population might provide a reservoir for infection for those who have not yet obtained immunity, naturally or through vaccination (Lindblade et al., 2013). Although these considerations must be carefully weighed when designing future interventions, it is indisputable that a better understanding of the mechanisms underlying clinical immunity will be enhance vaccine and therapeutic development efforts. This paper will next review what is known thus far about the development of clinical immunity to malaria, and suggest that the study of myeloid cells, particularly monocytes, might be a fruitful avenue for identifying uncharacterized protective mechanisms.

Tolerance, Exhaustion and Malaria

In general, a host can protect itself from an infectious disease by three basic mechanisms: avoidance, resistance, and tolerance (Medzhitov et al., 2012). Avoidance of pathogens reduces the risk of exposure to the pathogen. Resistance is a reduction of pathogen burden once infection is established. Tolerance is defined as reducing the impact of infection on host fitness, while not affecting pathogen burden (Medzhitov et al., 2012). Central tolerance, clonal selection against self-reactive T cells, is extremely well accepted and described in humans. The mechanisms underlying peripheral tolerance, or the dampening of harmful immune responses to a pathogen, are less well understood in the context of infection.

The mechanisms of peripheral tolerance are varied and include immunoregulation by tolerogenic T cells and antigen-presenting cells (APCs), anergy, and exhaustion. Immunomodulation takes many forms, and includes the production of inhibitory cytokines, lack of responsiveness to typically inflammatory PAMPs, and the expansion of tolerance-promoting cell subsets. Anergy and exhaustion both specifically pertain to lymphocyte function. T cell anergy is an induced hypo-responsive state in naïve T cells, when they are met with either low co-stimulatory or high co-inhibitory activation. Exhausted T cells are effector T cells with decreased cytokine expression and effector function, which are resistant to reactivation (Crespo et al., 2013). Exhausted T cells have been well described in cancer, autoimmunity, and chronic infections (Pauken and Wherry, 2015). In particular, in the past few years, exhaustion has begun to be suggested as a major driver of asymptomatic *Plasmodium* infection.

T cell Functions in Malaria Infection

CD4+ T cells have long been thought to have a key role in the immunity to and control of *Plasmodium* parasites. In general, intracellular protozoan parasite infections require the development of an inflammatory Th1 response, characterized by the production of interferon gamma (IFNγ) and tumor necrosis factor (TNF), to stimulate the activation and generation of microbicidal molecules by phagocytes (Engwerda et al., 2014). CD4+ T cells play an important role in initiating antibody production and driving cytokine production during this inflammatory response. CD4+ T cells are essential for promoting B cell antibody responses, and the adoptive transfer of CD4+ T cells along with B cells is necessary to transfer protective immunity in mice (Meding and Langhorne, 1991). CD4+ T cells are also indispensable for parasite restriction. The depletion of CD4+ T cells in *Plasmodium* infected mice leads to chronic, high level parasitemia which cannot be controlled (Podoba and Stevenson, 1991). One mechanism by which CD4+ T cells might promote parasite restriction is through their role in IFNγ production. IFNγ is produced first by NK cells and then by CD4+ T cells during the course of infection (Baccarella et al., 2013; Kim et al., 2008). IFNγ has been heavily studied for its role in control of *Plasmodium* parasites and disease pathogenesis. While IFNγ is undoubtedly necessary for the control of *Plasmodium* parasite load in mice (Langhorne et al., 1990), its role in producing the clinical symptoms of malaria is unclear. In studies of previously malaria-naïve individuals, fever strongly correlated temporally with IFNγ production (Walther et al., 2006). Conversely, the strength of parasitized erythrocyte induction of IFNγ production also tends to be correlated with decreased

risk of fever and clinical malaria episodes [reviewed in (McCall and Sauerwein, 2010)], although one recent study did not find an association between IFNγ producing CD4+ T cells and protection from malaria disease in a cohort of highly exposed children (Jagannathan et al., 2014a). One potential explanation for the discrepancy between IFNγ's association with fever and IFNγ's association with clinical protection is that although IFNγ can cause downstream activation of pyrogens, it is not a pyrogen itself, so other modulators downstream are dampening its induction of pyrogens, or their actions. It is likely that modulation of immune responses such as this leads to the natural development of clinical immunity.

CD8+ T cells have traditionally not been a focus of study during blood-stage infection; since erythrocytes do not express MHC Class I molecules, it has been thought they evade CD8+ T cell detection. However, studies in a mouse model of infection have shown that the depletion of CD8+ T cells during blood-stage infection led to slightly delayed clearance of the parasites (Podoba and Stevenson, 1991). Additionally, in a different mouse model of infection, transfer of either CD4+ or CD8+ T cells from mice that were highly immunized against a lethal malaria infection conferred complete protection to *Plasmodium*-naïve donors (Imai et al., 2010). These results suggest that both CD4+ and CD8+ T cells are important for the control of repeated *Plasmodium* infection.

In contrast to the protective role played by CD4+ T cells as a whole, the CD4+CD45RO FOXP3- memory cell subset seems to have a very different role in immunity to malaria. It has long been clear that the anti-inflammatory cytokine, interleukin 10 (IL-10) serves to limit immunopathology during *Plasmodium* infection in mouse models of infection (Kossodo et al., 1997). More recently, in a study of Malian children it was observed that there was a shift from inflammatory cytokine production, such as IL-1β, IL-6 and IL-8, to anti-inflammatory cytokines, such as IL-10 and TGFβ, following a febrile episode of malaria. This shift from pro-inflammatory to anti-inflammatory cytokines was hypothesized to be a partial explanation for why children in malaria endemic areas typically only experience mild febrile symptoms or have asymptomatic parasitemia (Crompton et al., 2014). Recently a source of IL-10 was identified as being CD4+CD45RO FOXP3- cells, which co-produce IFNγ and IL-10, rather than the more typical FOXP3+ T regulatory cells, as had previously been suspected (Illingworth et al., 2013). This subset of T cells was found to be expanded in highly exposed children living in malaria endemic areas, as compared to less exposed children from the same area, and inversely correlated with time since last malaria episode (Jagannathan et al., 2014a). Unfortunately, the levels of these T cells was not found to correlate with protection from future malaria episodes, and the IL-10 produced from these cells inhibits CD4+ T cell proliferation (Jagannathan et al., 2014a). It's likely that these cells have some role in inhibiting the immunopathology of malaria infection, but their presence certainly does not offer a full picture of malaria immunity.

Deletion of malaria-specific T cells

Both CD4+ and CD8+ malaria-specific cells are necessary for developing sterilizing immunity to malaria, and for the initial control of the parasites; however, in mouse models of malaria,

parasite-specific T cells undergo apoptotic cell death following infection [reviewed in (Stanisic et al., 2013)]. While some apoptosis is normal following infection, due to natural homeostatic mechanisms, there was evidence that immunity post-infection was diminished due to this depletion of parasite-specific cells. While this depletion of T cells may be detrimental to the development of sterilizing immunity, it may serve to limit immunopathology. A recent human study found that loss of a subset of γδ T cells was associated with repeated infections, and correlated to a reduced likelihood of symptoms during subsequent *P. falciparum* infections (Jagannathan et al., 2014b). Additionally, the ability of malaria specific γδ T cells to produce TNF and IFNγ decreased as prior malaria incidence increased (Jagannathan et al., 2014b). In addition to supporting the above possibility that immunoregulation serves to limit pathology, the above study suggests that this occurs not only during the contraction phase of acute infection, but also as a result of cumulative exposure. Conversely, there is likely a trade off with parasite control, as these cells are known to have parasite killing functions. It is currently unclear what cellular mechanisms or interactions are driving this selective loss of malaria-specific T cells.

T cell exhaustion and PD1

One mechanism of promoting eventual T cell death that has been well described in chronic disease and cancer is PD-1 dependent exhaustion of T cells [reviewed in (Keir et al., 2008)]. T cell co-stimulation has traditionally been described as a mechanism for activation of T cells. In this model, a primary, TCR specific signal is needed, as well as a second, antigen independent co-stimulatory signal, provided by the APC. Without this second co-stimulatory signal, the T cell will fail to activate, and will become anergic and resistant to future activation by the antigen. Recently, it has become appreciated that this second signal can either be co-stimulatory or inhibitory. One of the most widely studied inhibitory signals in this family of molecules is the programmed death 1 (PD-1) receptor and its ligands, PD-L1 and PD-L2 (Keir et al., 2008). PD-1 can be expressed on activated T cells, as well as B cells, NKT cells, activated monocytes and dendritic cells, although its functions are best understood on T cells (Keir et al., 2008). Notably, PD-1 is not expressed on resting T cells, but can be upregulated within a few hours of stimulation.

It is known that PD-L1 is highly expressed on monocytes, with lower relative expression on activated T cells, plasmacytoid dendritic cells (pDCs), myeloid DCs, as well as vascular epithelium, and other non-hematopoietic cells. PD-L2 expression is more restricted, but is inducibly expressed on monocytes, macrophages, DCs and bone-marrow derived mast cells (Keir et al., 2008).

Ligation of T cell PD-1 with its ligands (especially when coupled with low levels of TCR stimulation) has marked deleterious effects on T cell function and survival. During exhaustion, the loss of effector function of antigen-experienced T cells begins with a loss of proliferative ability. This is followed by loss of IL-2 production and cytotoxic abilities, then by loss of TNF and IFNγ production, and eventually cell death (Rodrigues et al., 2014).

In models of chronic disease, a careful balance of PD-1/PD-L1 signaling is necessary to balance immune clearance with immunopathology. For example, in a model of adenovirus infection in mice lacking PD1 signaling, these mice cleared the virus more quickly than wildtype controls, but also suffered more hepatocellular injury (Iwai et al., 2003).

Conversely, a number of viruses have exploited the PD-1: PDL-1 pathway to evade immune detection. T cell exhaustion in viral infections has been associated with high viral loads and high and persistent antigen loads (Wherry, 2011).

In contrast to viral infections, T cell exhaustion during parasitic infections is associated with low and controlled antigen loads, suggesting that exhaustion may be predominantly promoted by the body as a defense mechanism, rather than by the parasite as an evasion mechanism (Rodrigues et al., 2014). In chronic parasitic diseases, exhaustion tends to occur following an initial adaptive immune response to control the parasite burden. This has been described in *T. gondii* and *L. donovani* infections, as well as a mouse model of *Plasmodium* infection (Butler et al., 2012; Gigley et al., 2012).

PD-1 in malaria

PD-1 and other inhibitory receptors have been shown to play a role in mouse models of malaria infection. In the *P. yoelii* mouse model of infection both CD8+ and CD4+ T cells become exhausted within 30 days of blood stage infection. Blockade of PD-1 and inhibitory receptor LAG-3 restored CD4+ T cell function and accelerated parasite clearance (Butler et al., 2012). In a different mouse model of chronic infection, *P. chabaudi,* PD-1 expression of CD8+ T cells was found to mediate the loss of parasite specific CD8+ T cell functions and number, leading to less efficient clearance of the parasites (Horne-Debets et al., 2013a). Additionally, PD-1 has been reported on T follicular helper cells during *P. yoelii* infection, and the blockade of both PD-L1 and LAG-3 increased humoral immune responses and increased numbers of germinal center B cells, suggesting that T cell exhaustion also affects B cell function (Butler et al., 2012). While this evidence shows that PD-1 engagement leads to impaired parasite clearance, there is also evidence that these inhibitory mechanisms may protect from severe pathology. In a murine model of cerebral malaria, the blockade of PD1 leads to increased incidence of CD8+ T cell infiltration into the brain and cerebral malaria mortality (Hafalla et al., 2012).

In studies of human infection, children who have been persistently exposed to *Plasmodium falciparum* infection, regardless of current infection status, express a larger proportion of atypical B cells, as compared to unexposed or historically exposed controls (Illingworth et al., 2013). These exposed children also had higher levels of PD-1 and PD-1 and LAG-3 positive CD4+ T cells. Interestingly, only currently asymptomatically infected children showed higher levels of PD-1+ CD8+ cells (Illingworth et al., 2013).

These studies suggest that PD-1 signaling, along with other similar pathways, may be a major driver of clinical immunity to malaria, and may also serve to aid parasite escape from the immune system. However, the cells driving this exhaustion phenotype have not been studied,

and it is unknown which cells and ligands promote exhaustion and T cell loss in *Plasmodium* infection.

Myeloid Cells and Exhaustion

The myeloid cell compartment has been understudied in malaria, particularly in the development of natural immunity to malaria. As stated above, monocytes are the highest known expressers of PD-L1, and also, one of the only inducible expressers of PD-L2, along with other antigen presenting cells (Keir et al., 2007). In chronic hepatitis C virus (HCV) infection, it was recently shown that an altered ratio of PD-L1 to CD86 on the intermediate monocyte subpopulation correlated with chronic infection and immune suppression, as opposed to viral clearance (Zheng et al., 2014). Additionally, lower levels of intermediate monocytes were correlated with higher viremia, suggesting that these monocytes are involved in viral suppression (Zheng et al., 2014). PD-1:PD-L1 signaling has also been implicated in T cell dysfunction during HIV infection, and the major source of stimulation of PD-1 comes from monocyte expression of PD-L1 (Porichis and Kaufmann, 2012). It is possible that monocytes play a role in promoting tolerance to malaria, either through PD-1 or other tolerogenic mechanisms; however, this has not yet been investigated.

Tolerized monocytes in malaria

It is likely that monocytes themselves become tolerized during malaria infection. In order to understand the changing roles of monocytes during acute and chronic malaria infection, it is first necessary to understand the varied functions and subsets of these cells.

Monocyte Functions

Monocytes constitute approximately 10% of leukocytes in humans. They are part of the mononuclear phagocyte system (MPS), along with dendritic cells and macrophages. It was originally thought that monocytes served primarily as precursors to dendritic cells and macrophages; however it has become increasingly clear that at steady-state, macrophages and dendritic cells arise from their own precursor cells (Tamoutounour et al., 2013), with the exception of certain compartments such as the lamina propria of the intestines and the skin (Italiani and Boraschi, 2014). Rather than being derived from monocytes, it has been shown that a large proportion of macrophages are seeded embryonically (Epelman et al., 2014) and self-renew through adulthood (Sieweke and Allen, 2013). Additionally, it has been shown in humans that many macrophage populations are intact in adults with monocytopenia due to autoimmune conditions (Bigley et al., 2011). It should be noted however, that during certain inflammatory conditions monocytes can differentiate into select subsets of dendritic cells and macrophages, including TNF and iNOS producing dendritic cells (Tip-DCs) [reviewed in (Geissmann et al., 2010)]. No longer considered as primarily macrophage precursors, monocytes have now been recognized as a diverse, heterogeneous class of cells, with subsets serving a wide range of functions in states of homeostasis and disease.

Human blood monocytes have conventionally been divided into two subgroups based upon their levels of the surface marker, CD16. However, in recent years, a third monocyte population has been defined based on relative levels of both CD14 and CD16. It has now become widely accepted to group monocytes based on both CD14 and CD16 levels, classifying them into classical monocytes, which are $CD14^{++}CD16^{-}$; intermediate monocytes which are $CD14^{++}CD16^{+}$; and nonclassical monocytes which are $CD14^{lo}$ $CD16^{+}$, according to the International Consensus on Monocyte Nomenclature (Ziegler-Heitbrock et al., 2010). With the advent of genome analysis, it has become increasingly clear that these three monocyte subsets play different roles in both normal human physiology and in disease states.

Human monocytes subsets as compared to those in the mouse

Most initial work in defining monocyte subset functions has been done in mice. Mouse monocytes and human monocytes are not defined by the same markers. In general, mouse "inflammatory" monocytes, defined as being $ly6c^{hi}$, share inflammatory properties with classical and intermediate monocytes (Ingersoll et al., 2010), while non-classical human monocytes share similar patrolling functions with murine $Ly6c^{lo}$ "alternative" or "patrolling" monocytes (Geissmann et al., 2003). Both human classical monocytes and mouse inflammatory monocytes express high levels of CCR2 and low levels of CX3CR1, while non-classical and patrolling monocytes show the reverse pattern (Italiani and Boraschi, 2014). The proportions of these monocytes vary greatly between mice and humans. While patrolling monocytes constitute about half of the circulating monocytes in mice, $CD16^{+}$ monocytes typically account for less than 15% of circulating monocytes in humans (Passlick et al., 1989).

Monocyte Subset Functions

Classical Monocytes

Most monocyte functional studies have been done in mice. In mice, $Ly6c^{hi}$ monocytes are thought to be most similar to $CD14^{++}CD16^{-}$ classical monocytes in humans. Generally, classical monocytes are thought to be the drivers of inflammatory responses, especially during bacterial infection, although this role may be shared with intermediate $CD14^{++}CD16^{+}$ monocytes. Unsurprisingly since CD14 is involved in lipopolysaccharide (LPS) sensing, these monocytes respond with cytokine production to bacterial signals (Gama et al., 2012). These monocytes are considered to be the primary respondents to infection and injury (Italiani and Boraschi, 2014). They express high levels of CCR2, which binds to CCL2 (MCP1) and allows monocytes to extravasate to the site of insult. CCL2 is produced by fibroblasts, endothelial cells and epithelial cells in response to inflammatory cytokines or microbial molecules (Kurihara et al., 1997; Serbina and Pamer, 2006). They are also reported to be the superior phagocytes of the monocyte subpopulations (Wong et al., 2012a; Zawada et al., 2011). Mouse studies have shown $Lyc6^{hi}$ monocytes to replenish some macrophage compartments, including the skin, small intestine, and partial populations of the heart and lung (Italiani and Boraschi, 2014). It has been well described in mouse models of infection that classical monocytes can home to the site of

infection and differentiate into TNF producing DCs and Tip-DCs, which help suppress infection, but are not necessary for adaptive immune priming, with the exception of a model of *L. major* skin infection [reviewed in (Ingersoll et al., 2011)].

Intermediate Monocytes

The functions of intermediate, $CD14^{++}CD16^{+}$ monocytes are just beginning to be delineated. Initially, they had been grouped with nonclassical monocytes as $CD16^{+}$ monocytes; however, there is increasing evidence that they have their own distinct genetic signature. The intermediate monocytes express many genes at an intermediary level between classical and nonclassical monocytes; however, they uniquely have high levels of antigen presenting genes such as HLA-DR (Schmidl et al., 2014). Based upon genetic and functional analysis, the main functions of these monocytes appears to be promoting T cell proliferation and stimulation, and production of reactive oxygen species for pathogen killing (Wong et al., 2012a). Like classical monocytes, they express high levels of CCR2, which allows them to home to both sites of infection and to the lymph nodes. Genetic profiling has shown that intermediate monocytes express the highest levels of MHC II processing and presentation genes (Wong et al., 2011), suggesting that they play an important role in T cell priming and response (Yang et al., 2014). Like classical monocytes, their CD14 expression allows them to be superior responders to bacterial signals (Gama et al., 2012). Similarly, mouse studies have reported that $Ly6c^{hi}$ monocytes can enter non-lymphoid tissues without differentiation, then upregulate MHC-Class II expression and recirculate to the lymph nodes, where they activate T cells (Jakubzick et al., 2013). When compared to nonclassical monocytes, intermediate monocytes from healthy individuals are enriched for genes involved in ROS production, phagocytosis and antigen presentation (Zawada et al., 2011), although it is unclear how these subsets compare to classical monocytes, which were not included in this study. Previous studies of all $CD16^{+}$ monocytes have shown cytokine production in response to pathogens, labeling the entire $CD16^{+}$ compartment as “inflammatory” monocytes [reviewed in (Ziegler-Heitbrock, 2007)]. Additional studies distinguishing between the $CD14^{++}$ intermediate monocytes and $CD14^{lo}$ nonclassical monocytes showed that these inflammatory cytokines are likely produced by intermediate monocytes, including a study that reports intermediate monocytes as being the superior producers of TNF, IL-1β and IL-6 in response to LPS in vitro, as opposed to classical monocytes (Cros et al., 2010). One study renamed intermediate monocytes as “inflammatory” based on a subset of human data, with the desire to align with the mouse literature (Antonelli et al., 2014a); however, this naming might add to the confusion of monocyte subsets, rather than alleviate it. Although not as well, a subpopulation of intermediate and nonclassical monocytes expressing TIE-2, the receptor for angiopoetin-1, has also been implicated in angiogenesis (Wong et al., 2012a).

Nonclassical monocytes

Nonclassical monocytes, defined in humans as $CD14^{lo}CD16^{+}$, are considered to be “patrolling” monocytes. They are thought to be functionally equivalent to $ly6c^{lo}$ monocytes, and patrol the

luminal surface of the vascular endothelium (Auffray et al., 2007; Cros et al., 2010). They are reliant on anti-apoptotic signaling through CX3CR1 for their survival, and in mice lacking CX3CR1 a reduced number of ly6c^{lo} monocytes are seen (Auffray et al., 2007; White and Greaves, 2012). Although they have been reported as poorly phagocytic by at least one review (Wong et al., 2012a), *Cros et al. 2010* have shown that they have the ability to phagocytose particles *in vitro*, and gene expression studies have shown enrichment for phagocytosis related genes (Schmidl et al., 2014). They primarily produce inflammatory cytokines in response to nucleic acids via TLR7 and TLR8, but unsurprisingly are poor LPS responders (Cros et al., 2010). These monocytes may be the alarm sounders of the blood vessels, producing local inflammatory cytokines to recruit classical monocytes and neutrophils to mount a larger immune response, as was seen in a murine model of *listeria monocytogenes* (Auffray et al., 2007). The authors of a recent, comprehensive review of monocyte functions suggest that patrolling monocytes likely have a higher activation threshold than classical monocytes, allowing them to produce sufficient cytokines and chemokines to coordinate endothelial repair, but not enough to initiate a strong inflammatory reaction (Italiani and Boraschi, 2014). However, other reviews suggest that their sensitivity to TLR7/TLR8 ligands may implicate them in autoimmune disease such as rheumatoid arthritis (Yang et al., 2014).

Nonclassical monocytes also serve a major function in tissue healing and angiogenesis, and have been found to coordinate myocyte and skeletal muscle healing, as well as wound healing, following the initial inflammatory phase of infection (Arnold et al., 2007; Crane et al., 2014; Nahrendorf et al., 2007). Interestingly, in a mouse model of sterile wound healing it was shown that Lyc6^{hi} monocytes were initially recruited to the wound to mount an inflammatory response, and were then recruited back to the bone marrow, where they differentiated into ly6c^{lo} monocytes to promote tissue healing and angiogenesis (Nahrendorf et al., 2007).

Shifting Proportions of Monocytes during acute and chronic infections

Interestingly, the relative proportions of the CD16^{+} monocyte subpopulations have been found to be increased in a large number of acute and chronic infections. Both non classical and intermediate monocytes have been seen to increase in tuberculosis infection (Castaño et al., 2011), as well as in chronic hepatitis B and C, tuberculosis, non HAART suppressed HIV-1 patients, and septic patients [reviewed in (Wong et al., 2012a)]. Alternatively, only intermediate monocytes were found to be elevated in dengue fever, and autoimmune disease such as Crohn's disease, Eales' disease and rheumatoid arthritis [reviewed in (Wong et al., 2012a)]. Nonclassical monocytes alone were found to be elevated in statin-treated patients with coronary artery disease (Tallone et al., 2011). It is currently unclear what the function of these expanded subsets are in disease, although based on mouse studies and gene expression at steady state, one might guess that intermediates play a role in T cell priming and antigen presentation to mount the immune response, while nonclassical monocytes may serve to limit immunopathology with wound healing effects. However, gene expression profiling and function

studies of the individual monocyte subsets during disease would greatly augment our understanding.

Monocyte subsets in malaria

Given the implications that monocytes may have an important role as tolerizers of T cells, promoting natural immunity, via PD-1 signaling or other signaling pathways, it is likely monocytes play a role in the development of clinical immunity to malaria infection; however, currently, not much is known about monocyte function in human malaria disease. During *P. vivax* infection, the proportion of monocytes of total blood cells increased in infected patients, although absolute numbers of leukocytes decreased. These monocytes expressed lower levels of HLA-DR and adhesion molecules CD31 and CCR7, suggesting their ability to activate the adaptive immune system may have been impaired. In contrast to this, gene analysis showed that classical monocytes of infected individuals expressed higher levels of *IFNG* and other pro-inflammatory genes, while intermediate monocytes showed the highest levels of *IL6* and *IL10,* along with co-stimulatory molecule CD80. Nonclassical monocytes showed the highest transcript level of *TNF*, although in culture, intermediate monocytes were shown to be superior producers of TNF in response to infected red blood cells. Intermediate monocytes also displayed elevated phagocytosis of infected reticulocytes, elevated ROS production and increased levels of adhesion molecules, suggesting that during acute infection these monocytes may be important for parasite control (Antonelli et al., 2014a). Another study reported that the proportion of classical monocytes is lower in Africans living in malaria endemic areas, as opposed to Caucasian American adults, suggesting that shifting monocyte subsets may be implicated in immunity to malaria (Appleby et al., 2013).

We understand even less about what role monocytes might play during chronic malaria infection. As seen above, during acute infection, monocytes are capable of producing inflammatory and pyrogenic molecules in response to *Plasmodium* parasites. However, asymptomatic infection is characterized by the absence of clinical symptoms, including the hallmark fever. It is likely that changes are occurring in these monocytes which decrease either their sensing of *Plasmodium* molecules or their production of pyrogens. These monocytes may be becoming tolerized over time, changing future innate responses to the parasites. This concept is similar to trained innate immunity, in which innate immune responses can be enhanced over multiple exposures to pathogens (Netea et al., 2011). Epigenetic mechanisms can then lead to changes in PAMP receptor levels and differences in monocyte populations (Netea et al., 2011). In the opposite direction, endotoxin shock is a well described phenomena in which organisms will not response to a second endotoxin challenge after mounting a strong inflammatory response to a first challenge (Biswas and Lopez-Collazo, 2009). Like trained innate immunity, TLR induced chromatin modification has been implicated in endotoxin shock (Foster et al., 2007).

Overall, our current understanding of the roles of specific monocyte subsets in the context of disease, especially *Plasmodium* infection, is incomplete at best. Given the suggestion that

monocytes may have an important role in tolerizing T cells, further understanding of monocyte changes during this development of clinical immunity will be integral to our picture of natural immune development. Additionally, evidence suggests that monocytes may become tolerized themselves. Understanding this phenomenon is the first step towards promoting or blocking this tolerance in future vaccine development. Careful characterization of monocyte frequencies in malaria endemic areas, specifically focusing on correlations between frequencies and clinical parameters of exposure and immunity is the obvious next step in understanding the changes that take place during repeat infection and immunity. Additionally, gene expression analysis of monocyte subsets of individuals living in these endemic areas will greatly contribute to our understanding of the roles of monocytes in disease, and their contribution towards the development of clinical immunity in malaria infection.

Part II: The Role of Monocytes in the Development of Clinical Immunity to Malaria

INTRODUCTION

Malaria, caused by parasites of the genus *Plasmodium,* is a leading cause of infectious morbidity and mortality worldwide (WHO Global Malaria Programme, 2012). Malaria disproportionately affects children, with 78% of malarial deaths occurring in children under 5 years old, making it the fourth leading cause of childhood death worldwide (WHO Global Malaria Programme, 2012). Children are also at higher risk of severe complications from malaria, which can result in lasting neurological dysfunction (Idro et al., 2005; MacKintosh et al., 2004). The high incidence of malaria in endemic regions, coupled with this high morbidity and mortality, exacts a tremendous economic toll (Sachs and Malaney, 2002). An effective, long lasting vaccine would be of great value in reducing disease burden. However, previous vaccine attempts have only achieved moderate success, in part due to our limited understanding of protective immunity as it pertains to malaria.

Naturally acquired immunity is critical for modulating morbidity and mortality from malaria in endemic areas, where individuals may be exposed to hundreds of infectious mosquito bites per year. With repeated exposure to *Plasmodium,* children eventually develop protection against severe disease - a semi-immune state known as clinical immunity to malaria. Clinically immune individuals are susceptible to *Plasmodium* infection, but remain minimally symptomatic or asymptomatic during infection, with much lower risk of severe complications. In contrast, the goal of current vaccine candidates is to generate sterilizing immunity, which is protection from infection with *Plasmodium* parasites. Interestingly, It has recently become appreciated that clinical immunity represents the vast majority, if not all, of naturally acquired immunity (Tran et al., 2013b). As part of a large cohort study of children in Uganda, in a highly endemic region in Uganda, it was demonstrated that while malaria incidence decreases with age, the prevalence of asymptomatic parasitemia steadily increases with age (Figure 1). While clinical immunity is extremely important to the natural development of malaria immunity, little is currently known about the immunological changes that occur during acquisition of immunity, which allow the host to remain asymptomatic while infected.

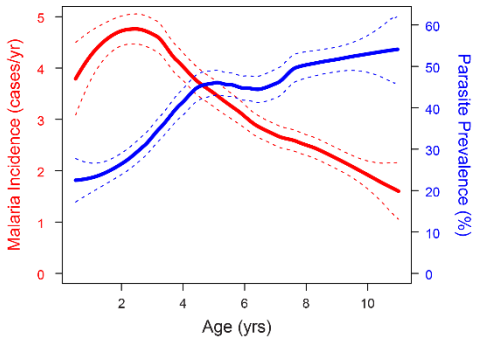

Figure 1. Malaria incidence and parasite prevalence in Tororo, Uganda. *Incidence of symptomatic malaria shown in red (left axis), parasite prevalence shown in blue (right axis). Figure generated by B. Greenhouse*

Clinical immunity to malaria is likely a careful balance of immune responses to control parasitemia while avoiding excess inflammation, which itself likely drives the most severe complications of malarial disease such as cerebral malaria and severe anemia leading to anoxic damage (Hansen, 2012; Hansen and Schofield, 2010; Riley et al.). One mechanism of clinical immunity to *Plasmodium* infection is likely the development of tolerance to certain *Plasmodium* antigens. Tolerance is defined as the lack of response or diminished response of the immune system to normally immunogenic molecules, typically after repeated or chronic exposure. Tolerance was initially studied as a process to inhibit in human disease processes known for their immune evasion, such as cancer, chronic hepatitis infection and HIV (Day et al., 2006; Mapara and Sykes, 2004; Racanelli and Rehermann, 2003; Stoop et al., 2005). However, plant biologists have long described tolerance as a protective process in plants, limiting damage from herbivore and parasitic insult (Schafer, 1971; Strauss and Agrawal, 1999). In the past decade, human immune tolerance has been recognized as a potentially immunoprotective state, serving to limit host pathology from inflammation during various pathogenic infections, including malaria (Boots, 2008; Gigley et al., 2012; Johnston et al., 2014; Råberg et al., 2007; Schneider and Ayres, 2008b; Wakelin, 1996).

It is likely that this development of tolerance during *Plasmodium* infection is multifactorial, encompassing changes in both cell type frequencies and functions. Recent studies have described mechanisms which likely contribute to the production of tolerance in the adaptive immune compartment, including a loss of *Plasmodium* specific gamma delta T cells during chronic *Plasmodium* infection (Jagannathan et al., 2014b), the shift to co-production of both pro-inflammatory IFNγ and anti-inflammatory IL-10, rather than pyrogenic TNFα by *Plasmodium* specific CD4+ T cells (Jagannathan et al., 2014a) and the increased activity of T regulatory cells (Hansen and Schofield, 2010). Additionally, recent work suggests that specific mechanisms of tolerance that temper lymphocyte function, such as exhaustion, also promote the establishment of clinical immunity (Butler et al., 2012; Couper et al., 2008; Horne-Debets et al., 2013b). Exhaustion is characterized by diminished T cell cytokine production, and potentially apoptosis, in response to chronic antigen stimulation of the T cell receptor (Rodrigues et al., 2014). However, the molecular drivers of exhaustion have just begun to be characterized in human disease.

Recent data from our group in a chronic mouse model of asymptomatic *Plasmodium chabaudi* infection shows that the nonclassical monocyte population expands and upregulates molecules that promote T cell exhaustion (Fontana, et al. manuscript in preparation). These findings suggest that during *Plasmodium* infection, these monocytes might suppress T cell functions such as cytokine production to protect against severe inflammatory pathologies, at the cost of preventing the development of acquired immunity. If nonclassical monocytes are also suppressive in human infection, inhibiting these suppressive functions might be a way to bolster adaptive immune responses and sterilizing immunity.

Additionally, a hallmark symptom of malaria is fever, which is driven by the production of pyrogenic cytokines released by monocytes, in response to broadly conserved ligands on malaria parasites. During symptomatic infection these molecules stimulate signaling through toll-like receptors and other pathways to elicit cytokines. It has been observed that classical monocytes produce the pyrogen, TNF, in response to stimulation by *Plasmodium* parasites *in vitro* (Stanisic et al., 2014). During clinical immunity, characterized by asymptomatic infection, the absence of fever suggests that these monocyte-encoded sensing pathways are inhibited. The down-regulation of TLRs has been proposed as a contributor to this tolerance (Boutlis et al., 2006b); however, this and other mechanisms of innate immune tolerance have never been studied in the context of human asymptomatic *Plasmodium* infection. The mechanisms by which this tolerance occurs remain unclear. Additionally, this dampened recognition of parasites may be a key factor in the slow development of natural immunity and the ineffectiveness of many vaccines.

Based on these observations, we hypothesized that broad changes in monocyte population frequencies and transcription would occur between those children who have developed clinical immunity and are maintaining an asymptomatic infection, and those who are symptomatically infected. However, thus far, very little work has been done to elucidate the role of monocyte subsets in the natural development of clinical immunity to malaria. In this study we seek to quantify changes in monocyte populations and identify immune response modifying and tolerance promoting changes in monocyte subsets during the development of clinical immunity to malaria. We utilize two established, well characterized cohorts of children living in Nagongera in Tororo County and Walukuba in Jinja County, Uganda – regions which greatly differ in the exposure to *Plasmodium* parasites and incidence of malarial disease.

METHODS

Cohort

Samples were collected from children in the Program for Resistance, Immunology, Surveillance, and Modeling of Malaria in Uganda (PRISM), an ongoing longitudinal observational cohort of 200 households across two study sites, Nagongera sub-county in the Tororo District, and Walukuba sub-county in the Jinja District. In Tororo malaria transmission is perennial, and with an annual entomological inoculation rate (aEIR) of 310 infectious bites per person, while in Walukuba malaria transmission is much lower, likely due to recent urbanization and pesticide usage, with an aEIR of only 2.8 bites. *Plasmodium falciparum* represents 94% of all *Plasmodium sp.* transmission in the area, with *P. malariae* constituting the remaining 6% (Clark et al., 2010; Pullan et al., 2010).

All children aged 0.5-10 years from 100 randomly sampled homes in each district were enrolled in August 2011. Cohorts are dynamic; children leave the study at age 11 and infants born into the same households enter the study at 6 months of age.

Parasite Monitoring and blood collection

Subjects are seen in the study for all medical care, allowing us to accurately determine the incidence of symptomatic malaria. Blood smears are obtained every 3 months to assess asymptomatic parasitemia. Blood samples (15mL) are also drawn every 3 months and during some symptomatic malaria episodes. Subjects have consented to the use of their blood in immunology studies.

Following blood draws, peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll gradients, counted, and immediately cryopreserved and stored long-term in liquid nitrogen, until use in flow cytometry or RNA-seq.

Flow Cytometry

Total PBMCs were thawed in the presence of DNase and immediately stained in FACS buffer with antibodies specific for the following targets: CD7 (clone 4H9), HLA-DR (clone L243), CD16 (clone CB16), CD14 (clone 61D3), CD19 (clone HIB19) from eBioscience, CD177 (clone MEM-166) from Biolegend and CD91 (clone A2MR-a2) from eBioscience. For flow cytometry, classical monocytes were identified as $CD177^{-}CD7^{-}CD19^{-}HLA\text{-}DR^{+}CD91^{+}CD14^{hi}CD16^{-}$; nonclassical monocytes were identified as $CD177^{-}CD7^{-}CD19^{-}HLA\text{-}DR^{+}$ $CD91^{+}CD14^{lo}CD16^{+}$, and intermediates were identified as $CD177^{-}CD7^{-}CD19^{-}HLA\text{-}DR^{+}$ $CD91^{+}CD14^{hi}CD16^{+}$. Cells were run on a LSRII (BD) or a FACSAria (BD). All analysis was done in Flowjo (Treeview).

Quantification of parasite load

Parasite load was quantified by PCR at the time of the blood draw.

Sorting for RNA isolation

For RNA isolation, monocyte populations were stained as described above. All monocyte subsets were isolated to high purity using two consecutive rounds of sorting on a FACSAria, using an event rate no higher than 5,000 events/second and sorting directly into an RNA preservative buffer on the second sort. Sorted cells were immediately snap frozen on dry ice and stored in a -80°C freezer until the time of RNA isolation.

RNA isolation

Cryopreserved sorted cells were thawed, and RNA was isolated using an RNAqueous Micro kit (Life Technologies) following manufacturer recommendations with the following modifications: lysis buffer/cell aliquots were initially mixed with 180ul of 200 proof RNase-free ethanol; the flow-through was reloaded onto the column to capture additional material with a second binding step; and the purified RNA was eluted twice with 6 µl 55^{o}C RNase-free water following a 2-min incubation. Isolated total RNA was vacuum concentrated to 2 µL and converted to pre-amplified cDNA libraries using template-switching reverse transcription (Matz et al., 1999; Petalidis et al., 2003) using the previously described Smart-seq2 protocol (Picelli et al., 2014), with the following modifications. Following PCR modification, PCR purification was not performed and DNA was quantified using Quant-iT Picogreen DNA quantification assay (Thermo-Fisher) and read on the Synergy 2 plate reader (Biotek). cDNA was normalized to

0.15ng/ul and 2ul were used for Nextera library preparation. Fragmentation was performed enzymatically using a Nextera XT DNA kit (Illumina, San Diego, CA), and barcoded samples were multiplexed, pooled, and purified and size selected to 200-400bp fragments using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA), using a ratio of 0.85X-1.05X for a double-sided cleanup. Libraries were quality-controlled for size distribution and yield using a Bioanalyzer 2100 with high sensitivity dsDNA assay (Agilent Technologies, Santa Clara, CA), and sequenced as 51 bp single-end reads on one lane of a HiSeq 4000 (Illumina) running in high-output mode at the UCSF Center for Advanced Technology (San Francisco, CA). Reads were demultiplexed with CASAVA (Illumina) and quality assessed using FastQC (Babraham Bioinformatics).

RNA-seq mapping and differential expression analysis

Reads were aligned to release GRCh37 of the human genome (downloaded from ENSEMBL). Reads were aligned using STAR version 2.4.2a (Dobin et al., 2013). All alignment programs were run using default parameters. Gene-level expression was modeled using the gene counter implemented in STAR version 2.4.2a (--quantMode GeneCounts) (Dobin et al., 2013). All statistical comparisons were pairwise comparisons, performed on counts data using DESeq2 version 1.2.10 (Love et al., 2014). The DESeq2 libraries were used within 64-bit R 3.0.2. Samples which were two standard deviations below the mean percentage or mean log number of reads mapped to the genome were excluded.

Principal component analysis

Principal components were calculated from complete outlier-filtered count data. Principal components were calculated on rlog transformed (Love et al., 2014), scaled, un-centered data using the prComp function in R.

Gene ontology analysis

Functional classification of differentially regulated genes based on Gene Ontology Biological Processes terms was performed using PANTHER Gene List Classification tool (Mi et al., 2013a, 2013b).

PubMed Scrape

Identification of immunoregulatory genes by a PubMed search was done using in-house code, which ran a PubMed search on all identified differentially expressed gene symbols and the following: “immunoregulation OR immunoregulatory OR exhaustion OR tolerance”. Genes which returned 30 papers or more were further investigated and displayed below. Among our 1722 differentially expressed genes, 92 met our criteria.

RESULTS

Quantification of monocyte populations

In order to determine changes in monocyte subsets during the development of natural immunity, we quantified the proportion of monocyte subsets in a total of 107 children from either low-exposure Walukuba or high-exposure Nagongera, who were either uninfected, asymptomatically infected, or symptomatically infected with *Plasmodium* parasites by flow cytometry (Figure 2 and Table 1).

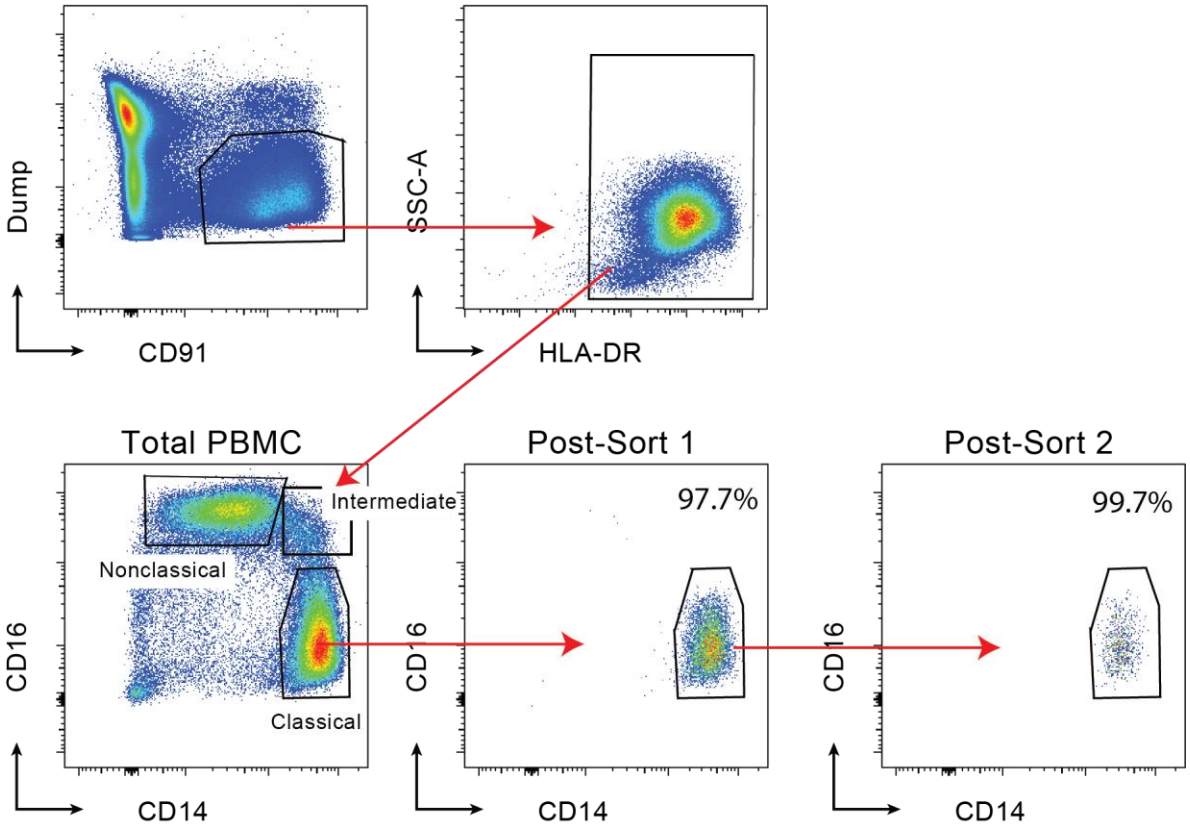

Figure 2. Monocyte isolation by flow cytometry and sequence read characteristics. *Gating strategy for isolation of monocyte subsets, and a representative demonstration of increasing purity of monocyte subsets upon successive rounds of flow cytometric sorting.*

	Walukuba	Nagongera Uninfected	Nagongera Asymptomatic Infection	Nagongera Symptomatic Infection
n subjects	33	42	18	14
age (months)	5.17 (1.5, 10.15)	5.43 (0.64, 10.64)	7.57 (2.11,10.64)	5.77 (1.74, 9.54)
aEIR (site specific)	2.8	310	310	310
incidence malaria (past year)	0.12 (0,1)	3.5 (0,10)	2.22 (0,6)	5.56 (2,10)
incidence asymptomatic parasitemia (past year)	0.06 (0,2)	0.52 (1, 3)	1.67 (0,4)	0.44 (0,1)

Table 1. Cohort characteristics for samples used in flow cytometry to identify percentages of monocyte subsets. *Mean and range shown*

We observed that children from high-endemicity Nagongera had consistently higher proportions of nonclassical monocytes, as well as intermediate monocytes, with a reciprocal drop in the proportion of classical monocytes (Figure 3). Additionally, both asymptomatic and symptomatically infected children from Tororo had higher proportions of nonclassical monocytes than uninfected children from both sites, and reciprocally lower classical monocytes. However, symptomatically infected and asymptomatically infected children did not have significantly different proportions of monocytes (Figure 3).

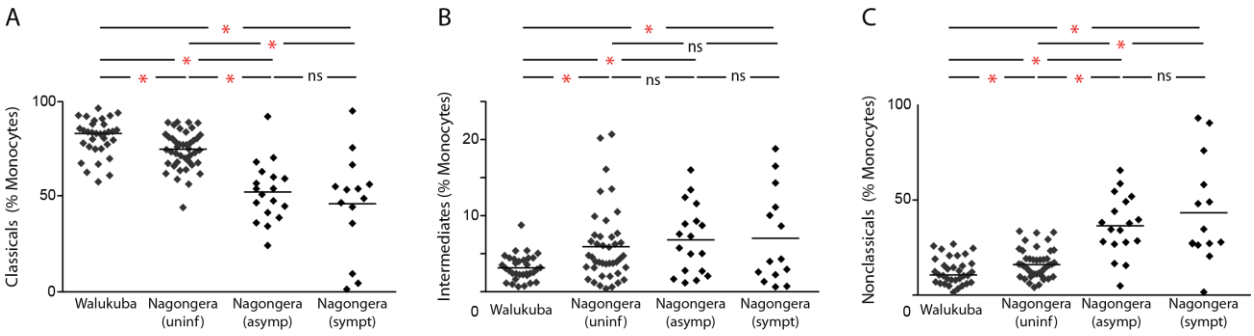

Figure 3. Children from a higher exposure area and Plasmodium infected children have significant changes in monocyte subset frequencies as compared to less exposed children. *Monocyte frequencies were determined by flow cytometry on cryopreserved PBMCs collected from uninfected Walukuba children, uninfected Nagongera or asymptomatically infected Nagongera children. Relative frequencies of classical (A), intermediate (B) and nonclassical (C) monocytes shown. * = p<0.05.*

Quantification of parasite load by infection status It is well documented that parasite levels tend to be lower in asymptomatically infected individuals than uncomplicated symptomatic individuals in high malaria transmission areas (Galatas et al., 2015). In concordance with these findings, we observed that the parasite load during asymptomatic infection was reduced as compared to that during symptomatic infection in Nagongera (Figure 4). Monocytes, and particularly nonclassical monocytes in our mouse model of asymptomatic *Plasmodium* infection (Fontana et al, manuscript in preparation), have been implicated in playing a role in phagocytosis of *Plasmodium* parasites and contributing to host restriction of the parasite via both phagocytosis and antibody-dependent cell inhibition (Antonelli et al., 2014b; Stevenson et al., 1989). Based on our findings that while the proportion of monocyte subsets did not change

during asymptomatic versus symptomatic infection, the parasite density was decreased during asymptomatic infection, we hypothesized that perhaps the functional role of the monocytes changed during asymptomatic infection, to promote restriction and limit inflammation.

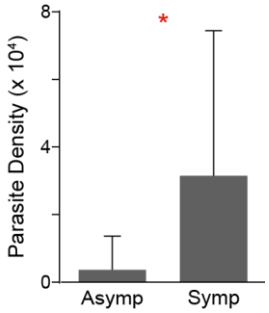

Figure 4. Parasite densities are significantly lower in asymptomatically infected children. *Parasite densities, as determined by PCR, are shown for Plasmodium infected children from Tororo. All available data from the larger cohort study are shown, representing 408 asymptomatic infections and 109 symptomatic infections (*= p<0.05, T-test).*

RNA-seq of monocyte subsets from Nagongera children

In order to investigate functional changes in the monocyte subsets during the development of clinical immunity, we profiled the transcriptomes of highly purified classical and nonclassical monocytes from children in Nagongera who were either uninfected, asymptomatically infected or symptomatically infected, via RNA-seq (Figure 2). We filtered the RNA-seq data by the percentage and number of reads mapped to the human genome. There were no significant differences between groups in the $\log_{10}$ reads mapped or percentage of reads mapped to the human genome (ANOVA, alpha = 0.05; Figure 5). Samples falling 2 standard deviations from the mean by either log reads mapped or percent reads mapped were excluded (Figure 5). Cutoffs were 4.74 and 33.838% respectively.

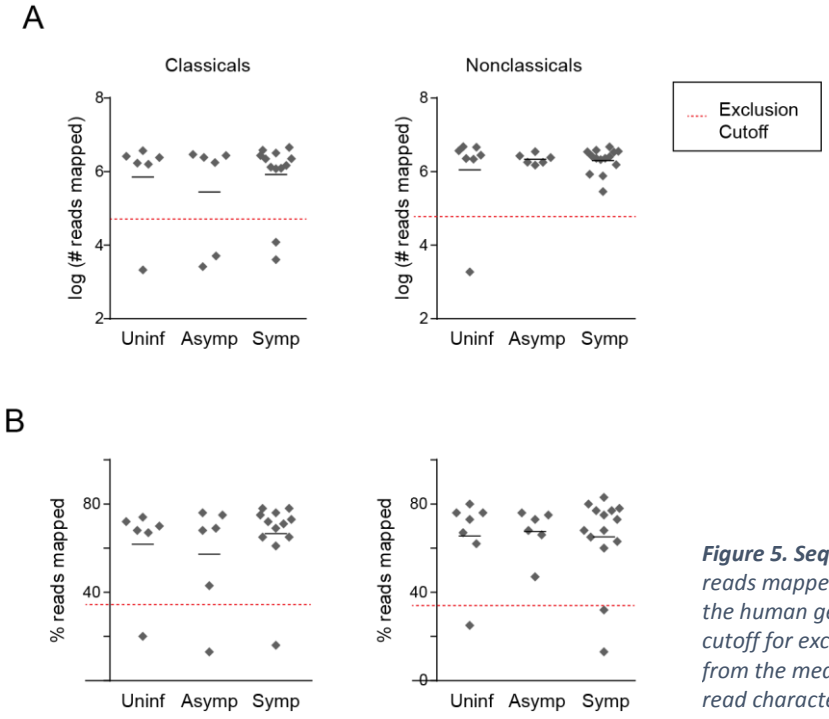

Figure 5. Sequence read characteristics. *Log of number of reads mapped (A) and percentage of reads mapped (B) to the human genome, by sample group. Red line shows the cutoff for exclusion from analysis, 2 standard deviations from the mean. Groups were not significantly different in read characteristics (ANOVA, alpha = 0.05).*

Following filtering, we had 24 nonclassical monocyte transcriptomes and 19 classical monocyte transcriptomes from Nagongera children (Table 2). Because children develop clinical immunity to malaria with cumulative exposure, which is tightly correlated to age, the mean age of the asymptomatic children was higher than both the uninfected and symptomatically infected children. We first wanted to use an unsupervised method to explore whether cell type and cohort would explain the largest variations between gene expression data of the samples. We performed principal component (PC) analysis on rlog transformed (Love et al., 2014), scaled, uncentered data. PC2 clearly delineated the samples based on cell type (with the exception of one outlier – cause unknown) and PC1 and 2 together seemed to cluster samples based on study group (including both cell type and cohort). Samples did not appear to be clustered by age category (Figure 6).

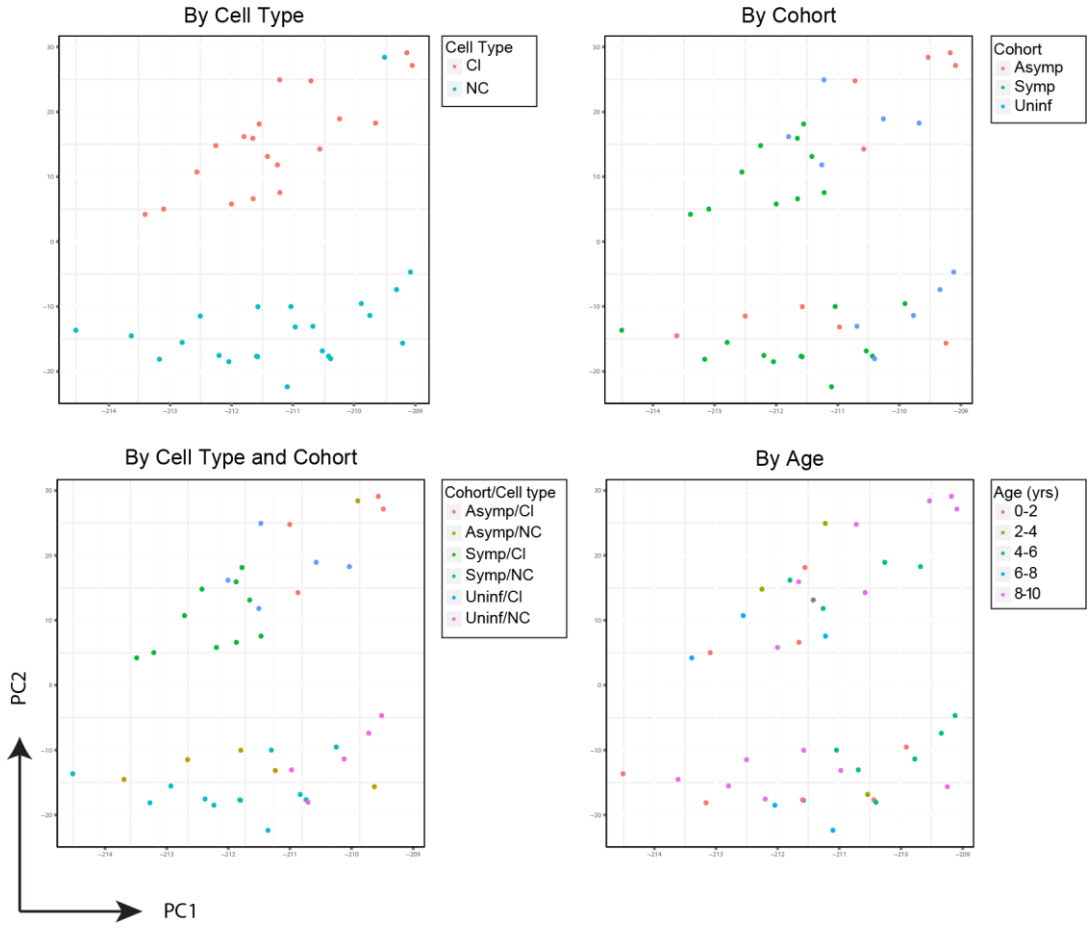

Figure 6. Principal component analysis of analyzed samples*. rlog transformed samples shown plotted against their first two principal components. Colors correspond to cell type, cohort, both cell type and cohort or age. Cl-classical monocyte, NC – nonclassical monocyte, Uninf – uninfected, Asymp – asymptomatic, Symp - symptomatic*

	Uninfected	Symptomatic	Asymptomatic
n subjects	10	14	8
age (years)	4.51 (4.00, 5.27)	5.50 (1.56, 9.37)	9.52 (9.36, 9.72)
incidence malaria (past year)	4 (1,9)	5.86 (1, 10)	2.75 (0,6)
incidence asymptomatic parasitemia (past year)	0.5 (0,2)	0.29 (0,3)	1.5 (0,5)
current parasite density	NA	38345 (8000, 120000)	2600 (160, 12400)

Table 2. Cohort characteristics for samples used in RNA-seq of monocyte subsets. *All samples taken from Nagongera children. Mean and range shown.*

We next sought to identify the genes which were significantly different between monocytes from the uninfected, asymptomatically and symptomatically infected children. To identify significantly differentially expressed genes (DEGs), we performed two-way comparisons of expression data using DESeq2 (Love et al., 2014). We identified 477 and 1206 DEGs between asymptomatically infected and symptomatically infected children in the nonclassical and classical monocyte populations, respectively. When comparing asymptomatic children to uninfected children, we detected 19 DEGs in the nonclassical monocytes and 268 DEGs in the classical monocytes. When comparing symptomatic children to uninfected children we detected 140 and 331 DEGs in the classical and nonclassical monocytes respectively (Table 3 and Figure 7).

Infection Status 1	Infection Status 2	Population	Number of DEGs
Asymptomatic	Symptomatic	Classical	1206
Asymptomatic	Symptomatic	Nonclassical	477
Asymptomatic	Uninfected	Classical	268
Asymptomatic	Uninfected	Nonclassical	19
Symptomatic	Uninfected	Classical	140
Symptomatic	Uninfected	Nonclassical	331

Table 3. Number of differentially expressed genes in each comparison

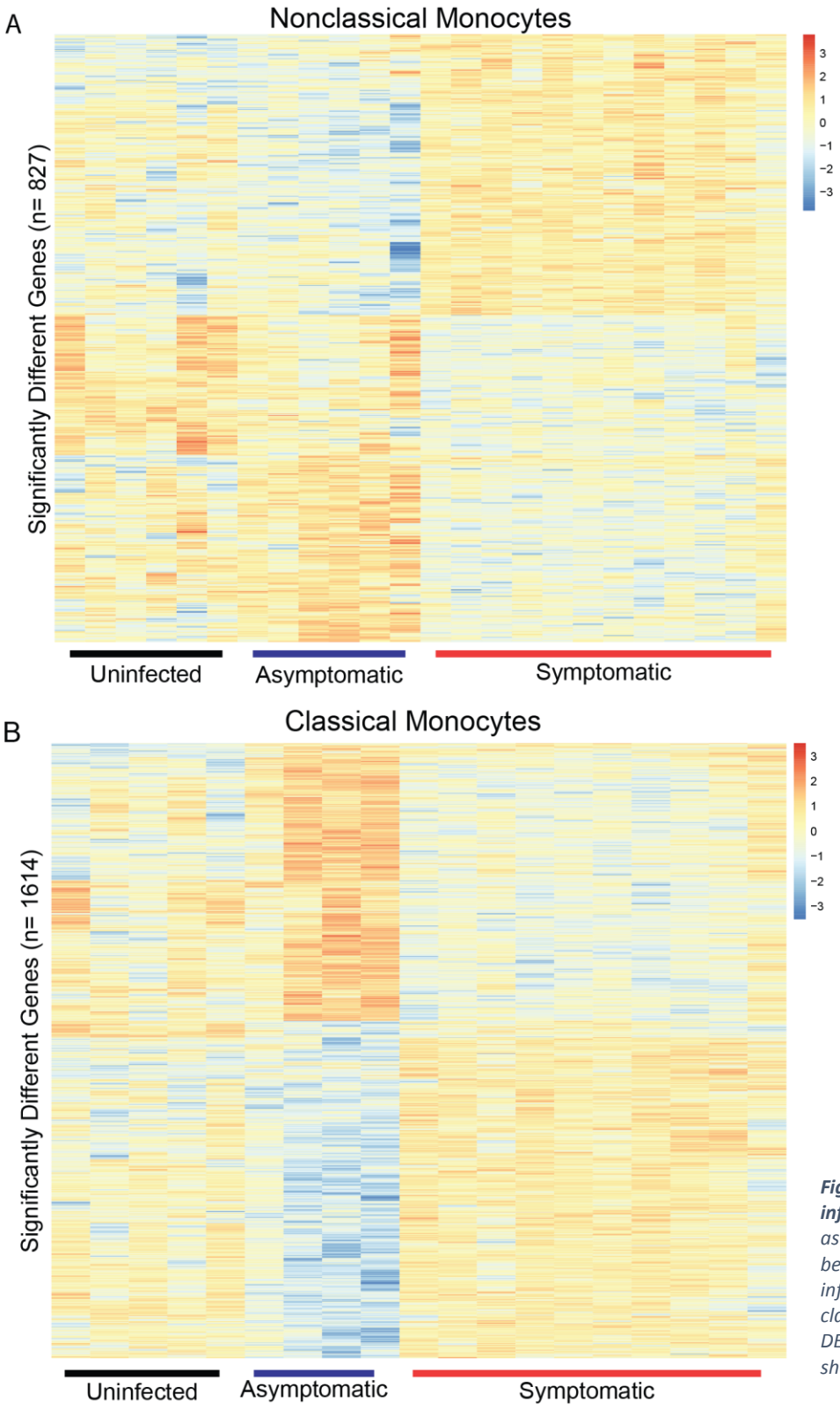

Figure 7. Differentially expressed genes by infection status. *Heat map of genes identified as being significantly differentially expressed between asymptomatic and symptomatic infection in nonclassical monocytes (A) and classical monocytes (B). Genes identified by DESeq2. Row normalized, rlogged values shown.*

Functional analysis of DEGs

In order to identify the biological functions of the differentially identified genes, we grouped the genes by gene ontology biological process label, using PANTHER analysis (Mi et al., 2013a, 2013b). The differentially expressed genes represented a wide swath of biological processes (Figure 8). Interesting to note, the relative proportions of genes representing each biological function did not show appreciable differences between the nonclassical and classical monocytes. Of greatest interest to us were those genes involved in immune system processes, which represented 5.82% and 4.67% of the genes differentially expressed in the nonclassical and classical monocytes, respectively (Figure 8).

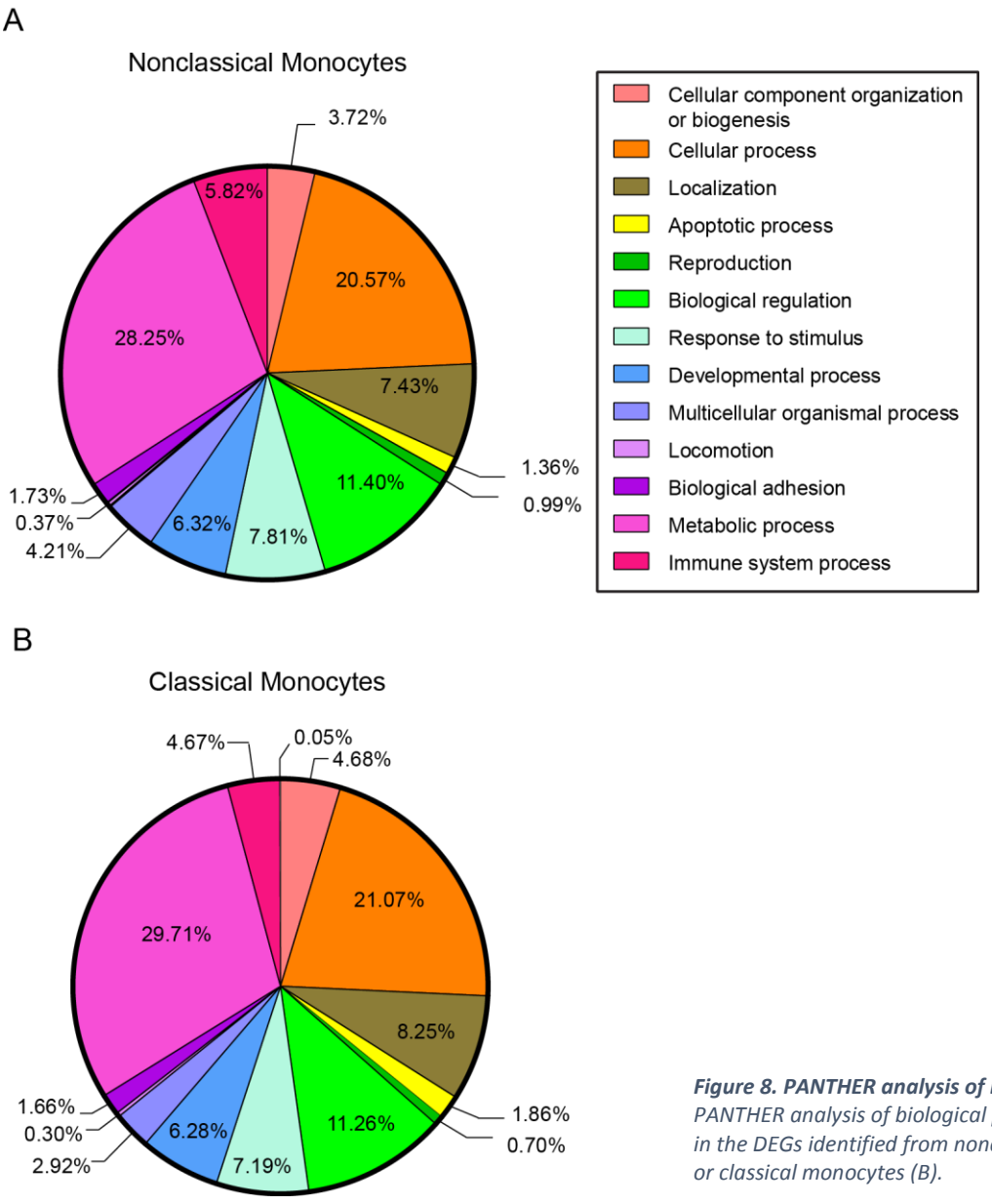

Figure 8. PANTHER analysis of DEG functions. *PANTHER analysis of biological processes represented in the DEGs identified from nonclassical monocytes (A) or classical monocytes (B).*

To further narrow these genes to those which might have immunoregulatory functions, we then used in-house generated scripts to perform a PubMed search of all identified DEGs in conjunction with terms related to immunoregulation and tolerance. 106 DEGs returned greater or equal to 30 papers on PubMed when searching for these immunoregulation-related terms (Table 4 and Figure 9).

Infection Status 1	Infection Status 2	Population	Number of PubMed Hits
Asymptomatic	Symptomatic	Classical	63
Asymptomatic	Symptomatic	Nonclassical	29
Asymptomatic	Uninfected	Classical	17
Asymptomatic	Uninfected	Nonclassical	2
Symptomatic	Uninfected	Classical	10
Symptomatic	Uninfected	Nonclassical	29

Table 4. PubMed Search Results. *Number of differentially genes in each comparison that returned greater than 30 hits in a PubMed search for the gene in conjunction with terms related to immunoregulation and tolerance*

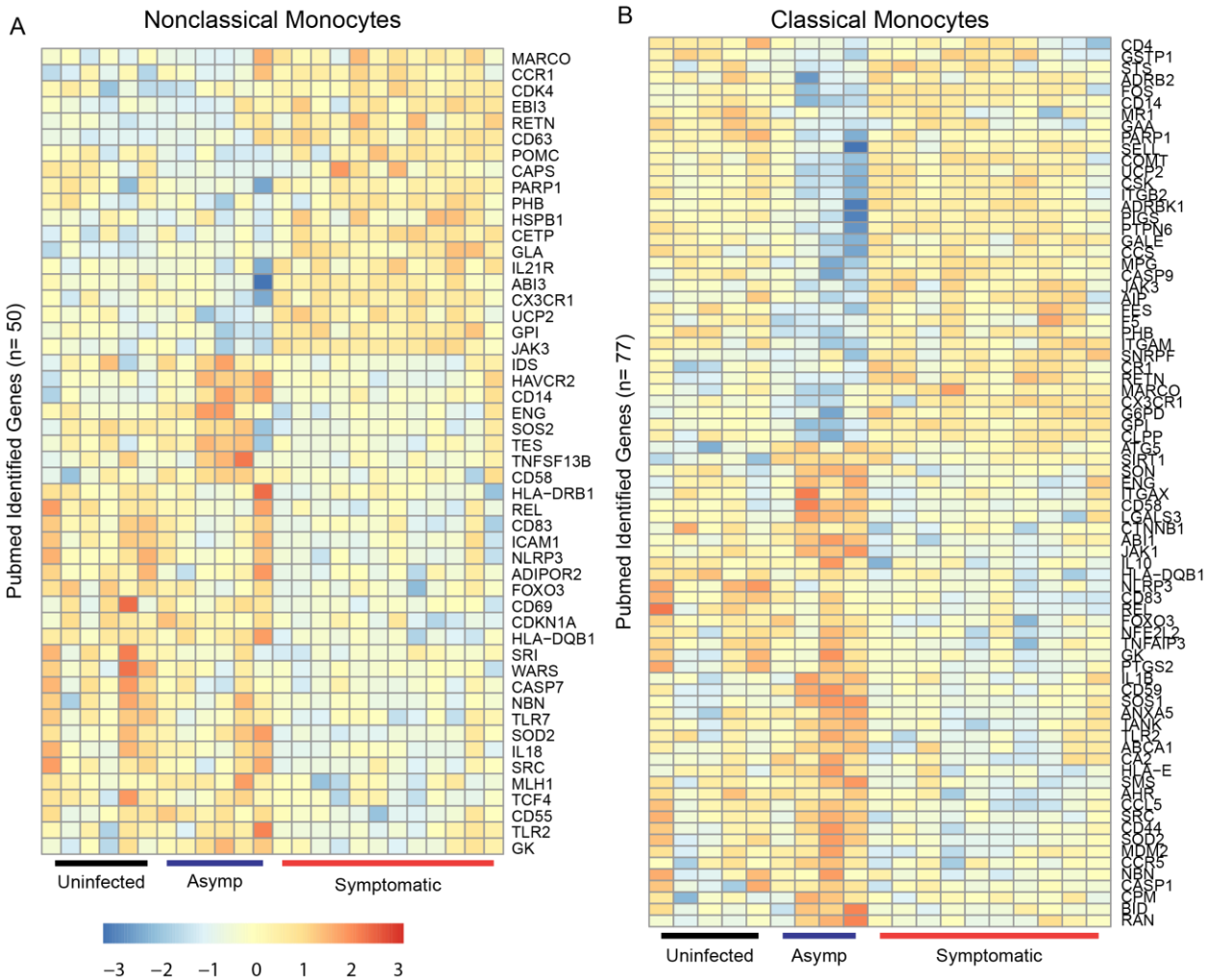

Figure 9. Immunoregulatory genes identified by PubMed. *Heat maps of the subset of differentially expressed genes that returned 30 or more search results in a PubMed search of terms related to immunoregulation and tolerance. Genes differentially expressed in nonclassical monocytes (A) and classical monocytes (B).*

Expression Levels of Immune Modifying Genes

Based on further literature search of the genes identified in the PubMed search, we decided to look more closely at the expression patterns of several categories of genes that may be involved in tolerization of monocytes, the production an anti-inflammatory cytokine environment, and the expression of tolerance inducing molecules.

HAVCR2 and SRC

We first examined the expression patterns of *HAVCR2* which encodes the protein TIM3 and has shown to both negatively regulate pro-inflammatory cytokine production and promote phagocytosis in myeloid cells (Han et al., 2013). Additionally, we examined *SRC*, a protein essential for TIM3 signaling (Maurya et al., 2014). We observed that *HAVCR2* is upregulated in nonclassical monocytes from asymptomatic children, as compared to either symptomatic or uninfected children. A similar pattern of expression is noted in classical monocytes, although these comparisons were not significant. *SRC* was also upregulated on classical monocytes during asymptomatic infection, whereas it was down-regulated in nonclassical monocytes during symptomatic infection (Figure 10).

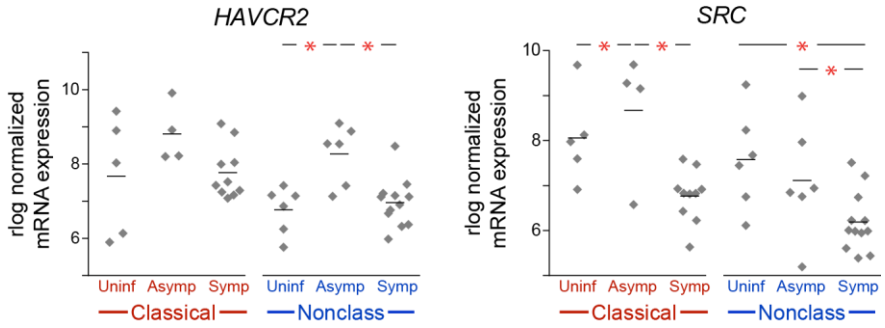

Figure 10. HAVCR2 and SRC are increased during asymptomatic infection. *Rlog normalized count data shown for each sample. (* = p<0.05, DESeq2 adjusted p-value)*

NF-kB signaling

We then looked at expression patterns of genes associated with nuclear factor-kappa B (NF-kB) signaling. NFkB signaling, along with subsequent downstream inflammatory cytokine production, is well established as being critical to initial defenses against *Plasmodium* infection; however, it is unclear if the maintainace of these responses contributes to or protects from severe malaria complications (Punsawad et al., 2012a; Tripathi et al., 2009; Zhu et al., 2005). Surprisingly, we saw a decrease in several NF-kB family transcription factors during symptomatic infection, including *NFKB1*, *NFKB2* and *REL*, in both nonclassical and classical monocytes (Figure 11A). However, we also saw several inhibitors of NF-kB signaling decrease during symptomatic infection – namely, *NFKBIA* (which encodes IkBa), *TNFAIP2* and *TNFAIP3* (which encodes A20). *NFKB1A* mRNA was significantly decreased in both classical and nonclassical monocytes during symptomatic infection, whereas *TNFAIP2* and *TNFAIP3* were only significantly decreased in symptomatic children in nonclassical and classical monocytes

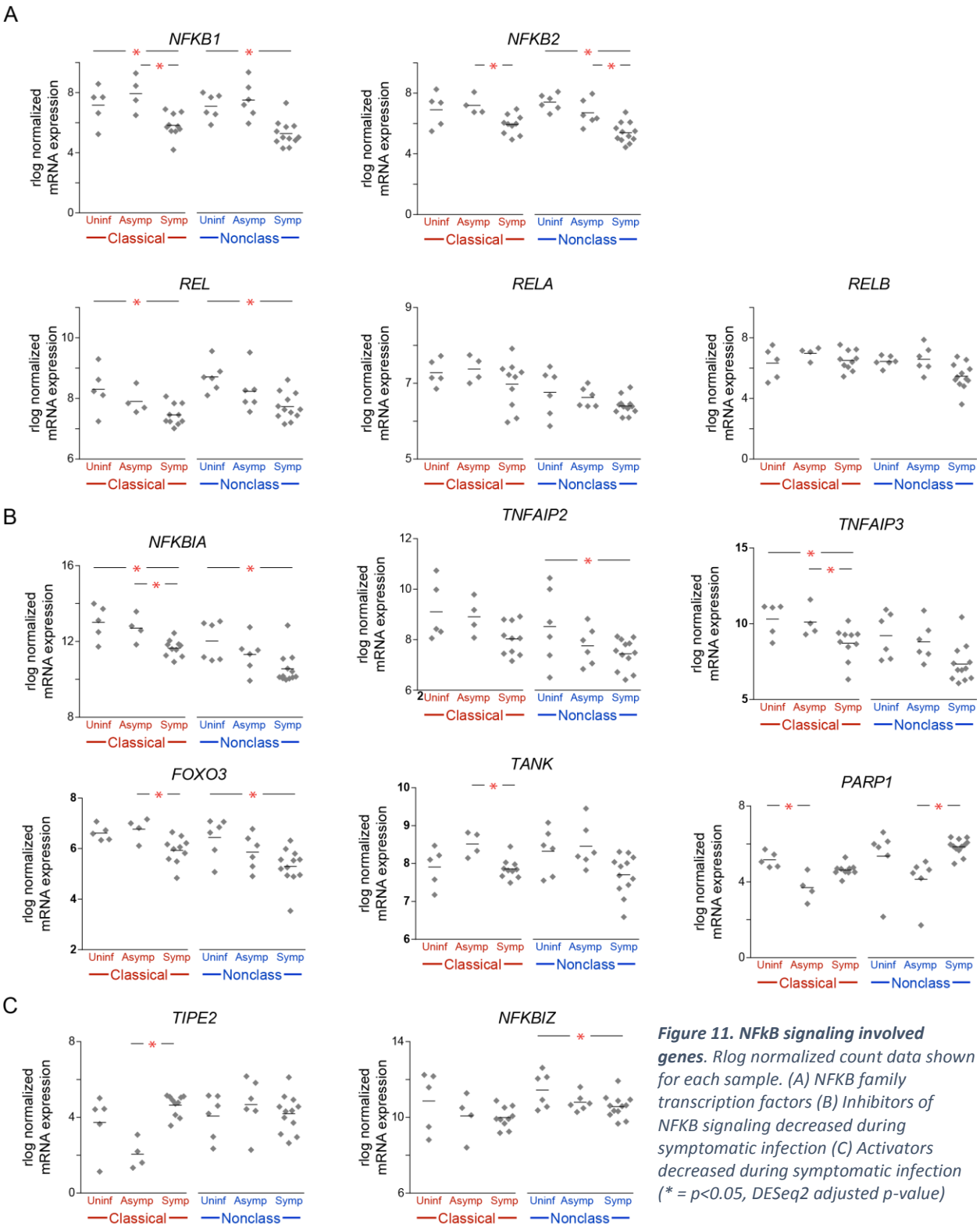

Figure 11. NFkB signaling involved genes*. Rlog normalized count data shown for each sample. (A) NFKB family transcription factors (B) Inhibitors of NFKB signaling decreased during symptomatic infection (C) Activators decreased during symptomatic infection (* = p<0.05, DESeq2 adjusted p-value)*

respectively (Figure 11B). *FOXO3*, which encodes a transcription factor that has been implicated in both reducing NF-kB activation and promoting tolerogenic programs in myeloid cells, was also decreased in monocytes from symptomatic children (Thompson et al., 2015; Watkins and

Hurwitz, 2012). In asymptomatic children, *PARP1*, which encodes a co-activator of NF-kB signaling (Ba and Garg, 2011), was down-regulated in both nonclassical and classical monocytes, whereas *TANK*, which encodes a known inhibitor of NF-kB signaling, was increased in classical monocytes from asymptomatic children (Wang et al., 2015) (Figure 11B). Converse to these patterns, *TIPE2*, which encodes another negative regulator of NF-kB signaling (Li et al., 2016), was decreased in classical monocytes from asymptomatic children, while *NFKBIZ*, which encodes an activator of a subset of secondary genes downstream of NF-kB, including IL-6 and CCL2, was down-regulated in symptomatic children (Hildebrand et al., 2013; Johansen et al., 2015) (Figure 11C).

TGF-β responsiveness

Tumor growth factor-beta (TGF-β), is a well-established immunoregulatory cytokine, the levels of which are thought to be correlated with less severe childhood malaria (Perkins et al., 2000), potentially by driving the production of immunoregulatory regulatory T cells (Clemente et al., 2011). In our data we identified several immunoregulatory genes known to be involved in the signaling of TGF-β or up-regulated in response to TGF-β signaling that were differentially

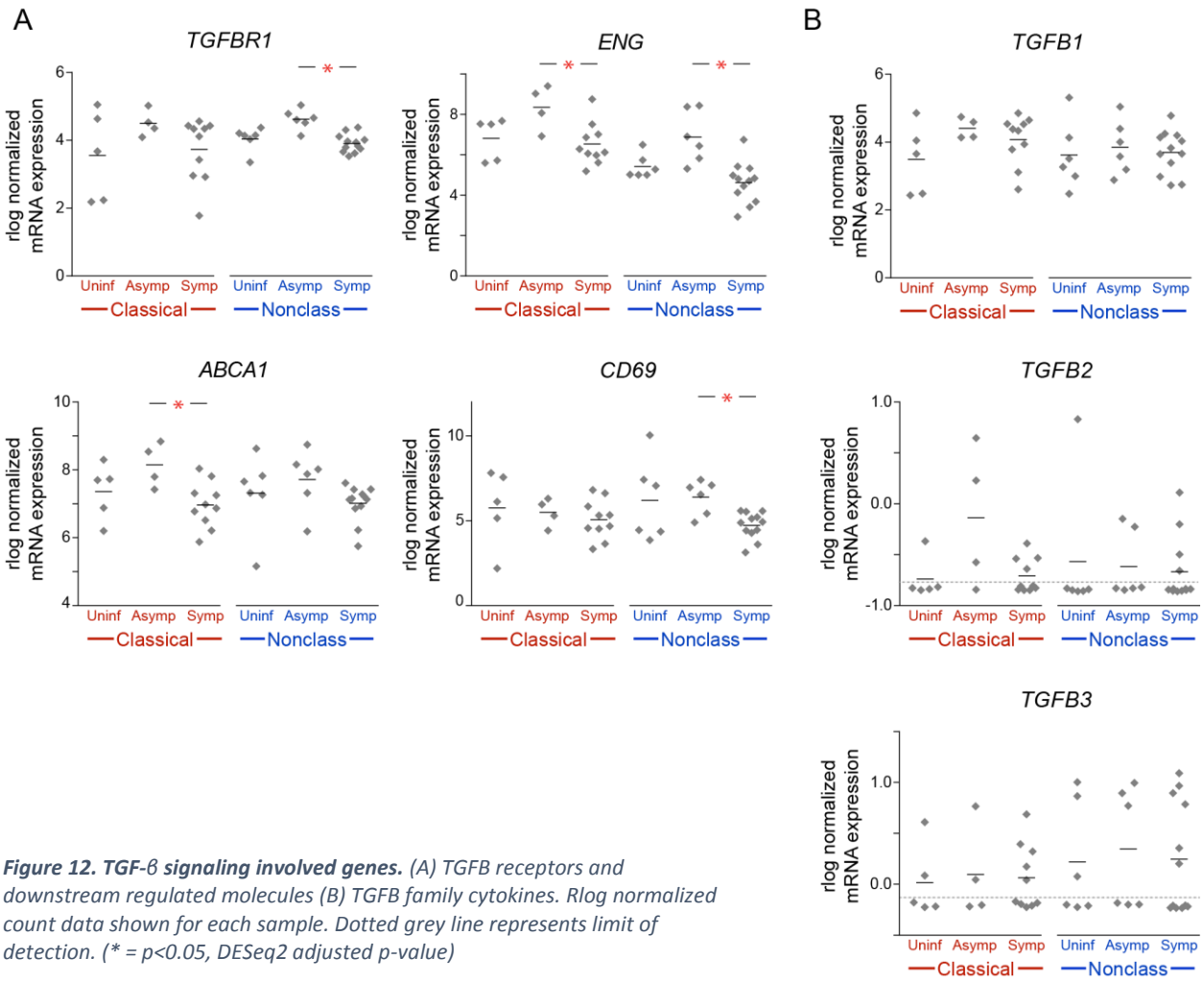

Figure 12. TGF-β signaling involved genes. *(A) TGFB receptors and downstream regulated molecules (B) TGFB family cytokines. Rlog normalized count data shown for each sample. Dotted grey line represents limit of detection. (* = p<0.05, DESeq2 adjusted p-value)*

expressed in the different infection states. Genes encoding two proteins known to be involved the TGF-β receptor complex were upregulated in asymptomatic children -- *TGFBR1*, a well characterized receptor of TGF-β (Massagué, 1998) and *ENG* (endoglin), a known auxiliary receptor for TFG-β superfamily molecules that has been shown to be essential for the resolution of inflammation and the function of myeloid cells (Peter et al., 2014) (Figure 12A). Additionally, several immunoregulatory genes downstream of TGF-β signaling were up-regulated in asymptomatic children. These include *ABCA1*, which was upregulated on classical monocytes from asymptomatic children and has been shown to encode as an anti-inflammatory receptor (Yin et al.) and *CD69*, which was increased in nonclassical monocytes from asymptomatic children (Figure 12A), and has been shown to be implicated in immunomodulation in other cell types (Sancho et al., 2005)**.** While TGF-β responsiveness appears to be modulated in asymptomatic infection, we did not detect differences in TGF-β RNA production during the different infection states (Figure 12B).

Production of cytokines

We observed several differentially regulated cytokines and cytokine receptors during asymptomatic and symptomatic infection. Expression of the gene encoding the anti-inflammatory cytokine IL-10 was increased in classical monocytes from asymptomatic children, while both monocyte subsets decrease *IL10RA* expression during symptomatic infection (Figure 13A). Additionally, expression of IL-4 receptor alpha (*IL4RA*), which is a receptor for anti-inflammatory IL-4, was increased on nonclassical monocytes from asymptomatic children (Figure 13C). Surprisingly, expression of mRNA encoding the pro-inflammatory cytokine, IL-1B, its receptor, IL-1R1, and IL-6R were all increased in classical monocytes from asymptomatic children, relative to symptomatic children (and uninfected children in the case of *IL1R1*), while expression of *IL18*, typically thought of as a pro-inflammatory, IFNγ-inducing cytokine gene, was decreased in symptomatic children (Figure 13B). Levels of *TNFA*, *IL6*, *IL8*, and *IL12B* mRNA were not found to be differentially expressed in monocytes in the various infection states (Figure 13). We were unable to detect *IL4* or *IL12A* mRNA (data not shown).

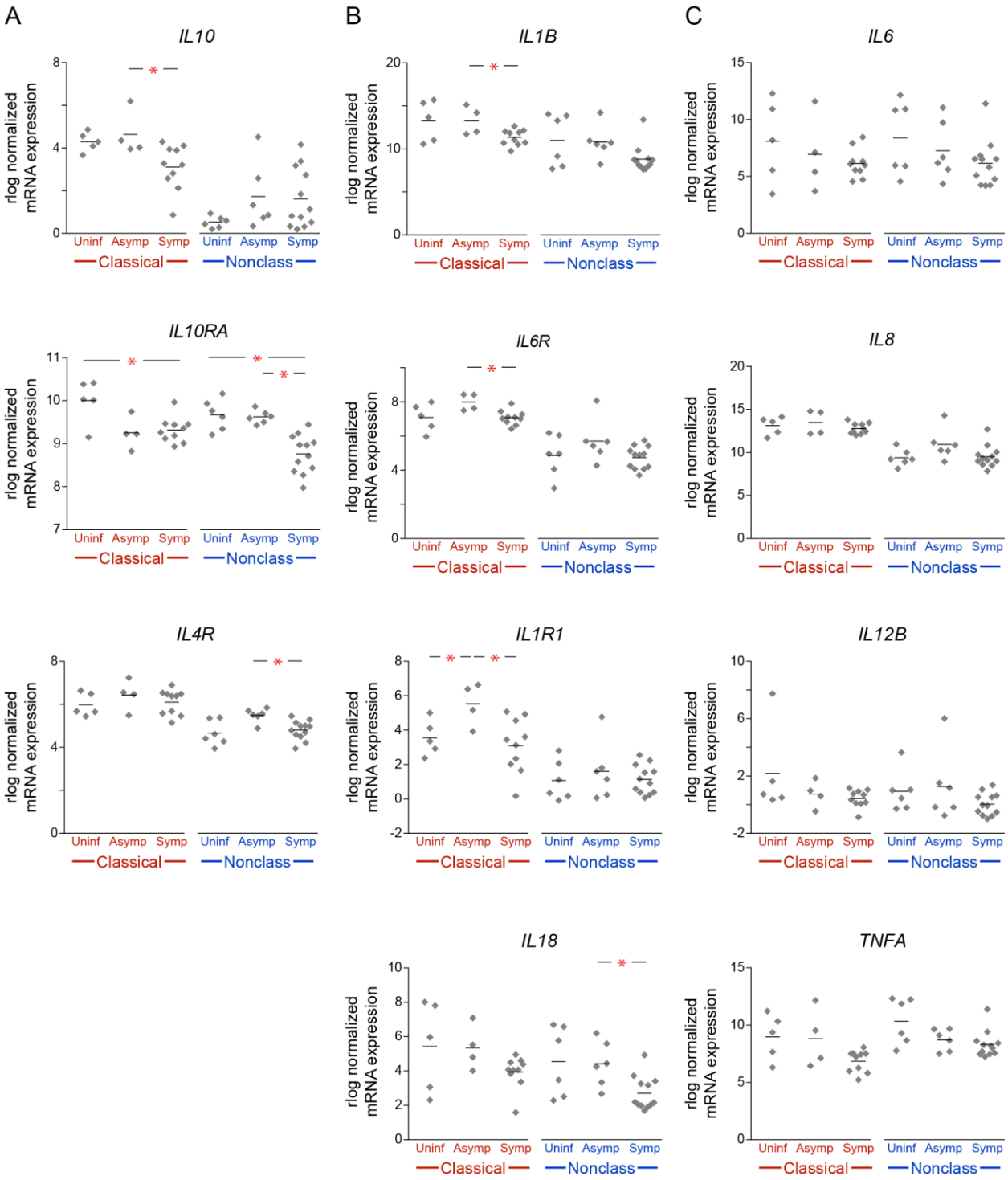

Figure 13. Cytokine signaling related genes. *(A) Significant anti-inflammatory cytokines and cytokine receptors (B) Significant inflammatory cytokines and cytokine receptors (C) non-significant expressed cytokines. Rlog normalized count data shown for each sample. (* = p<0.05, DESeq2 adjusted p-value)*

Tolerance inducing molecules

We also identified modulated expression of several cell surface proteins which are known to be tolerizing to other cell types. The first of these genes was *CD55*, also known as complement decay accelerating factor (DAF), the absence of which on antigen presenting cells during co-stimulation has been shown to enhance T cell proliferation, the generation of effector cells, and the production of inflammatory cytokines (Heeger et al., 2005; Lalli et al., 2007). The expression of *CD55* was lower in nonclassical monocytes from symptomatically infected children, and appears to be trending towards increased in asymptomatic children relative to uninfected children (Figure 14). The second tolerance inducing gene, *ANXA5*, was increased in classical monocytes from asymptomatic children (Figure 14).

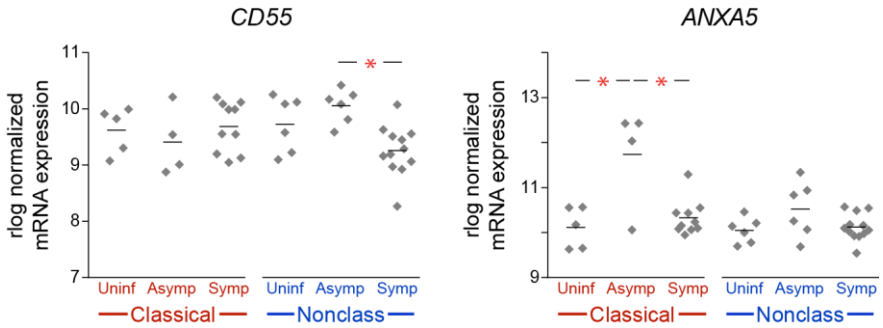

Figure 14. Differentially expressed known tolerance inducing molecules. *Rlog normalized count data shown for each sample. (* = p<0.05, DESeq2 adjusted p-value)*

Exploration of other known Immunomodulatory molecules

We also investigated the expression patterns of all genes in several classes of molecules known to modify immune system functions. We chose to examine all known genes in these classes, rather than limiting to those we identified as significantly different, since considering the expression patterns of these classes as a whole may provide more insight into the activation states of monocytes than considering these genes in isolation.

Human leukocyte immunoglobulin-like receptors

The human leukocyte immunoglobulin like receptor (LILR) family is a family of receptors which have both activating and inhibitory functions. These genes are grouped into 3 functional categories, cell surface bound and activating (LILRA1, 2, 4-6), cell surface bound and inhibitory (LILRB1-5) and soluble (LILRA3). The relative expression of these receptors has been implicated in several autoimmune diseases (Hirayasu and Arase, 2015); however, their expression in the context of *Plasmodium* infection has not been studied.

We observed that both *LILRA3* and *LILRA5* were increased in both nonclassical and classical monocytes from symptomatic children. Additionally, *LILRA1* was significantly increased in nonclassical monocytes from symptomatic children, and appears to follow a similar trend in classical monocytes (Figure 15). Interestingly, *LILRA1*, *LILRA3* and *LILRA5* all appear to be

decreased in asymptomatic children relative to uninfected children, and increased in symptomatic children relative to uninfected children, although this was only detected as significant in *LILRA5* expression in classical monocytes (Figure 15). *LILRA2* also appears to follow a similar pattern to mRNA encoding the other inflammatory LILRAs. As has been previously reported, we did not detect *LILRA4* expression in any monocyte population (Hirayasu and Arase, 2015). No differences were detected in the *LILRB* genes (Figure 15).

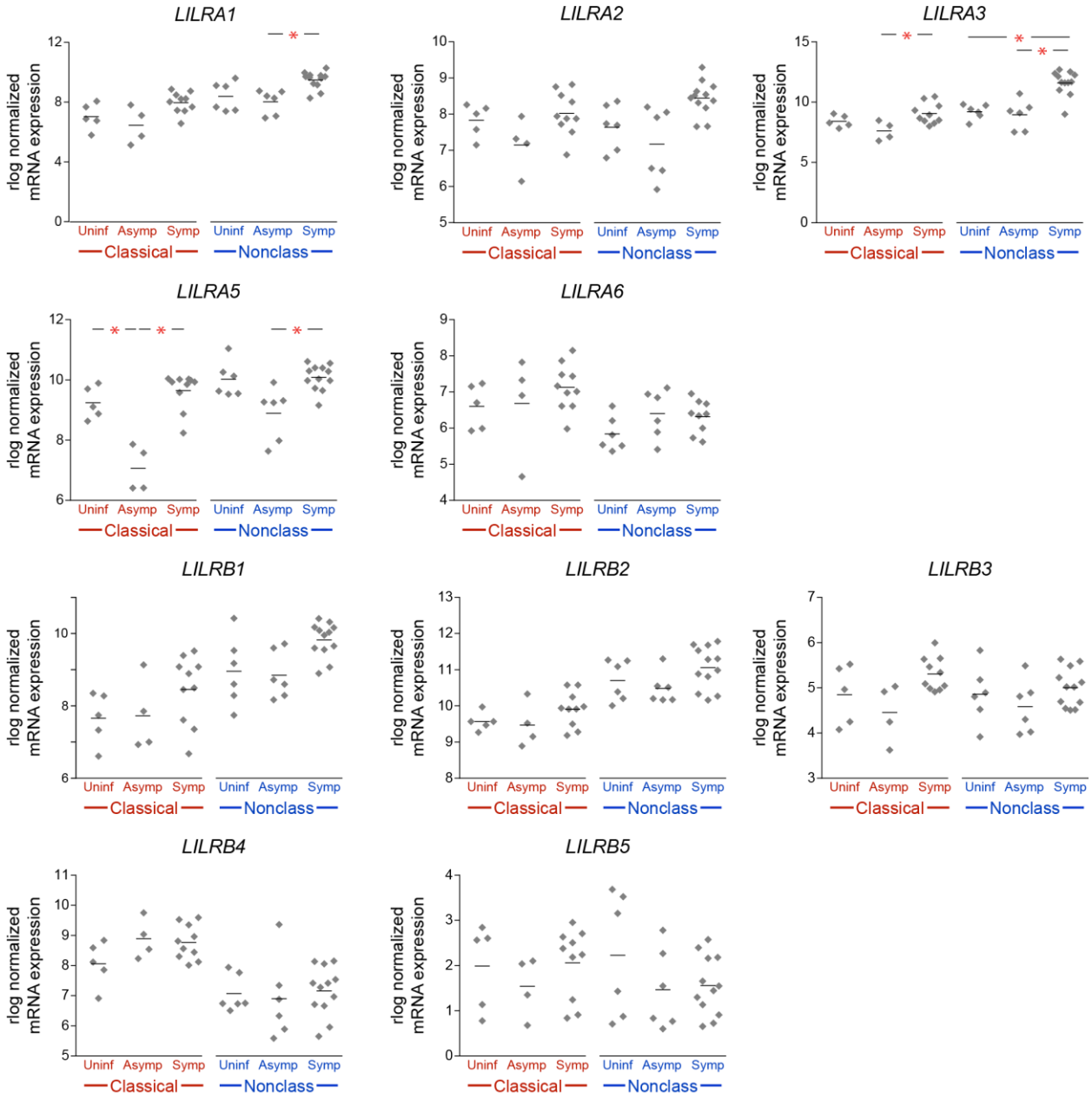

***Figure 15. Expression levels of LILR genes**. Rlog normalized count data shown for each sample. (* = p<0.05, DESeq2 adjusted p-value)*

Toll-like receptors (TLRs)

As discussed above, monocytes are known to express a family of pattern recognition receptors, the toll like receptors and several of these TLRs, TLR2, TLR4, TLR7 and TLR9, are known to recognize *Plasmodium* antigens and promote the production of pyrogenic and pro-inflammatory cytokines (Baccarella et al., 2013; Coban et al., 2005; Gowda et al., 2012; Krishnegowda et al., 2005). No differential expression of *TLR1*, *TLR4-6*, and *TLR8* or downstream adaptor molecule *MYD88* was observed (Figure 16), and we did not detect mRNA in most samples for *TLR3*, *TLR9* and *TLR10* (data not shown). Surprisingly, there was increased expression of *TLR2* in both classical and nonclassical monocytes from asymptomatic children, as compared to symptomatic children. Conversely there was a decrease in *TLR7* expression in symptomatic children in nonclassical monocytes (Figure 16). Similarly, a downstream adaptor of *TLR3* and *TLR4*, *TICAM1* (TRIF) was significantly decreased in both classical and nonclassical monocytes from symptomatic children.

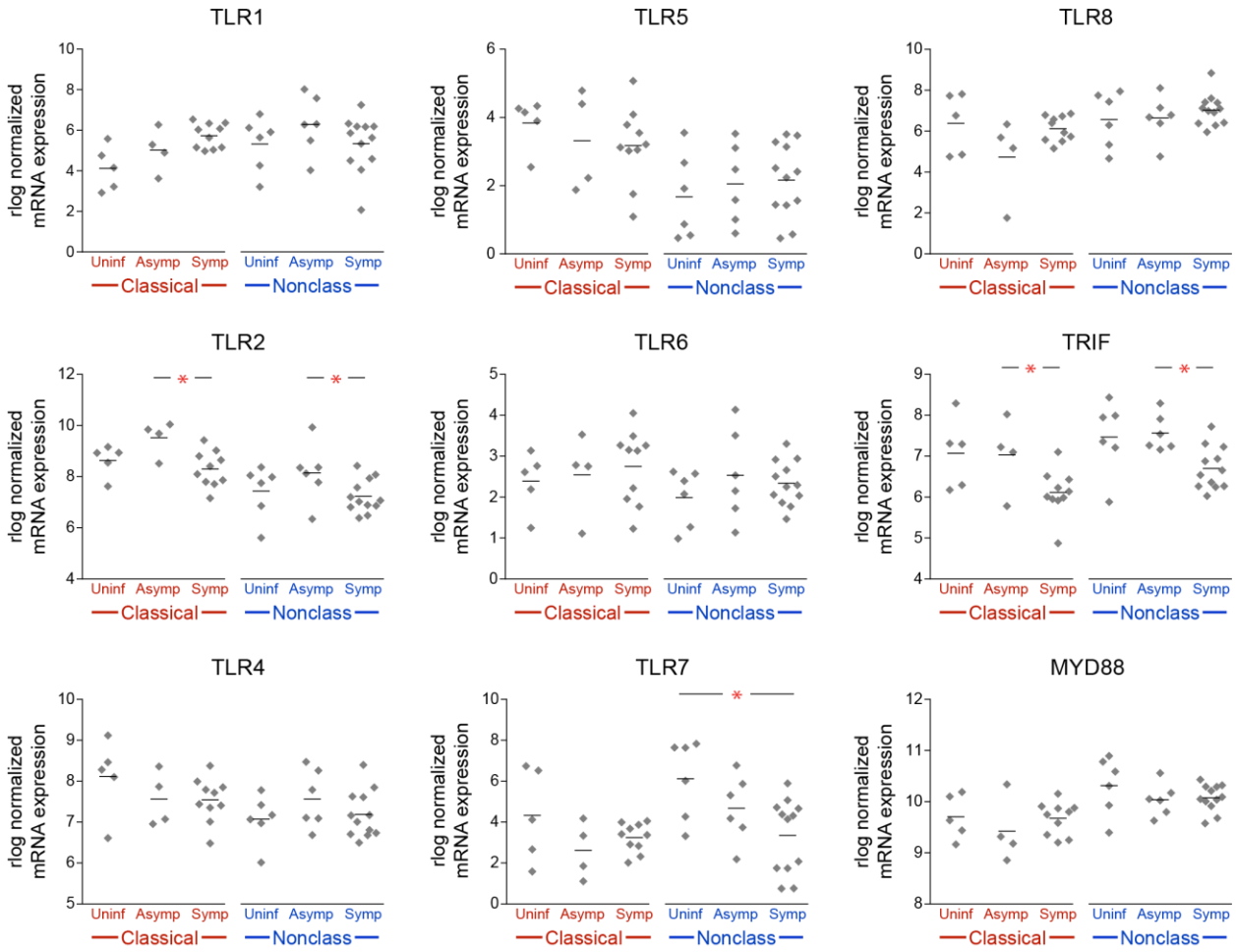

Figure 16. Expression levels of TLR signaling pathway molecules. *Rlog normalized count data shown for each sample. (* = p<0.05, DESeq2 adjusted p-value)*

Activating and inhibitory co-stimulation molecules

Monocytes can either activate or inhibit T cells by presenting a recognized antigen along with either co-stimulatory molecules or inhibitory molecules. In particular, the co-stimulatory molecules, CD80 and CD86, have been shown to promote an adaptive immune response against *Plasmodium* in vaccine candidate studies (Hui and Hashimoto, 2007); however, it has been shown *in vitro* that exposure to infected erythrocytes prevented the up-regulation of these activation molecules on antigen-presenting cells (Elliott et al., 2007), suggesting that this may be a mechanism of *Plasmodium* immune evasion. While we did not observe significant differences in mRNA of these activation markers, there was an apparent trend toward reduction of CD80 in nonclassical molecules from symptomatic children (Figure 17A). Monocytes also express inhibitory molecules, which prevent T cell activation when co-presented with an antigen. In particular, monocytes can express ligands for the programmed death 1 receptor (PD1), stimulation of which has been shown to promote a tolerogenic state in malaria infection (Butler et al., 2011). The tolerogenic state promoted by PD1 inhibits clearance of parasites, but may also protect against immunopathology. We observed that *CD274* and *CD273* which encode PD-L1 and PD-L2 respectively, the ligands of PD1, were variably expressed on both monocyte subsets, and no significant difference was appreciated (Figure 17B).

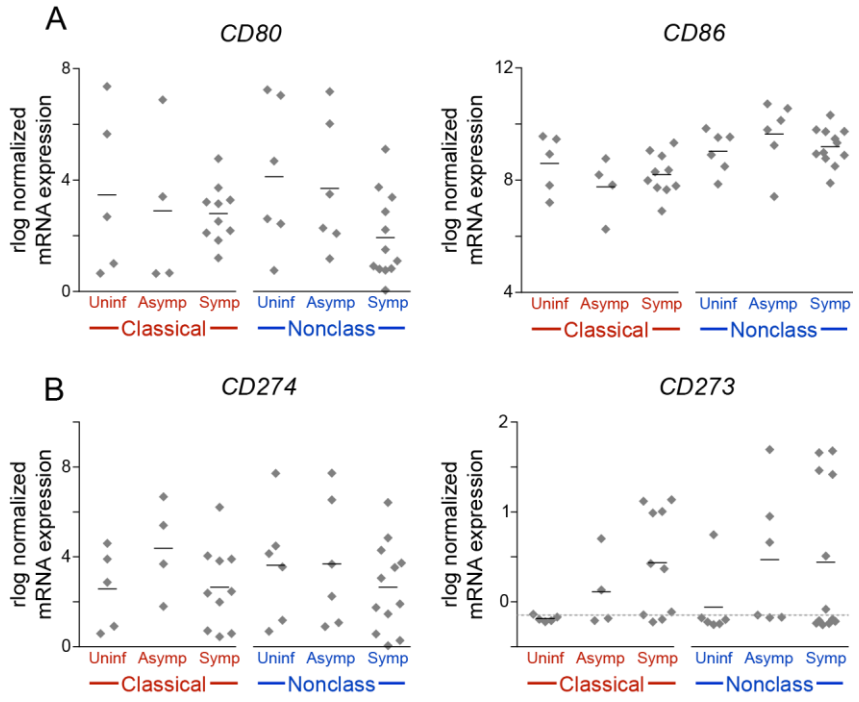

Figure 17. Expression levels of co-stimulatory molecules. *Activating (A) and inhibitory (B) co-stimulatory mRNA expression. Rlog normalized count data shown for each sample. Grey dashed line represents limit of detection.*

DISCUSSION

In this study we have identified that the relative proportion of monocyte subsets is modulated due to both exposure levels of *Plasmodium* infections and current infection status. The CD16+ subsets of monocytes, nonclassical monocytes and intermediate monocytes, are increased in children in a high-endemicity area of Uganda as compared to those in a low-exposure area. Nonclassical monocytes are further expanded in children with a current *Plasmodium* infection, regardless of whether or not the children are displaying clinical symptoms. This data suggests that the change in proportions of monocyte sub-populations is related to chronic exposure to *Plasmodium* parasites, but not to immune status. These findings are consistent with the observations that CD16+ monocytes expand in other models of inflammation or infection (Castaño et al., 2011; Nockher and Scherberich, 1998; Sheel and Engwerda, 2012; Ziegler-Heitbrock, 2007); however, the functions of these monocytes vary by disease process (Sheel and Engwerda, 2012; Wong et al., 2012b).

While the proportion of monocyte populations did not differ between asymptomatic and symptomatic infection, broad transcriptional changes occurred between asymptomatic and symptomatic infection, suggesting that monocytes changed both their internal responsiveness to immunogenic *Plasmodium* ligands, and played a role in altering the functions and responsiveness of other immune cells through changes in cytokine production and expression of cell surface molecules known to induce tolerance.

We identified several changes in monocyte gene expression which suggest that monocytes alter their responsive to *Plasmodium* ligands during asymptomatic infection. There was an increase in both *HAVCR2* and *SRC* expression during asymptomatic infection. These molecules have been known to maintain tolerance during chronic HCV infection, maintain T cell exhaustion and inhibit myeloid cytokine production while enhancing phagocytosis (Ferris et al., 2014; Han et al., 2013; Zhang et al., 2011). These changes suggest that monocytes may maintain a similar anti-inflammatory but pro-phagocytic role during asymptomatic infection. Further supporting an anti-inflammatory state during asymptomatic infection, monocytes appear to increase their sensitivity to TGF-β signaling, a well-studied anti-inflammatory cytokine. During asymptomatic infection we saw the increase of mRNA encoding the TGF-β receptors, TGFBR1 and endoglin, as well as mRNA encoding the immunoregulatory molecules ABCA1 and CD69, which are known to be induced downstream of TGF-β and function as an anti-inflammatory receptor and Immunomodulatory molecule, respectively. It should be noted that while cell membrane bound endoglin increases a cell's responsiveness to TGF-β, there is a soluble form of endoglin which has been shown to block anti-inflammatory responses and contribute to the pathology of severe malaria (Dietmann et al., 2009; Silver et al., 2011). We are unable to distinguish between the two forms of endoglin by mRNA; however, the increase in endoglin transcript during asymptomatic infection suggests that it may play an anti-inflammatory role in these children.

We additionally saw an increase in mRNA encoding pro-inflammatory LILRAs during symptomatic infection, which was not present in asymptomatic infection. LILRs have not

previously been studied in the context of *Plasmodium* infection. The observed changes suggest that LILRAs may be important mediators of the immune response during *Plasmodium* infection, increasing during symptomatic infection to drive the inflammatory response, and being down-regulated during asymptomatic infection. Notably, *LILRA3*, which was also increased during symptomatic infection, encodes the only soluble LILR and has been reported to be a negative regulator of the other inflammatory LILRAs. The deletion of *LILRA3* has been linked to numerous autoimmune diseases (An et al., 2010; Hirayasu and Arase, 2015), however this role has recently been debated in the context of systemic lupus erythematosus (Du et al., 2015). It is possible that during symptomatic *Plasmodium* infection it is co-regulated with the other LILRAs as a negative regulator to prevent pathogenic, over-inflammatory processes (such as is seen in cerebral malaria).

Although the aforementioned data suggests that during asymptomatic infection monocytes decrease their responsiveness to *Plasmodium* antigens during asymptomatic infection, this is likely an over-simplification of the process. Rather, monocytes likely change the type of response that they produce in response to *Plasmodium,* both by regulating which antigen receptors are expressed and modifying the downstream responses to these receptors. For example, *TLR2* was increased in asymptomatic infection. This is similar to a previously published study, which showed increased expression of *TLR2* and increased TLR2 responsiveness in children asymptomatically infected with *Plasmodium falciparum* as compared to monocytes from symptomatic children. These monocytes from asymptomatic children produced increased levels of both IL10 and TNF (Hartgers et al., 2008). On the other hand, *TLR7*, which has been shown to play a role in initiating an early pro-inflammatory cytokine immune response to *Plasmodium* in mouse models of infection (Baccarella et al., 2013), was decreased during symptomatic infection. One study examining susceptibility to malaria infection showed that children who were less susceptible to malaria were able to maintain TLR7 signaling during *Plasmodium* infection, whereas more susceptible children had dampened responses to TLR ligands (Arama et al., 2011). It is possible that a basal level of innate immune activation is beneficial during asymptomatic parasitemia, to allow for parasite restriction, and that increased signaling by IL-10, IL-4 and TGF-β serve to block the inflammatory actions of TNF, and it is the relative proportions of anti-inflammatory and pro-inflammatory cytokines produced downstream of the TLRs that drive disease outcomes.

Supporting the hypothesis that it is the relative levels of pro and anti-inflammatory cytokines, rather than absolute levels, that lead to asymptomatic infection and restriction or symptomatic infection, we saw increased levels of mRNA for both anti-inflammatory IL-10, as well as IL-1B and IL-18, which are typically thought of as pro-inflammatory, during asymptomatic infection. Interestingly, it has recently been reported that IL-1β signaling promotes monocyte differentiation to macrophages with increased phagocytic ability in a model of mycobacterial infection (Schenk et al., 2014) and in a mouse model of *Plasmodium* infection, it was shown that IL-18 was critical for parasite restriction and survival (Singh et al., 2002). It is possible that the phagocytic effects of IL-1β and IL-18 are essential for parasite control during asymptomatic

infection, while the deleterious inflammatory effects are blocked by increased IL-10 and TGF-β signaling.

The relative levels of cytokines produced in response to TLRs may be modulated by NF-kB signaling. We observed both changes in expression of NF-kB transcription factors, as well as molecules known to modulate NF-kB-dependent responses during asymptomatic infection. It is well described that NF-kB can induce a wide variety of target gene expression patterns based on stimulus, cell type, and the other proteins currently present in the cell, leading to only a small subset of NF-kB-dependent genes being activated in response to any given stimulus (Smale, 2010, 2011). Even during asymptomatic infection, monocytes likely need to maintain a balance of NF-kB signals to promote parasite restriction and limit parasite derived pathology. Either an absence of NF-kB-dependent signaling and related pathways or over-activation could push the balance toward severe malaria (Punsawad et al., 2012b; Tripathi et al., 2009; Watkins and Hurwitz, 2012). Differential expression of NF-kB-regulating genes may fine-tune the downstream NF-kB-dependent responses, including cytokine responses, to optimize for limiting both parasite-dependent and inflammation-dependent pathology during *Plasmodium* infection.

Additionally, we identified the up-regulation of two molecules known to be tolerogenic to other immune cells on monocytes during asymptomatic infection, which may provide a link between monocytes and the widely tolerogenic state that has been described in other cell types during asymptomatic infection. *CD55* expression, encoding DAF, was maintained during asymptomatic infection, but decreased during symptomatic infection. The absence of DAF on antigen-presenting cells during co-stimulation has previously been shown to enhance T cell proliferation and the generation of effector cells (Heeger et al., 2005). Additionally, T cells stimulated with DAF-deficient APCs produce more inflammatory cytokines, such as IFNγ and IL-12 (Lalli et al., 2007). In a murine model of CMV infection it was seen that mice deficient in DAF had greater pathology from infection, likely due to increased cytokine production and CD8+ T cell infiltration (Bani-Ahmad et al., 2011). It is possible that when transitioning from responding symptomatically to *Plasmodium* to asymptomatic infection that monocytes lose their ability to decrease surface DAF, and thus become less potent stimulators of T cells. Similarly, *ANXA5* was increased during asymptomatic infection. ANXA molecules have been shown to be increased on apoptotic cells and induce a tolerogenic state in dendritic cells once they have phagocytosed the apoptotic cell. In vitro, ANXA5 is shown to reduce the production of pro-inflammatory cytokines and activation markers by dendritic cells (Linke et al., 2015). Although we see a similar shift in the proportions of monocyte subsets during both symptomatic and asymptomatic infection, it is possible that the decreased proportion of classical monocytes is due in part to tolerance-promoting apoptotic classical cells during asymptomatic infection.

Several limitations of this study should be discussed. The differential gene analysis patterns described here are descriptive, and we are unable to make statistical statements about the overall state of the monocytes at different infection states (e.g., are the monocytes on the whole expressing tolerogenic gene programs during asymptomatic infection?). Our ability to

make such a statement is limited by the dearth of knowledge about the transcription programs expressed by monocytes in other tolerogenic states, such as chronic HBV infection or cancer. With RNA-seq becoming more affordable and accessible, these studies should become more numerous. An exciting possibility for the future is to compare the transcriptomes of monocytes and other cell types in both inflammatory and tolerogenic disease processes, to better characterize the molecular drivers of these immune states.

Additionally, this study was confounded by age differences in our various study groups. Because asymptomatic infection is more likely with age, asymptomatic children were older than the other cohorts. Due to sample availability, we were unable to use age-matched controls for our uninfected group. Ideally, subsequent studies would be designed as longitudinal studies, with the same child being used as their own control over time.

Finally, we only measured mRNA transcript levels in this study, which do not take into account post-translational regulation of mRNA and protein level regulation of the molecules discussed. Particularly when discussing cytokine transcripts, it should be noted that post-translational modification and RNA binding has been shown to regulate the levels of cytokines that are produced and secreted (Mino and Takeuchi, 2013), and thus measures of translated cytokines may reveal differences in cytokine regulation that we are unable to appreciate by RNA-seq. Further studies looking at levels of protein expression, function and cytokine secretion would greatly contribute to our understanding of how these observed changes of transcript levels manifest at a functional level during infection.

Despite these limitations, our study has still provided an exciting first step to understanding the changing functions of monocytes during the development of immunity to malaria. We have identified many exciting differentially regulated genes, extending our understanding of immunity to malaria and providing targets for future study and therapeutics.

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