Investigating the generation and maintenance of immunological memory to malaria infection

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Declaration

I, *Maria Lourdes M. Macalinao*, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed

April 2024

Abstract

It is well established that sterile immunity against malaria is hardly ever achieved by natural infection, and that the generation and maintenance of *Plasmodium*-specific immunological memory wanes in the absence of infection. Although both cell-mediated and antibody-mediated immune responses have been demonstrated to be critical for protection against malaria infection, the underlying mechanisms remain incompletely understood. This PhD thesis aims to address knowledge gaps in our understanding of the development and maintenance of immunological memory to malaria.

The main goal of this PhD project is to investigate the induction and maintenance of cellular and humoral immune memory responses to malaria infection. The study utilized 2 approaches. First, using mouse models, I examined the immune responses to acute and chronic blood-stage malaria, with a focus on $CD4^+$ T cells, which play a major role in both cell-mediated and humoral responses against malaria. *Plasmodium*-specific responses were characterized through $CD4^+$ T cells from T-cell receptor transgenic mice, PbT-II, and analyzed along with other immune cells. The role of the regulatory cytokine IL-27 in the development of immunological memory was investigated. Results showed that early and transient IL-27 inhibition allowed for better PbT-II cell maintenance during chronic infection. Moreover, IL-27 modulation of Th1 memory development had a suppressive effect on the protective capacity of both the cell-mediated and antibody-mediated immunity.

Next, through human studies, I determine the effect of differential malaria exposure on immunological memory. Using antibody data from three areas of varying endemicity in the Philippines, I first evaluated different methods of analyzing multiplex serology data. The simultaneous analyses of *Plasmodium*-specific antibody responses did not only provide accurate estimates of recent and historical malaria exposure, but also provided important implications for the impact of declining transmission on the maintenance of long-lived antibodies and memory cells. A human cohort study protocol is further described with the intention of pursuing further investigation on malaria-specific B and T cell memory responses in areas of varying malaria transmission.

Taken together, the findings from this PhD project provided important insights relating to the longevity of immune responses to malaria, contributing relevant information for devising new approaches for interventional and vaccine strategies in the context of malaria elimination.

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Abbreviations

7AAD	7-amino-actinomycin D
AMA1	apical membrane antigen 1
AMB	atypical memory B cells
ANOVA	analysis of variance
APCs	antigen-presenting cells
API	annual parasite index
ASCs	antibody secreting B cells
AUC	area under the ROC curve
B5 TCR Tg	B5 (MSP-1)-specific T cells from TCR transgenic mice
BCR	B cell receptor
CIDR1a	cysteine-rich interdomain region 1a
CQ	chloroquine
CRFs	case report forms
DAMPs	damage-associated molecular patterns
DBS	dried blood spot sample
DCs	dendritic cells
DoH	Department of Health
dpi	days post-infection
EBP	erythrocyte binding protein
ECM	experimental cerebral malaria
ELISA	enzyme-linked immunosorbent assays
ELISPOT	enzyme linked immunospot
Etramp5.Ag1	early transcribed membrane protein 5
FACS	fluorescence-activated cell sorting
FMM	finite mixture model
GBM	generalized boosted models

GC	germinal center
GEXP18	Gametocyte exported protein 18
GLM	generalized linear models
GPI	glycosylphosphatidylinositol
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IATF	Inter-Agency Task Force
Igs	immunoglobulins
ip	intraperitoneally
Ips	indigenous peoples
iRBCs	infected RBCs
iv	intravenously
kNN	k-Nearest Neighbor
LCMV	lymphocytic choriomeningitis virus
LLPCs	long-lived plasma cells
mAbs	monoclonal antibodies
MACS	magnetic-activated cell sorting
MBCs	memory B cells
MFI	median fluorescence intensity
MHC	major histocompatibility complex
ML	machine learning
MPECs	memory-precursor effector cells
MSP 2 Dd2	Dd2 allele of MSP2
MSP1	merozoite surface protein
MSP2 CH150/9	CH150/9 allele of MSP2
MTA	Materials Transfer Agreement
NegPop	negative population (model)

NK	natural killer cells
NKT	natural killer T cells
NPP	4-Nitrophenyl phosphate disodium salt hexahydrate
NU	Nagasaki University
OD	optical density
PAMPs	pathogen-associated molecular patterns
PB	peripheral blood
PbA	Plasmodium berghei ANKA
PbHsp90	P. berghei heat shock protein 90
PBMCs	Peripheral blood mononuclear cells
Pcc	Plasmodium chabaudi chabaudi AS
Pf	Plasmodium falciparum
Pf	Plasmodium falciparum
PfAMA1	Pf apical membrane antigen-1
PfGLURP R2	glutamate rich protein
PfMSP119	19KDa fragment of Pf merozoite protein 1
PfSEA1	schizont egress antigen
pi	post-infection
PRRs	pattern recognition receptors
Pv	*P. vivax*
Pv DBPRII	*P. vivax* region II, Duffy binding protein
PVDF	polyvinylidene fluoride
Pv RBP1a	*P. vivax* reticulocyte binding protein 1a
RBP2b	reticulocyte binding protein 2b
RDT	rapid diagnostic test
RF	Random Forest
ROC	receiver operating characteristics

RSV	relative spot volume
SCR	seroconversion rates
scRNA-seq	single-cell RNA sequencing
SD	standard deviation
SDZ	sulfadiazine
SL	Super Learner
SLECs	short-lived effector cells
SP	spleen
SRR	seroreversion rates
SVM	Support Vector Machine
TCM	central memory cells
Tcmp	T central memory precursor
TCRs	T cell receptors
Teff	effector T cells
TEM	effector memory T cells
TF	transcription factors
Tfh	T follicular helper
Th1	T helper 1
TLRs	Toll-like receptors
Tmem	memory T cells
Treg	regulatory T cells
UMAP	Uniform Manifold Approximation and Projection
uRBCs	uninfected RBCs
WHO	World Health Organization
WT	wild-type

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Chapter 1 : Introduction

The immune system is composed of a variety of cells and molecules in key organs, which provide the protection against foreign infectious agents that invade a host. There is a constant threat of disease from exposure to pathogens and other antigens that stimulate the host immune response, and while anatomic and chemical barriers such as the skin, stomach secretions, and antimicrobial peptides provide the first lines of defense against antigens, other innate and adaptive components of the immune system are necessary to provide non-specific and specific defenses (Delves *et al*, 2017, Murphy and Weaver, 2017). Should the immediate barriers fail, the innate immune system also relies on the presence of conserved pathogen-associated molecular patterns (PAMPs) in invading microbes as well as damage-associated molecular patterns (DAMPs) in damaged tissue that innate immune cells can recognize through their pattern recognition receptors (PRRs). This interaction allows for the rapid activation of elimination mechanisms. When the innate immune responses are not enough to totally eliminate the pathogen, the adaptive immune system comes into play, wherein antigen-specific lymphocytes proliferate and differentiate into effector cells to specifically target the infectious agents, and would also form and maintain memory cells that allow for faster antigen-specific responses upon re-encounter with the same antigen. This property typically attributed to the adaptive immune system, known as immunological memory, enhances the efficiency of antigen-specific responses, and this concept forms the basis for vaccination as an intervention to control infectious diseases (Murphy and Weaver, 2017). It is then important that we improve our understanding of how adaptive or acquired immune responses are mounted against specific diseases, such as in malaria, to be able to provide better insights into vaccine development.

1.1   Malaria: biology and disease burden

Malaria is a disease of public health importance caused by the Apicomplexan protozoan parasite *Plasmodium.* The five species that cause malaria infection in humans are *Plasmodium falciparum*, *P. vivax, P. malariae, P. ovale,* and the zoonotic *P. knowlesi. Plasmodium* parasites have developed a complex life cycle (Fig 1.1) requiring an invertebrate vector and a susceptible vertebrate host. In humans and rodents, parasites are transmitted through a blood meal of infected female *Anopheles* mosquitoes, wherein after sporozoites are transmitted by mosquitoes to the vertebrate host, parasites are transported to the liver (pre-erythrocytic stage) where they invade hepatocytes, undergo schizogony, and release merozoites that can invade red blood cells. This erythrocytic stage is the pathogenic stage
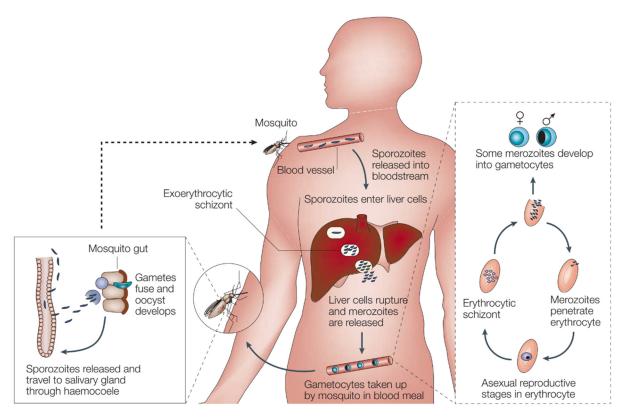
Figure 1.1. Life cycle of Plasmodium falciparum in the mosquito vector and the human host. Adapted from Stevenson and Riley (2004).

of the infection (clinical malaria), wherein the hallmark symptom of fever due to the proinflammatory cytokine production brought by the innate immune response is observed. Some of the blood-stage merozoites develop into sexual-stage gametocytes, which can be taken up by a mosquito in a blood meal to complete their life cycle (Stevenson and Riley, 2004, Hafalla *et al*, 2011, Cowman *et al*, 2016, Rivera-Correa and Rodriguez, 2017).

1.1.1 *Plasmodium falciparum* and *Plasmodium vivax*

The distribution and impact of malaria vary across regions based on seasonal patterns and local transmission intensity. *P. falciparum* and *P. vivax* are the two most prevalent species responsible for the majority of malaria-related illness in humans. While *P. falciparum* is the most virulent, and is considered the primary cause of mortality mainly in children under 5 years old, *P. vivax* is the most widespread globally. *P. falciparum* is concentrated mostly in sub-Saharan Africa, and also observed in some parts of Asia and Latin America, while *P. vivax* is found in Asia, South America, the Middle East, and some parts of Africa (Cowman *et al*, 2016, Battle *et al*, 2019, Weiss *et al*, 2019). Although there is considerable progress in the control of disease burden for both *P. falciparum* and *P. vivax* infections across the globe, this

has been due mostly to control efforts that focus on *P. falciparum.* It has been observed that with the significant decrease in malaria incidence in countries aiming for elimination, just like in the Asia-Pacific region, there is a subsequent increase in proportions of *P. vivax* infections, suggesting that current control measures had less effect on *P. vivax* transmission (Adams and Mueller, 2017, Price *et al*, 2020).

As the two primary causes of human malaria, *P. falciparum* and *P. vivax* infections are characterized by unique features in their life cycles and clinical manifestations. *P. falciparum* infections result in higher parasitemia (20,000-500,000 parasites/uL on average vs 20,000/uL for *P. vivax*) due to the preferential invasion of *P. vivax* of blood reticulocytes (<1.5% of blood). This higher parasitaemia has been associated with excessive and persistent inflammation, which are key factors leading to severe pathology and the onset of complications such as cerebral malaria and severe malarial anemia for *P. falciparum* (Mota and Rodriguez, 2017b), while *P. vivax* infections are commonly found to be commonly asymptomatic or submicroscopic in varying endemic settings, likely due to the observed significantly more rapid acquisition of clinical immunity for this infection (reviewed by Adams and Mueller (2017)). However, severe vivax malaria can also be observed in some cases despite the perception that it is a benign illness (Olliaro *et al*, 2016). The most notable difference between these two prevalent *Plasmodium* species is the ability of *P. vivax* (also *P. ovale*) to form dormant hypnozoites during the liver stage, which can remain undetected for extended periods and lead to clinical relapse (Antinori *et al*, 2012). Relapses due to liver hypnozoites are said to contribute to more than 80% of observable *P. vivax* blood-stage infections in vivax malaria-endemic regions (Adams and Mueller, 2017). The radical cure for vivax malaria, or the treatment for the elimination of all parasite stages, require the use of anti-hypnozoite drugs such as the 8-aminoquinolines primaquine and tafenoquine (Price *et al*, 2020).

The wider geographic range for *P. vivax* can be attributed to its ability to develop at lower temperatures and also at faster rates in *Anopheles* vectors (Olliaro *et al*, 2016). The incubation period from the mosquito infection to the symptomatic blood stage in humans takes about 2-10 days, with the pre-patent period lasting 9 days for *P. falciparum* and 11-13 days for *P. vivax* (Antinori *et al*, 2012). *P. falciparum* takes a longer time (about 8-12 days) to form gametocytes compared to other species. Since *P. vivax* can rapidly develop infectious gametocytes (as early as 3 days post-infection, even before symptom onset), there is a tendency for its increased transmissibility (Cowman *et al*, 2016, Henry *et al*, 2019). Moreover, despite *P. falciparum* infections leading to higher parasitemia, fever onset can be observed at a lower

threshold for *P. vivax* – at as early as during subpatent *P. vivax* infection levels, further risking missed early infections (Antinori *et al*, 2012, Olliaro *et al*, 2016, Adams and Mueller, 2017). With these difference in the trends in *P. falciparum* and *P. vivax* transmission and biology, it is increasingly being recognized that vivax malaria elimination presents unique challenges that warrant targeted intervention strategies (WHO 2021).

1.1.2 The global burden of malaria

In 2021, there were an estimated 247 million malaria cases with 619,000 deaths worldwide, and populations in more than 80 endemic countries remain at risk of the disease (WHO 2022a). The disease has a wide geographic distribution, but in terms of burden, the African region contributes more than 90% of the total malaria cases worldwide. Following the WHO African region, the South-East Asian region and Eastern Mediterranean region contributed about a combined 8% of the global malaria burden.

The successful reduction of cases in elimination countries can be attributed to the increase in coverage for bed nets and indoor residual spraying, as well as the use of artemisinin-based therapies. The impact of these interventions has paved the way for endemic countries to get closer to achieving their goals of malaria elimination (Fowkes *et al*, 2016, WHO, 2020). Although these various interventions have been in place for several years, a highly efficacious vaccine for malaria that can control disease onset and transmission remains a critical missing piece for the goal of malaria eradication (Langhorne *et al*, 2008, Hafalla *et al*, 2011, Crompton *et al*, 2014). Moreover, due to the recent COVID-19 pandemic, there were disruptions to the implementation of health services for malaria control in certain areas, and about 13 million cases from 2019 to 2021 were attributed to this (WHO 2022a). Still, most countries were able to mitigate the impact of the pandemic and keep the numbers of cases and deaths from worsening.

An important milestone that exhibited the resolve for the global malaria eradication efforts was the designation in 2021 by the World Health Organization of the RTS,S/AS01 as the first malaria vaccine for widespread use for children in the African region, and more recently of R21/Matrix-M as the second malaria vaccine to be recommended (WHO 2021, WHO 2022b, WHO 2023). With the goal of reducing the disease burden and mortality caused by falciparum malaria in the sub-Saharan Africa, where 95% of cases and deaths occur, there were initial introductions for routine immunization in Ghana, Kenya and Malawi starting 2019. Their cohort evaluation found a 30% decrease in severe malaria cases and 21% in hospitalization with parasitaemia (WHO 2021). Efficacy studies of the vaccine showed that it conferred

protection against clinical malaria with only about 30-50% efficacy in African infants and children although sterile protection was achieved in 50% of malaria-naïve adults (Casares *et al*, 2010). Also, since the immune responses induced by the RTS,S vaccine have only a modest protective effect, and do not affect the gametocyte stage, there is the possibility of mosquito infectivity and continued transmission, such that a next-generation vaccine that targets different stages of malaria is warranted. Still, the benefits outweigh the risk, and the impact of the RTS,S vaccine rollout shows how vaccination is a critical tool for malaria eradication (Zavala, 2022). The decades-long endeavor for the generation of better vaccines against malaria that can induce long-lasting immunity continue to be a challenging one, and becomes all the more important as countries are targeting elimination.

1.1.3 Malaria epidemiology in the Philippines

There is an increasing number of countries targeting zero indigenous malaria cases, with considerable potential for expediting their elimination goals through the implementation of robust health programs. Most of the countries in the Asia-Pacific region are targeting malaria-free certification by 2030, and are employing intervention strategies that involve stratifying their areas for subnational elimination (WHO 2011). Such is the case for the Philippines, where the current health system is following a devolved structure (Espino *et al*, 2004). Malaria remains a public health issue in the Philippines, with an estimated 4,200 cases reported in 2021. About 80% of these cases are *P. falciparum,* 16% *P. vivax*, and the remaining are from other species, mixed infections, or imported cases (WHO 2022a). Only a total of 2 out of 81 provinces and regions in the country are reporting local cases in 2022, with >90% of these malaria cases concentrated in the province of Palawan.

The country has seen a reduction of cases by 95% and deaths by 98% since 2005. The Philippines' Department of Health, with partial aid from international organizations, has implemented several malaria control and elimination programs, including the distribution of insecticide-treated bed nets, indoor residual spraying, and prompt diagnosis and treatment of infected individuals (World Health Organization, 2014). Despite these efforts, the persistent transmission of malaria in the few endemic provinces in the country remains a challenge. Most of the recent malaria research in the country focuses on epidemiology, molecular surveillance, and therapeutic efficacy studies (Fornace *et al*, 2018b, Stresman *et al*, 2020, Reyes *et al*, 2021), with immunological studies limited to quantifying antimalarial antibody responses (van den Hoogen *et al*, 2020a).

1.2 Immune responses to *Plasmodium* infection: Overview

The optimal outcome of an immune response to an infection is successful pathogen control without the detrimental effects from induced pathology. The different components of the innate and adaptive immune system have distinct roles in balancing between these pathological and protective immune responses during an infection. This interplay is much more apparent in immune responses to intracellular protozoan infections like *Plasmodium*, which are capable of immune evasion with its complex nature and diverse antigenic repertoire (Langhorne *et al*, 2008, Scholzen and Sauerwein, 2013).

This complexity of the *Plasmodium* life cycle is one of the many immune evasive mechanisms that makes the clearance of this parasite a challenging feat for the immune system. With the parasite's capability for expression of variant antigens, they are able to escape immune surveillance, which can lead to the failure to control the infection (Struik and Riley, 2004, Achtman *et al*, 2005, Zabriskie, 2009). The nature of the induction of the innate and adaptive immune responses would then dictate the level of host inflammatory responses and severity of pathology. Aside from fever, malaria infection in humans would typically present non-specific clinical symptoms such as chills, headache, dizziness, nausea, and abdominal discomfort among others, and may progress to complicated or severe malaria, wherein cerebral malaria and severe anemia can cause death (Compans *et al*, 2005, Cowman *et al*, 2016, Milner, 2018). Typical malaria infections in humans present as acute symptomatic infections in non-immune individuals, but become chronic after repeated infections (Moormann *et al*, 2019). Splenomegaly, or the enlargement of the spleen, can be induced in response to repeated infections, which suggests the organ's major involvement in the development of immune responses (Engwerda *et al*, 2005, Ghosh and Stumhofer, 2021). The host responses are species- and stage-specific, such that malaria pathogenesis should be understood in varied contexts.

1.2.1 Innate immunity to malaria

The cells of the immune system can be divided into 2 main classes – lymphoid cells and myeloid cells. The lymphoid cells refer to the antigen-specific lymphocytes, while the myeloid cells encompass most of the cell types with functions (mainly) in the innate immune system, which include neutrophils, macrophages, mast cells, and dendritic cells (Delves *et al*, 2017, Abbas *et al*, 2018). The innate immune system has cells that can recognize PAMPs expressed in pathogens as well as DAMPs derived from damaged tissue through PRRs to maintain homeostasis in the body. These interactions allow for a rapid activation of innate immune cells,

which include macrophages, neutrophils, monocytes, natural killer (NK) cells, and dendritic cells (DCs), and trigger responses such as phagocytosis, cell-killing mechanisms, and cytokine production (Delves *et al*, 2017, Mota and Rodriguez, 2017a). Some of these cells function as antigen-presenting cells (APC), which capture and process antigens to activate T lymphocytes and initiate T cell-mediated responses. In the context of malaria, the main APC subsets are the dendritic cells (DCs) and macrophages, with DCs identified as crucial APCs that can activate naive T cells specific for the malaria antigens, and that can efficiently produce the necessary cytokines and chemokines in response to the malaria antigens. DCs prime CD4^{+} T cells during the symptomatic blood stage, and play the key role of initiating and regulating the overall antimalarial immune response through its interactions with cells of both the innate and adaptive immune system (Gowda and Wu, 2018).

Innate immune responses are important in dictating the nature of the subsequent acquired immune responses and the associated disease pathology. The immune responses initiated by innate immune cells such as antigen-presenting DCs and macrophages set up the environment for the adaptive immune response (T cells, B cells, and antibodies) through the production of proinflammatory cytokines such as tumor necrosis factor (TNF) and interferon-γ (IFNγ) (Urban *et al*, 2005, Yui and Inoue, 2020). The PAMPs that have been identified from the *Plasmodium* parasite include the glycosylphosphatidylinositol (GPI)-anchor molecule, hemozoin, *Plasmodium* nucleic acids as well as host-derived DAMPs such as uric acid and heme, which are recognized by PRRs such as Toll-like receptors (TLRs), and increase the production of inflammatory cytokines and chemokines for parasite elimination (Gazzinelli *et al*, 2014, Cai *et al*, 2020). The cellular sources of cytokines during the initial response to infection also include γδ T cells, whose IFNγ production could contribute to the activation of macrophages, dendritic cells, neutrophils, and monocytes (Stevenson and Riley, 2004, Dantzler and Jagannathan, 2018). With the progress of infection also comes the increase of anti-inflammatory cytokines that can gradually downregulate and balance out the inflammatory responses to prevent further damage experienced by the host (Stevenson and Riley, 2004, Gowda and Wu, 2018, Cai *et al*, 2020).

During liver stages, hepatocytes can detect *Plasmodium* RNA in the cytosol, and produce type I interferons (IFNs). Studies in mice suggest that the presence of type I IFN signaling aids in the recruitment of NK cells, natural killer T (NKT) cells and DCs (Liehl *et al*, 2014, Miller *et al*, 2014, He *et al*, 2020). The activity of the recruited APCs primes the adaptive immune response, specifically the CD8^{+} T cell responses, to eliminate liver-stage parasites. On the

other hand, for the symptomatic blood stage infection, which involves the exponential growth of the parasites, innate immune responses are more easily induced than during the silent liver-stage infection. Macrophages can rapidly act on reducing parasite burden through phagocytic clearance (Gazzinelli *et al*, 2014), while mainly DCs produce cytokines and chemokines, with their activity crucial in bridging the innate and adaptive immune responses (Gowda and Wu, 2018). The subsequent production of chemokines and cytokines in response to the progression of infection, and the overreaction of innate immune cells through pro-inflammatory responses show how innate immune responses contribute to development of malaria symptoms (Gazzinelli *et al*, 2014, Montes de Oca *et al*, 2016, Minkah *et al*, 2019).

Although innate immune responses are traditionally considered not to have memory, more recent studies delving into their mechanisms suggest that innate immune cells are capable of adapting responses after an encounter with a pathogen, through a functional reprogramming that is mediated by epigenetic and metabolic remodeling. This concept, termed as trained immunity, is observed to be able to provide better protection not only against homologous reinfection but wider cross-protection against other unrelated pathogens (Netea *et al*, 2020). Another trained innate immune mechanism observed following a re-encounter is known as tolerance, which involves the decrease in inflammatory responses (Schrum *et al*, 2018). Despite these observed changes in responses due to previous pathogen encounter, innate immunity does not provide specific protection mediated by adaptive immunity, which is regulated by lymphocytes expressing rearranged antigenic receptors.

1.2.2 Acquired immunity to malaria

Adaptive (or acquired) immune responses during the initial phase of *Plasmodium* infection will involve the clonal expansion of *Plasmodium*-specific T and B lymphocytes, which will lead to their differentiation into effector cells that play essential roles for controlling the disease (Murphy and Weaver, 2017, Kurup *et al*, 2019).

Repeated infections are required to attain both anti-disease immunity and anti-parasite immunity, with acquisition observed to be more gradual for the latter, and the risk of morbidity and mortality decreases with age in endemic areas, likely due to the frequent and continuous exposure (Struik and Riley, 2004, Marsh and Kinyanjui, 2006, Doolan *et al*, 2009). Individuals who have not been exposed to malaria will most likely develop symptoms upon their first malaria infection, and the disease may even become severe and fatal (Langhorne *et al*, 2008). As early as the 1900s, observations of malaria-endemic populations by Robert Koch led to

the still-upheld assumption that although natural immunity against malaria can be achieved, repeated exposure to the parasite is necessary (Koch, 1900). This is partly because malaria parasites have evolved immunomodulatory mechanisms against host immune responses that makes the development of naturally acquired immunity to malaria suboptimal as has been established through decades of studies (Doolan *et al*, 2009, Scholzen and Sauerwein, 2013). This suggests that the hallmark feature of the adaptive immune system, which is the capacity to develop immunological memory, seems to be impaired for malaria, although not necessarily impossible to achieve (Struik and Riley, 2004).

Naturally acquired immunity to malaria has been categorized into anti-disease or clinical immunity, which protects against clinical disease; anti-parasite immunity, which gives adequate control of parasitaemia; and premunition, which allows for protection against new infections through a maintenance of a low-dose or chronic asymptomatic infection (Doolan *et al*, 2009, Scholzen and Sauerwein, 2013, Cowman *et al*, 2016). Sterilizing immunity, or total protection against reinfection, seems to be never fully achieved even in individuals living in areas of high transmission, wherein the extent of protection would be prevention of severe disease and high parasite density. This latter observation is of major concern, as progress in malaria control and elimination is hampered by the lack of an efficacious vaccine (Langhorne *et al*, 2008, Vaughan and Kappe, 2012, Cockburn and Seder, 2018).

The antigen-specific immune responses are composed of the cell-mediated and antibody-mediated (humoral) components. Antigen recognition for B cells and T cells are through immunoglobulins (Igs) and T cell receptors (TCRs), respectively, whose variable regions are able to strongly bind to specific antigens. Immunoglobulins come in the form of the membrane-bound B cell receptor (BCR), or the secreted form – better known as the antibody that is produced by plasmablasts and plasma cells. Antibodies are able to directly bind to antigens and perform effector functions such as recruitment of other immune cells or molecules to clear the pathogen. On the other hand, TCRs cannot directly bind to a pathogen, but instead recognize a short peptide fragment from a specific antigen that is bound to and presented by major histocompatibility complex (MHC) molecules (Murphy and Weaver, 2017).

In both extraerythrocytic and erythrocytic stage infections, humoral immunity through antibody production plays a crucial role in pathogen clearance upon reinfection (Scholzen and Sauerwein, 2013), which is enhanced by the accumulation of an antibody repertoire with various antigenic targets resulting from continuous exposure (Compans *et al*, 2005, Rogers *et al*, 2021). The cell-mediated responses orchestrated by helper CD4^{+} T cells (Achtman *et*

al, 2005, Hafalla *et al*, 2011) and to a lesser extent cytotoxic CD8^{+} T cells (Junqueira *et al*, 2018) also contribute to the adaptive immune response, where the action of pro-inflammatory (*e.g.,* TNF, IFNγ, IL-2) and anti-inflammatory (*e.g.,* TGFβ, IL-10) cytokines produced in response to infection aid in parasite clearance, and dictate clinical manifestations of the disease, or lack thereof (Good *et al*, 2005). These protective mechanisms of the cell-mediated and humoral immunity are explained in more detail in the subsequent sections.

1.2.3 Immunological memory to malaria infection

Immunological memory, or the recall response of the adaptive immune system, allows for an efficient response upon re-exposure to antigens. Upon pathogen clearance, most effector T and B lymphocytes undergo apoptosis, with a small proportion that will remain as long-lived memory cells, which will represent the immunological memory cell pool that can facilitate an enhanced antigen-specific recall response (Amanna *et al*, 2007, Gasper *et al*, 2014, Hviid *et al*, 2015, Ryg-Cornejo *et al*, 2016, Ly and Hansen, 2019). Some of the plasma cells develop to long-lived plasma cells (LLPC) and continue to produce antibodies (Brynjolfsson *et al*, 2018, Silveira *et al*, 2018). This protective immunity is observed in response to many viral and bacterial pathogens such as measles, tetanus and mumps, wherein a single exposure to the pathogen induces long-term to life-long immunity (Amanna *et al*, 2007, Ryg-Cornejo *et al*, 2016, Ly and Hansen, 2019). Learning the nature of the adaptive immune response against pathogenic infections is the guiding principle that informs vaccine development.

In malaria, adults living in endemic areas to not develop sterile immunity suggesting that natural immunity against the pre-erythrocytic (skin and liver) stages is difficult to acquire, if it does develop (Cockburn and Seder, 2018). Despite the observed lack of protective immunity at this stage, pre-erythrocytic stage of malaria infection does induce immune responses from the host, particularly CD8^{+} T cells and antibodies (Zuzarte-Luis *et al*, 2014, Holz *et al*, 2016, Kurup *et al*, 2019). For the erythrocytic stage of malaria infection, the main cell subsets that play integral roles in the immune response are CD4^{+} T cells and B cells for both the primary infection and during memory response, with only a minimal role for CD8^{+} T cells (Good, 2001, Crompton *et al*, 2014). The spleen, a secondary lymphoid organ involved in erythropoiesis and hematopoiesis, is a site where immune cells are activated and memory lymphocytes develop following a *Plasmodium* infection. T and B cell responses are generated in respective T and B cell zones in the white pulp of the spleen while splenic red pulp contains macrophages that remove infected RBCs from circulation (Engwerda *et al*, 2005, Ghosh and Stumhofer, 2021).

RBCs are devoid of MHC class I and II molecules to directly present antigens to T cells. As such, to control blood-stage malaria, CD4^{+} and CD8^{+} T cell responses rely on antigen presentation by myeloid cells such as DCs and macrophages (Moormann *et al*, 2019). In particular, helper CD4^{+} T cells have an essential role in coordinating other aspects of the immune response, specifically for providing help to B cells for antibody production, as well as preparing the necessary inflammatory cytokine environment for CD8^{+} T cell killing and antigen recognition by innate immune cells. CD4^{+} T cells differentiate into diverse functional subsets depending on the polarizing cytokine environment, and during malaria infection, the main effector subsets are found to be T helper 1 (Th1) and T follicular helper (Tfh) cells (Lönnberg *et al*, 2017, Jian *et al*, 2021). The Tfh subset is required to provide the necessary signals and crucially help antigen-specific B cells in the development and maintenance of germinal center (GC) reactions within the peripheral lymphoid organs, which then lead to efficient class-switching and affinity maturation of antibodies, as well as generation of antibody-producing long-lived plasma cells (LLPCs) and memory B cells (MBCs) (Wykes *et al*, 2017).

Antibody production by B cells contribute critically to long-term protective responses, as evidenced by the protective ability of a passive transfer of serum IgG antibodies from malaria-immune adults that were able to treat young patients with symptomatic malaria (Cohen *et al*, 1961). Numerous field studies in endemic areas have also shown that higher antibody levels against blood-stage malaria antigens correlated well with better protection and clinical immunity (as reviewed by Ryg-Cornejo *et al* (2016)). These antibodies are maintained by continuous production by LLPCs that reside in the bone marrow, and continually produce high-affinity antibodies even in the absence of antigen. LLPCs emerge from GC responses of antibody-secreting cells (ASCs). *Plasmodium*-specific MBCs likewise are maintained in chronic infection, and can undergo rapid effector differentiation into antibody-producing plasma cells that can also produce high quality antibodies; however, numerous studies suggest that protective antibody responses against *Plasmodium* infections are not acquired easily (Crompton *et al*, 2014, Hviid *et al*, 2015, Fowkes *et al*, 2016, Cockburn and Seder, 2018, Ghosh and Stumhofer, 2021). This is discussed further in Section 1.7.

1.3 Malaria immunology: lessons from model systems

Studying immune responses to diseases in humans have certain limitations. As the only immune cells we can readily study in humans are peripheral blood, gaining access to secondary lymphoid organs through the use of mouse models contribute important

information that can be further explored in the human setting (Achtman *et al*, 2005). Immunological studies make use of mouse models and non-human primate models to study fundamental mechanisms of immunity in a controlled experimental environment (Rénia *et al*, 2002, Langhorne *et al*, 2011, Zuzarte-Luis *et al*, 2014, Kimura *et al*, 2016). In malaria studies, the use of rodent plasmodia, namely *P. berghei, P. vinckei, P. chabaudi*, and *P. yoelii,* allowed for extensive studies of malaria infection, that is said to have significant conservation of genetic and phenotypic traits from mouse to humans (Rénia *et al*, 2002, Stephens *et al*, 2012). However, their relevance to human infections have always been up for debate, as the disease is not a natural pathogen in mice, and a single mouse parasite strain cannot mimic all of the clinical features and immune responses observed in humans(Langhorne *et al*, 2002, Stevenson and Riley, 2004). Although it has apparent limitations, mouse studies have greatly contributed to the current understanding of the nature of malaria infection – the parasite biology, host response, as well as mechanisms of immunopathology (Langhorne *et al*, 2011, Zuzarte-Luis *et al*, 2014) (Langhorne *et al*, 2011, Stephens *et al*, 2012).

Each rodent malaria strain is used to mimic different clinical manifestations of the disease in an experimental setting, while exhibiting different morphologies and RBC tropisms. The *P. berghei* model is used to observe severe and cerebral malaria in susceptible mice such as C57BL/6 (B6), with the *P. berghei* ANKA (*PbA*) strain as the more widely used experimental cerebral malaria (ECM) model of human cerebral malaria (de Souza *et al*, 2010). Severe malaria can also be observed using the *P. yoelii 17YM* and *17XL* strains, which induce high levels of parasitemia leading to death. Non-lethal strains include *P. yoelii 17XNL, P. chabaudi chadami,* and *P. vinceki petterei.* Regarding RBC tropisms, *P. berghei* infects mature RBCs and reticulocytes*, P. yoelii 17XNL* infects reticulocytes, and *P. chabaudi* and *P. vinckei* have strains that preferably infect mature RBCs. These strain differences have been utilized to study different aspects of the pathogenesis and immune mechanisms that could provide explanations for observations in humans (Langhorne *et al*, 2002, Schofield and Grau, 2005, Wykes and Good, 2009)*.

Another commonly used strain is the *Plasmodium chabaudi chabaudi* AS (Pcc), which is said to be similar to the human parasite *P. falciparum (*Pf)*. In particular, the similarities in immune responses to Pcc compared with human Pf responses has made it the typical model used for studying immune mechanisms and inflammation-related pathogenesis of malaria (Stephens *et al*, 2012). Insights on the mechanisms of innate and adaptive immune responses have been obtained through studies in the mouse models, from the invasion of sporozoites in the host

epithelial surface, to the host response to parasite activities in the liver and blood (reviewed by Zuzarte-Luis *et al* (2014). Through the use of imaging, molecular techniques, and classical immunological techniques such as flow cytometry, we have achieved better understanding of the immunological responses to malaria infection. Notable examples of this include the discovery that sterile protection can be achieved for liver-stage malaria through immunization with radiation-attenuated sporozoites (Nussenzweig *et al*, 1967), as well as the critical role of CD4^{+} T cells in controlling parasitaemia (Langhorne *et al*, 2002).

Assessment of the immune cell subpopulations during the course of Pcc blood-stage infection suggests that although CD4^{+} T cells and B cells play essential roles in the response during the acute phase, other hematopoietic cells also play critical roles as shown that the depletion of innate immune such as DCs, red pulp macrophages and monocytes during the chronic stage resulted in failure to control parasitaemia, suggesting their critical role during parasite recurrence (Chen *et al*, 2022). This observation is supported by several other studies looking into the effects of the depletion of specific cell subsets during malaria infection. These studies revealed the importance of different immune cell populations aside from CD4^{+} T cells such as the NK cells during the acute phase, as well as the requirement of both CD4^{+} T cells and B cells for complete parasitaemia control during the chronic phase (Stevenson and Riley, 2004).

The requirement of CD4^{+} T cells for immune protection were studied in more detail through the Pcc infection model. Infection of C57BL/6 (B6) mice (wild-type /WT mice) with blood-stage *Pcc* infection leads to a peak parasitaemia at around 7-9 days post-infection (pi), wherein T cell expansion is also expected to peak along with antibody production by B cells, after which the contraction phase of the immune response will occur (Stephens *et al*, 2012). Since the first wave of parasitaemia occurs before sufficient levels of protective IgG antibodies, the resolution of the acute phase of infection relies heavily on the CD4^{+} T cell response and other antibody-independent mechanisms (Stevenson and Riley, 2004). CD4^{+} T cell activation is induced via presentation on MHC-class II molecules of Pcc antigens by bone marrow-derived dendritic cells, macrophages and B cells (Langhorne *et al*, 2002). Using Pcc chronic infection model, it was shown that long-term protective immunity depends not on the antibody levels and B cells, but on the memory CD4^{+} T cells generated (Meding and Langhorne, 1991, Achtman *et al*, 2007, Freitas do Rosario *et al*, 2008). With this study focusing on characterizing immune responses during the blood-stage infection, the following sections will delve into the immune mechanisms of CD4^{+} T cells and B cells, as elucidated

through mouse studies.

1.4 CD4^{+} T cell differentiation during malaria infection

CD4^{+} T cells have the plasticity to further differentiate into multiple subsets depending on the activation by polarizing cytokines in the environment (Perez-Mazliah and Langhorne, 2014, Kurup *et al*, 2019). The differentiation of CD4^{+} T cells into its distinct subsets (T helper 1/Th1, T follicular helper/Tfh, T regulatory/Treg cells, etc) was also observed through Pcc studies, which have been confirmed to occur in human immune responses to malaria as well (Kurup *et al*, 2019). Signals from specific pathogens induce the expression of cytokines by dendritic cells, and other innate immune factors that affect differentiation of naïve T cells. Th1 differentiation is driven mainly by IL-12 and IFNy inducing Tbet, Th2 by IL-4 inducing GATA3, Th17 by TGFβ and IL-6 inducing RORγT, and Tfh by the induction of Bcl6 (Crotty, 2011, Sallusto *et al*, 2018).

During *Plasmodium* infection, CD4^{+} T cells play a significant role in controlling malaria infection not just in the aspect of cell-mediated immunity through its T helper 1 (Th1) subset, but also humoral immunity – through its follicular helper T (Tfh) subset that are needed for B cell responses and antibody production (Doolan *et al*, 2009, Perez-Mazliah and Langhorne, 2014, Kurup *et al*, 2019). Other subsets of note are the FOXP3^{+} regulatory T cells or Tregs, as well as IL-10-producing type I regulatory (Tr1) cells that act on limiting the severity of disease (Kurup *et al*, 2019), and the IL27-producing regulatory T cells, termed Tr27 cells, which had been shown to have an inhibitory role during a malaria infection (Kimura *et al*, 2016). This plasticity of CD4^{+} T cells and their roles in the generation of immune memory and protection are still incompletely understood.

The subset of IL-12 driven Th1 cells – that produce inflammatory cytokines such as IFNy and TNF – was identified as the major subset controlling the parasitaemia during the peak of Pcc infection (Stephens *et al*, 2012). It has also been shown that CD4^{+} T cells develop to follicular helper T (Tfh) cells, which are involved in the antibody production by B cells (Crotty, 2011, Kurup *et al*, 2019) (Stephens *et al*, 2009, Silveira *et al*, 2018, Perez-Mazliah *et al*, 2020). Although clinical immunity may develop, parasitaemia may still persist, and this is attributed to the Th1 cells that favor immunosuppression. Through its production of IL-10 and IFNy, the differentiation into Tfh subsets could be hindered, and this in turn affects the proliferation of B cells (Hansen *et al*, 2017, Silveira *et al*, 2018).

The phenotype and function of immune cells such as T cells can be determined using flow

cytometry analysis at specific time points during the immune response. Characterization using known phenotypic markers for distinguishing CD4$^+$ T, CD8$^+$ T, B cells, and other immune cells, as well as markers of T cell activation, differentiation, proliferation, memory, exhaustion and transcription factors, provide important information regarding the functional properties of immune cells (Okada *et al*, 2008, Mahnke *et al*, 2013, Ndungu *et al*, 2013). For the major CD4$^+$ T cell subsets in response to malaria, Th1 cells secrete IFNγ and express Tbet, Ly6C, CXCR3 and CXCR6 (Kim *et al*, 2001, Marshall *et al*, 2011, Soon *et al*, 2020); while Tfh cells are characterized by their expression of CXCR5, PD1, Bcl6, Icos, and produces IL-21 (Kim *et al*, 2001, Crotty, 2011, Soon *et al*, 2020). The status of differentiation and maturation of T cells – from naïve to terminally differentiated – can also be determined using these markers at the protein or transcriptomic level, as well as its state of exhaustion or senescence.

1.4.1 Memory T cell development

The main characteristics of memory T and B lymphocytes are its generation after a contraction phase of the primary adaptive response, its long-term maintenance upon resolution of infection, and its increase in numbers after pathogen re-exposure to improve protection. CD8$^+$ T cell memory formation has been progressively described in numerous studies over the years (Kaech and Cui, 2012, Jameson and Masopust, 2018); on the other hand, memory CD4$^+$ T cells and B cells are less well understood. In the case of CD4$^+$ T cells, their differentiation into diverse functional subsets makes characterization of memory more challenging. As CD8$^+$ T cell memory subsets are better defined, some CD4$^+$ T cell studies determine the similarities in known T cell signatures with these lymphocytes (Hope *et al*, 2019, Kurup *et al*, 2019).

Studies on the Pcc model suggest that memory CD4$^+$ T cell fate commitment occurs early during the activation of naive cells. A widely used characterization of memory T cells first described in human circulating memory T cells (Sallusto *et al*, 1999), categorized memory subsets as effector memory (T$_{EM}$) and central memory cells (T$_{CM}$), which has been used to describe other model systems as well. These subsets can be distinguished through their cell surface markers, including lymph node-homing receptors such as CD62L and CCR7, which are expressed by T$_{CM}$ cells, thereby allowing them to home to secondary lymphoid tissue. T$_{CM}$ cells are also thought to possess stem cell-like properties, and are capable of self-renewal (Mahnke *et al*, 2013). In contrast, T$_{EM}$ cells are CD62L$^-$ and CCR7$^-$, and are capable of rapid effector function upon re-exposure, with preferential homing to peripheral lymphoid tissue

(Pepper and Jenkins, 2011). T helper 1 (Th1) cells and T follicular helper (Tfh) cells are identified as the more relevant effector subsets that may have potential mechanisms for immune memory generation (Zander *et al*, 2017, Opata *et al*, 2018, Yap *et al*, 2019, Carpio *et al*, 2020, Kunzli *et al*, 2020, Stephens *et al*, 2020). The phenotypes of memory T cells have been described in the different T cell lineages such as Th1 and Th2, albeit less so for Th17, Tfh and Treg lineages, owing to their plasticity, through which even lineage-committed cells can still gain additional functions or reprogramming depending on the environment (Gasper *et al*, 2014, Sallusto *et al*, 2018). It has been suggested that Th1, Th2 and Th17 cells that survive the contraction phase can become Tem cells, while Tfh cells with no lineage commitment can become Tcm cells (Pepper and Jenkins, 2011). Collectively, CD4+ T cell differentiation shows complexity such that characterizing the Th subsets that develop in response to pathogens such as malaria can be a challenge.

Aside from the use of CD127 expression as a marker to identify memory precursors and terminally differentiated subsets in CD8+ T cells, KLRG1 expression is also used to describe CD127^{lo}KLRG1^{hi} short-lived effector cells (SLECs) and CD127^{hi}KLRG1^{lo} memory-precursor effector cells (MPECs) (Joshi *et al*, 2007, Martin and Badovinac, 2018). A recent study challenged the assumption that all CD127^{lo}KLRG1^{hi} cells are short-lived, as they characterized a long-lived effector subset within this phenotype (Olson *et al*, 2013, Hudson *et al*, 2019, Renkema *et al*, 2020). Although these markers are established for CD8+ T cells, it is not considered to be a direct correlate for CD4+ T cells, and their analogues in humans are yet to be identified (Lees and Farber, 2010).

Studies on the lymphocytic choriomeningitis virus (LCMV) infection model provides much of our earlier knowledge on CD4+ T cell differentiation, particularly for memory CD4+ T cells. Genomic approaches such as single-cell RNA sequencing (scRNA-seq) have recently been used to further elucidate on this area of study, which provided interesting insights into the differences in the dynamics of the immune responses during malaria infection. A recent scRNA-seq study by Ciucci et al (2019) provided insights on how CD4+ T cell memory against LCMV chronic infection develops using an unbiased transcriptomics approach. Consistent with previous observations that is also applicable to the malaria model, they also found *Id2* and *Tbx21*-expressing Th1, and *Cxcr5* and *Icos*-expressing Tfh subsets as effector subsets, but also identified a novel T central memory precursor population (Tcmp), which expressed memory-related signatures such as *Ccr7, Bcl2* and *Id3*, but not *Il7r*. The presence of other memory precursor subsets such as Tcmp observed during the acute stage of response

suggest that there is an early fate decision warranted for memory precursors (Harrington *et al*, 2008, Marshall *et al*, 2011, Opata and Stephens, 2013).

1.4.2 *Plasmodium*-specific memory CD4⁺ T cell responses

Looking into *Plasmodium*-specific T cell responses, these immune cells provide protection by having naïve T cells activated (primed), clonally expanded and differentiate into effector T cells upon antigenic stimulation. After the contraction, some T cells remain as circulating central memory or effector memory T cells that patrol the lymphoid tissues (Gray *et al*, 2018, Sallusto *et al*, 2018, Kurup *et al*, 2019, Yui and Inoue, 2020). Previous studies in malaria and viral infection models use the upregulation of integrins CD11a and CD49d as activation markers, and as surrogate markers to identify pathogen-specific CD4⁺ T cells; at memory phase, the CD11a^hi^CD49d^hi/+^ CD4⁺ T cell phenotype is classified as antigen-experienced, pathogen-specific CD4⁺ T cells in infection models of *Plasmodium* spp., LCMV, and *L. monocytogenes*, among others (Butler *et al*, 2011, Kimura *et al*, 2016, Sebina *et al*, 2016).

The current availability of malaria-specific TCR-transgenic mouse lines allows for investigation of malaria specific CD4⁺ T cell responses while minimizing the influence of TCR diversity on the cell fate commitment of CD4⁺ T cells (Stephens *et al*, 2005, Stephens and Langhorne, 2010, Fernandez-Ruiz *et al*, 2017, Opata and Stephens, 2017). *Plasmodium*-specific CD4⁺ T cell responses have been studied using MSP-1-specific TCR-transgenic mouse line B5 (Stephens *et al*, 2005). The studies revealed how MSP-1-specific T cells are protective during the course of a chronic malaria infection with *P. chabaudi*, and elucidated the differentiation and maintenance of malaria-specific CD4⁺ T cells from primary infection to memory states (Opata *et al*, 2015).

Using IL-7Rα (CD127), CD62L, CD44, and CD27 as the main cell surface markers to distinguish memory from effector subsets, Opata et al showed different memory T cell subpopulations during persistent Pcc infection. The main T cell populations observed to arise during chronic infection were the IL-7Rα⁻ Teff, CD44^hi^IL-7Rα^hi^ Tmem, CD44^hi^IL-7Rα^hi^CD62L^lo^ effector memory (Tem), as well as the CD44^hi^IL-7Rα^hi^CD62L^hi^ central memory (Tcm) cells. Moreover, the effect of chronic infection on protection was also observed, wherein chronically stimulated cells were able to confer better protection compared to drug-treated mice, with the maintenance of early effector and effector memory subsets. These observations could explain the better protective immunity observed in humans with continuous exposure to malaria (Stephens *et al*, 2005, Stephens and Langhorne, 2010, Opata *et al*, 2015, Opata *et al*, 2018). Teff and Tem populations had increased frequencies of the IFNγ⁺TNF⁺IL-2⁻ Th1

phenotype, and the generation of these memory T cell subsets during chronic infection with higher cytokine production but poor proliferative capacity could also explain the suboptimal maintenance of protective immunity against malaria (Opata and Stephens, 2017, Ibitokou *et al*, 2023).

A more recently developed MHC-II restricted TCR transgenic mouse, PbT-II, provides another avenue to focus studies on a *Plasmodium*-specific clonal population of TCR transgenic CD4$^+$ T cells in C57BL/6 mice (Fernandez-Ruiz *et al*, 2017, Enders *et al*, 2021). The PbT-II mouse line was developed from CD4$^+$ T cells from a *P. berghei*-infected mouse, whose TCR is specific for heat shock protein 90 epitope (PbHsp90$_{484-496}$) common to *P. berghei*, *P. chabaudi*, *P. yoelii,* and *P. falciparum* (Enders *et al*, 2021). Single cell RNA-seq analysis of PbT-II cells using *P. chabaudi* model showed clearly that *Plasmodium*-specific CD4$^+$ T cells proliferate and bifurcate to Th1 and Tfh cells at both population and single clone level during malaria infection (Lönnberg *et al*, 2017). Furthermore, tracking of these differentiated CD4$^+$ T cells and scRNA-seq analysis during chronc *P. chabaudi* infection demonstrated that these Th1 and Tfh trajectories have minimal lineage plasticity, while heterogeneity among effectors was partially reset in memory phase (Lönnberg *et al*, 2017, James *et al*, 2018, Soon *et al*, 2020). The findings from recent PbT-II studies have been vital in this current study, wherein we aimed to further characterize malaria-specific immunological memory in the context of how a specific cytokine such as IL-27 affects CD4$^+$ T cells.

1.5 Factors affecting immune memory responses during malaria infection

The current view on the development of naturally acquired immunity to malaria is that it is slow, requires repeated infections, almost never achieves complete (sterile) immunity, but the loss of exposure leads to loss of immunity (Freitas do Rosario *et al*, 2008, Langhorne *et al*, 2008, Bediako *et al*, 2016, Ryg-Cornejo *et al*, 2016). For some parasite antigens, such as PfEMP1 in Pf parasite, antigenic variation allows immune evasion through its shift of the expression of multiple genes (Scherf *et al*, 2008).

The magnitude of response of CD4$^+$ T cells depend on the number of naïve CD4 present in the primary infection during priming, as well as on TCR (T cell antigen receptor) signaling, co-stimulation and cytokine which plays a role in the fate commitment of these cells(Gasper *et al*, 2014, Soon and Haque, 2018). Chronic stimulation negatively affect memory T cell responses, wherein activation-induced apoptosis occurs, such that memory cells are unable to expand upon secondary infection (Opata and Stephens, 2017, Hope *et al*, 2019).

Previous studies in mouse and humans suggest that malaria infection affects the production of memory cells. In the case of memory B cells, findings from field studies point to a possible altering of memory B cell profiles by *Plasmodium* infection, as it has been observed that atypical memory B cells (AMB) are generated in malaria-infected individuals. These AMBs have a Tbet[+] expression, which negatively affect antibody production capabilities of these cells (Weiss *et al*, 2009, Scholzen and Sauerwein, 2013, Perez-Mazliah *et al*, 2020). This has been viewed as a cause for an impaired response to malaria; however, there has been conflicting findings on its function, wherein some studies report on the AMB's exhausted phenotype (Weiss *et al*, 2009, Portugal *et al*, 2017, Perez-Mazliah *et al*, 2018), while others show evidence that they can potentially contribute to protection (Muellenbeck *et al*, 2013), although no human studies have yet shown direct evidence (Perez-Mazliah *et al*, 2020). Antigen-specific MBCs are also found to express the co-inhibitory markers PD1 and LAG3, which affects the cells' antibody production and parasite clearing capabilities (Butler *et al*, 2011).

1.6 Cytokine regulation of malaria immune memory responses

Cytokines produced by innate and adaptive immune cells shape the inflammatory environment, and dictate the nature of infection (Raeber *et al*, 2018). As naïve T cells are activated through antigen encounter, they receive TCR signals (signal 1), co-stimulatory signals (signal 2) as well as cytokine signals (signal 3), that determine the nature of T cell activation, proliferation and differentiation, and B cells are known to require similar signaling requirements. The response to cytokine signals depends on various factors, such as the context of the cytokine signaling, the cell type and differentiation status of the cell receiving the signal, as well as the presence of other cytokines (Delves *et al*, 2017). Elevated expression of cytokines during *Plasmodium* infection affect the differentiation of lymphocytes, leading to unique T or B cell subsets with mixed differentiation profiles that are observed in various studies, such as hybrid Th1/Tfh cells, Th1-like memory cells, or Th2-like Tfh CD4^{+} T cells (Carpio *et al*, 2015, Zander *et al*, 2017, Chan *et al*, 2020). Since it is known that the cytokines present during the first antigen encounter of B and T cells have a large influence on the nature of their responses (Delves *et al*, 2017), this section discusses some of the cytokines that play critical roles in the regulation of immune responses during malaria infection, with a focus on CD4^{+} T cells.

During the primary infection, type I IFNs are the earliest cytokines observed to be produced, that can either provide anti-parasitic (cell-mediated) immunity or decrease it, and cause

immunosuppression depending on certain conditions and contexts (McNab *et al*, 2015, Gowda and Wu, 2018). In particular, type I IFN-dependent production of the anti-inflammatory cytokine IL-10 has been observed following blood-stage *P. falciparum* infection in humans (Montes de Oca *et al*, 2016), as well as in experimental murine malaria models (Haque *et al*, 2011, Zander *et al*, 2016). Aside from type I IFNs, DCs produce other cytokines in response to malaria infection such as IFNγ, TNF, IL-12, and IL-6, and chemokines CXCL1, CXCL2, CCL2, CCL5, CXCL9, and CXCL10, which are involved in targeting an efficient cell-mediated parasite clearance (Gowda and Wu, 2018). These initial immune mechanisms would dictate the immunoregulatory networks that would be in place to provide a bridge to adaptive immune responses.

During the course of infection, IL-2 is induced shortly after TCR activation in CD4^{+} T cells, and during the memory phase produced more by Tcm rather than Tem cells (Sallusto *et al*, 1999). TNF is another early effector cytokine, which induces the transcriptional program of CD4^{+} T cells for the upregulation of cytokines, chemokines and other immune molecules. Innate immune cells also produce IL-12 and IL-18. Increased IL-12 leads to the preferential differentiation of CD4^{+} T cells to a Th1 phenotype. An increased Th1 bias of memory T cells is regulated by T cell-intrinsic STAT3 signaling, which is activated by interferons and IL-6, and was observed to promote more protective recall responses (Carpio *et al*, 2020). On the other hand, there are regulatory or anti-inflammatory cytokines including IL-10, IL-27, TGFβ, IL-21 and type I interferons, which regulates T cell differentiation, and can consequently negatively affect antigen-specific recall responses. Moreover, presence of IL-21 leads to more Tcm phenotype, while IL-2 drives Tem differentiation (Gasper *et al*, 2014). Although type I IFN contributes to protective immunity, it is also involved in the modulation of Th1 development during the blood-stage of the infection, and can consequently worsen the disease outcome (Gazzinelli *et al*, 2014, Montes de Oca *et al*, 2016). It has been established through several studies that a high ratio of inflammatory versus regulatory cytokines, such as IFNy/IL-10, is an indicator of disease severity (Mota and Rodriguez, 2017a).

For the memory phase, studies on the memory CD8^{+} T cells have revealed that antigen-specific memory CD8^{+} T cells are maintained for a long time after the primary response without antigen exposure and that cytokines such as IL-7 and IL-15 play critical roles for its maintenance with signaling via their receptors CD127 (IL-7R) and CD122 (IL-15R), respectively (Surh and Sprent, 2008, Hope *et al*, 2019). This is also established in CD4^{+} T cells, showing the importance of these cytokines in memory development.

1.6.1 IL-27 activity during malaria infection

Previous studies suggest that the regulatory cytokine IL-27 is affecting the induction and maintenance of memory CD4$^+$ T cells after malaria infection (Gwyer Findlay *et al*, 2014, Kimura *et al*, 2016, Sukhbaatar *et al*, 2020). IL-27 is a heterodimeric immunoregulatory cytokine from the IL-12 family, composed of protein subunits Epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28 (or simply, p28), and receptor subunits WSX-1 (also known as IL-27Rα) and gp130. IL-27p28 is the main protein subunit that distinguishes IL-27, while EBI3 is known to partner with other cytokine subunits to form other heterodimeric cytokines, such as IL-35, which has EBI3 paired with IL-12p35 (Pflanz *et al*, 2002, Hunter and Kastelein, 2012). IL-27 is secreted mainly by dendritic cells and macrophages, and has been shown to exert pro- and anti-inflammatory effects depending on the disease (Yoshida and Hunter, 2015). CD4$^+$ T cells were also found to be minor producers of IL-27 (Kimura *et al*, 2016). Binding of IL-27 to its receptor triggers the activation of the Janus kinase (JAK), signal transducer and activator of transcription (STAT), and mitogen-activated protein kinase (MAPK) signaling pathways, which mediate cellular processes such as T cell activation and differentiation (Yoshida and Hunter, 2015).

Early studies on infection models of *Listeria monocytogenes* and *Leishmania major* suggested that IL-27 promotes Th1 immune responses. However, IL-27 has also been found to inhibit protective immunity and immunopathology during infections with *Toxoplasma gondii, Trypanosoma cruzi* and helminth infections by negatively regulating Th1 and Th2 responses (Yoshida and Hunter, 2015). Furthermore, IL-27 is found to inhibit IL-2 production by CD4$^+$ T cells (Owaki *et al*, 2006, Villarino *et al*, 2006), inhibit the generation of Th17 cells (Stumhofer *et al*, 2006), and promote IL-10 production by CD4$^+$ T cells (Awasthi *et al*, 2007). In a malaria infection model, IL-27-producing *Plasmodium*-specific CD4$^+$ T cells, Tr27, are generated during malaria infection and control immunopathology, indicating its important role in the immune response (Kimura et al, 2016). Studies using IL-27 receptor-deficient (*WSX-1$^{-/-}$*) mice have shown that in the absence of IL-27 signaling, CD4$^+$ T cells have enhanced immune responses during *Plasmodium chabaudi chabaudi* AS (Pcc) infection, leading to immunopathology (Findlay *et al*, 2010, Gwyer Findlay *et al*, 2014, Sukhbaatar *et al*, 2020). WSX1 signaling inhibits terminal differentiation into Th1 KLRG1$^+$ cells during malaria infection and regulates the pathogenic response (Villegas-Mendez *et al*, 2013, Gwyer Findlay *et al*, 2014). Moreover, a study in Mozambique showed that plasma IL-27 levels were upregulated in malaria-infected patients, which seemed to correlate with the level of parasitaemia, but had no relation to disease severity (Otterdal *et al*, 2020). Clarifying the mechanisms of IL-27 can

elucidate on immunological memory to malaria, and can inform future vaccine development. Its utility in determining immune status of populations will also be explored.

1.7 B cell and antibody responses during malaria infection

Antibodies produced by B cells are main effectors for secondary responses during blood-stage malaria infection. The progression of B cell maturation follows a sequential process, with various developmental phases to ultimately produce B lymphocytes that are capable of generating functional antibodies, also known as immunoglobulins. The membrane-anchored Igs, known as BCRs, are first developed in the bone marrow, wherein each B cell will have a unique BCR. Ig molecules are generally structured to have two light and two heavy polypeptide chains, wherein the structure of their heavy chains dictate effector function, and their quality may be reflected by their avidity, or overall binding strength to the antigenic epitope. BCRs can be categorized into 5 main isotypes or classes: IgM, IgD, IgG, IgA and IgE (Delves *et al*, 2017, Ssewanyana *et al*, 2017). Immature B cells that are not self-reactive are selected for survival, and migrate to secondary lymphoid organs such as the spleen, where they mature into naïve (mostly IgM^+IgD^+) B cells (Zabriskie, 2009, Murphy and Weaver, 2017). Presence of their cognate antigens activates naïve B cells, through T cell-dependent or independent mechanisms (Bortnick *et al*, 2012, Wong *et al*, 2020).

T_{FH} cells and GCB cells have essential roles in B cell-mediated memory generation (Crotty, 2014). In T cell-dependent B cell responses, germinal centers (GCs) are formed within the center of follicles bordered by T cell zones in secondary lymphoid organs, wherein B cells expressing high-affinity antibodies are selected to enter the GC pathway, and differentiate into antibody-secreting cells (ASCs) or plasma cells, and memory B cells, while others may differentiate into short-lived plasmablasts with low affinity for antigens (De Silva and Klein, 2015). Although it is widely believed that only short-lived plasma cells are generally induced from T cell-independent antibody production, there is evidence that long-lived plasma cells can also develop from T cell-independent responses (Weisel *et al*, 2016, Tomayko and Allman, 2019, Wong *et al*, 2020). Nonetheless, for humoral immunity to be induced effectively, antigen-specific $CD4^+$ T helper cells and GC reactions are most often required for B cell proliferation, affinity maturation and class-switching of produced antibodies of higher affinity (Dups *et al*, 2014, Perez-Mazliah *et al*, 2020, Baumgarth, 2021). B cells activated with the help of T_{FH} cells clonally expand and differentiate through 3 possible pathways in T cell-dependent responses – into short-lived plasmablasts, and GC B cells that later on become long-lived plasma cells (LLPCs) or memory B cells (MBCs) (De Silva and Klein, 2015, Weisel *et al*, 2016,

Ly and Hansen, 2019).

In the GC reaction process, activated B cells proliferate and mostly undergo isotype switching from IgM to IgG, IgA or IgE through class-switch recombination, and affinity maturation through somatic hypermutation of Ig genes. The diversification of the BCRs through isotype switching results in changes in effector function while retaining their antigen specificity (Brynjolfsson *et al*, 2018). After the elimination of pathogens, most of antigen-specific B cells as well as plasma cells undergo apoptosis, as typical of lymphocytes, and immune responses are downregulated. A portion of antigen-specific B cells survive and generate MBCs that remain quiescent and in circulation until their re-encounter with the antigen. In addition, a portion of plasma cells become bone marrow-residing LLPCs, which can keep producing circulating antibodies without antigenic stimulation, and can then be detected in the serum of those who were previously exposed by the pathogen (Nogaro *et al*, 2011, Hviid *et al*, 2015). Thus, serological evaluation of the presence of pathogen-specific antibodies in samples can provide a reliable metric to estimate pathogen exposure within a population.

In the murine malaria model, it has been observed that the memory B cell population following malaria infection consisted of 3 MBC subsets of the IgM, IgG and IgD isotypes (Krishnamurty *et al*, 2016, Brown *et al*, 2022). Although IgG antibodies (particularly IgG1 and IgG3) are considered the most abundant and typically contributes the most to anti-malarial protective responses (Compans *et al*, 2005), it has been reported that IgM+ MBCs are also observed during the memory phase of malaria infection, and are also capable of proliferation and antibody production in both mouse and humans (Krishnamurty *et al*, 2016, Hopp *et al*, 2021, Thouvenel *et al*, 2021, Brown *et al*, 2022). Some of these studies suggest that IgM+ MBCs are capable of producing IgM-derived antibodies with higher avidity even than those derived from IgG+ MBCs (Thouvenel *et al*, 2021).

The antigen-specific MBCs and LLPCs are known to persist for long periods of time in different infection environments; however, it is said to be inefficiently produced in the case of malaria infections (Scholzen and Sauerwein, 2013, Ryg-Cornejo *et al*, 2016, Silveira *et al*, 2018, Perez-Mazliah *et al*, 2020). Although repeated exposure to antigens typically lead to protective antibody responses against malaria (Struik and Riley, 2004), it has been observed in human studies that *P. falciparum* infection can interfere with B cell function, wherein an increased exposure to malaria antigens did not result in an increased affinity maturation and avidity of malaria-specific antibodies, with some studies even observing an inverse relation of antibody avidity to level of transmission intensity (Ssewanyana *et al*, 2017), as well as no

observed increase of malaria-specific MBCs in areas of higher transmission (Wipasa *et al*, 2010, Ndungu *et al*, 2013, Jahnmatz *et al*, 2022).

1.7.1 Longevity of memory B cell and antibody responses in malaria

There is a debate on the longevity of antibody responses following malaria infection. Immune memory generation in malaria seems to be dysfunctional (Hviid *et al*, 2015, Ryg-Cornejo *et al*, 2016, Ly and Hansen, 2019, Perez-Mazliah *et al*, 2020). This is due in part to the observation that *Plasmodium*-specific antibody levels rapidly decrease following acute malaria infections – suggesting that the response is short-lived and suboptimal (Kinyanjui *et al*, 2007, Weiss *et al*, 2010, Perez-Mazliah *et al*, 2020). Although the mechanisms that drive the variability of responses to specific malaria antigens remain unclear, what is apparent is that there are several factors that can influence the longevity, specificity and durability of antibody responses produced against malaria infections. This would include the nature of the infection, as the differing biology of the *Plasmodium* species (as discussed in an earlier section), such as for *P. falciparum* and *P. vivax*, would elicit a different breadth of species-specific antigens, which the variability of the antibody pool would depend on (Achtman *et al*, 2005, King *et al*, 2015). Antibody development can also be affected by the differences in the antigens, as their presentation to the immune system can influence the functionality of the antibodies produced and their corresponding decay rates, which will be specific for the alleles of these polymorphic and variant antigens (Achtman *et al*, 2005, Mugyenyi *et al*, 2017).

Previous studies suggest that humoral immune memory to malaria infection develops relatively short-lived plasmablasts instead of generating LLPCs and MBCs (Achtman *et al*, 2005, Wipasa *et al*, 2010, Portugal *et al*, 2013). Several studies provided evidence that antibody and MBCs capable of inducing immune responses are maintained despite infrequent exposure, but results vary depending on study populations (Wipasa *et al*, 2010, Ndungu *et al*, 2013, Tran *et al*, 2013, Fowkes *et al*, 2016, Mugyenyi *et al*, 2017, Perez-Mazliah *et al*, 2020). Dorfman et al (2005) provided the first report on the levels of human antibody and B cell responses against malaria infection, and their results suggested that B cell memory can be maintained but not in all exposed individuals. Immunogenetics of populations affects variation among individuals. Immune responses to malaria infection as well as vaccine could be affected by factors such as the human leukocyte antigen (HLA) alleles, as suggested by a study of the RTS,S vaccine responses potentially being affected by the heterogeneity of HLA genotypes (Nielsen *et al*, 2018).

Compared with tetanus toxoid that was able to maintain antigen-specific MBCs that correlated

with antibody levels, some malaria-exposed Kenyan children and adults did not have circulating MBCs despite having detectable antimalarial antibodies (to antigens merozoite surface protein 1 / MSP119, apical membrane antigen 1 / AMA1, and cysteine-rich interdomain region 1a / CIDR1a)(Dorfman *et al*, 2005). Other studies showed that long-lived MBCs can be maintained even after years of no exposure or antigen stimulation, such as in the adults of a low transmission setting in Thailand (Wipasa *et al*, 2010), in Kenya (Ndungu *et al*, 2013), and in previously exposed Swedish travelers (Ndungu *et al*, 2013). However, there is still the question of why this observation does not seem to be consistent.

1.7.2 Application of serological tools for measuring malaria exposure

Measurement of antibody production through serological tools can identify past exposure to diseases, and depending on the availability of antigen-specific serological markers (Corran *et al*, 2007, Drakeley and Cook, 2009), could potentially distinguish recent and historical exposure by using dried blood spot or serum samples (Corran *et al*, 2008, Wu *et al*, 2019). Since LLPCs maintain production of antibodies, which remain in circulation for years after the initial infection, and their presence and levels appear to differ among malaria antigens to which they bind, the persistence of malaria-specific antibodies can provide insights into whether an infection is currently active or historical, contingent on the stability and duration of antibody persistence (Lynch *et al*, 2016). Historical exposure markers can be detected even when the infection had been naturally cleared or treated, while recent exposure markers can potentially identify asymptomatic exposure, which can estimate a possibly current disease transmission at a subclinical level (Helb *et al*, 2015, van den Hoogen *et al*, 2020c, Wu *et al*, 2020a).

The most common detection method used for analyzing serological measures is the enzyme-linked immunosorbent assays (ELISA). This assay conventionally measures a single analyte at a time, and quantitative measures are analyzed with statistical models such as the sero-catalytic models, which incorporate age to estimate malaria transmission intensities (Corran *et al*, 2007, Sepulveda *et al*, 2015). Recently, serological evaluations have shifted to multiplex approaches, such as the multiplex bead-based assays, and protein microarray, which allows for a high-throughput screening of numerous antigenic targets at a time, even from different pathogens (malERA Refresh Consultative Panel, 2017, Chan *et al*, 2022, Oulton *et al*, 2022). Sero-surveillance studies for *P. falciparum* and *P. vivax* previously focused on antibodies representing cumulative exposure such as AMA1 and MSP1$_{19}$, but with the availability of serological markers associated with recent exposure, there is now the potential to detect

changes, if any, in recent malaria transmission (Helb *et al*, 2015, Longley *et al*, 2020). For *P. falciparum* exposure, the identified recent exposure markers were PfGLURP R2 (glutamate rich protein), Etramp5.Ag1 (early transcribed membrane protein 5), and GEXP18 (Gametocyte exported protein 18), and were used for serological assessment studies in Africa and the Caribbean (Helb *et al*, 2015, van den Hoogen *et al*, 2020c, Wu *et al*, 2020a). Longley *et al* (2020) also recently identified a panel of 8 *P. vivax* recent exposure markers, which included reticulocyte binding protein 2 (RBP2b) and $MSP1_{19}$ among others, that can identify individuals at risk of P. vivax relapses. The study also assessed mathematical models such as logistic regression and machine learning approaches such as random forests for classifying recent vivax malaria infection (Longley *et al*, 2020). The application of these recent exposure markers in novel surveillance approaches can potentially inform targeted intervention strategies, and can also be applied for disease outbreak predictions (Cook *et al*, 2011, Greenhouse *et al*, 2019, Longley *et al*, 2020, Surendra *et al*, 2020).

Numerous studies showed the utility of measuring serum antibody levels to malaria-specific antigens in estimating malaria transmission intensities in elimination settings (Corran *et al*, 2007, van den Hoogen *et al*, 2015, Dewasurendra *et al*, 2017, Idris *et al*, 2017a, Simmons *et al*, 2017, Keffale *et al*, 2019). The use of serosurveillance allowed for observing how intensity of transmission correlates with the longevity of antibody responses (Drakeley *et al*, 2005, Bousema *et al*, 2010, Biggs *et al*, 2017). Knowledge of the underlying mechanisms of the maintenance of IgG antibody responses to malaria-specific antigens, as well as the correlation between assay responses and immune protection, may offer insights into the development of naturally acquired immunity within populations (King *et al*, 2015, Proietti *et al*, 2020).

1.8 Immunity to malaria: relevance for elimination (Added value of current study)

Unlike other diseases, clinical malaria requires repeated exposure to *Plasmodium* infection for naturally acquired humoral and cellular immunity to develop, suggesting that the immune memory response is impaired (Achtman *et al*, 2005, Langhorne *et al*, 2008, Fowkes *et al*, 2016, Ryg-Cornejo *et al*, 2016, Ly and Hansen, 2019). Insights on how malaria evades our immune system is important for vaccine development. What makes it even more difficult to identify antigenic targets for vaccination is the presence of different stages of the *Plasmodium* life cycle that would require different types of immune responses, and also the inherent ability of the parasite to have antigenic variation and allelic polymorphisms (Good, 2001, Moormann

et al, 2019).

Understanding the immune responses generated by the human body to control and eliminate the parasite is crucial to the development of effective vaccines and treatments. Studies using rodent malaria models have provided important insights into the immunological mechanisms involved in controlling malaria, as well as the development of new treatments and vaccines. Over the past few decades, extensive research has been conducted to examine the immunological mechanisms involved in generating malaria-specific memory responses. However, as with most models, it may be difficult to translate the findings to the human context. Nonetheless, devising ways to interpret the observations in the mouse model should always be a welcome challenge.

Of interest for countries aiming for elimination is evaluating the impact of a decrease in transmission on the immunity or vulnerability of a population, especially in differing endemic settings (Fowkes *et al*, 2016, Mugyenyi *et al*, 2017, WHO 2017b). For the Philippines in particular, it is important to know how the changing malaria situation could be affecting the immune status of endemic populations in Palawan, which is considered to be the last frontier to conquer to achieve malaria elimination in the country.

Ultimately, it is hoped that this PhD study can contribute to a better understanding of immune mechanisms to malaria for the development of successful vaccines, as well as aid in devising targeted intervention strategies for elimination. While mouse models are able to provide insights into immune mechanisms of malaria, it remains essential to analyze the link of the important findings from the mouse model to the context of human immune responses. Despite the limited access to immune cells in human studies, longitudinal studies of malaria-infected individuals with well-timed sample collections are able to address these limitations(Achtman *et al*, 2005, Wipasa *et al*, 2010, Fowkes *et al*, 2016).This project then aims to contribute to this currently limited area of study.

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Chapter 2 : Research Aims and Objectives

The main aim of this PhD project was to investigate the induction and maintenance of immunological memory responses to malaria infection. This PhD project addressed the need to evaluate the mechanisms of modulation of humoral and cellular immune memory responses to malaria, which can inform the development of future intervention strategies.

The study utilized two approaches – a mouse model for the analysis of underlying immune memory mechanisms against malaria infection, and human studies to investigate the factors affecting the maintenance of immune memory. The specific objectives of the study are the following:

1. To analyze the induction and maintenance of malaria-specific memory CD4$^+$T cells in the blood-stage infection of malaria in a Pcc infection model, and investigate the role of cytokine IL-27 in the modulation of malaria-specific cellular and humoral memory responses:

    a. To distinguish the phenotypic and functional characteristics of *Plasmodium*-specific memory cells maintained in the presence or absence of IL-27 signaling;

    b. To determine the effect of chronic stimulation on the maintenance of *Plasmodium*-specific immune memory cells; and

    c. To describe the mechanisms of IL-27 in the modulation of immune memory responses

2. To examine the effect of varying transmission levels and differential exposure to natural human malaria infection on the generation and maintenance of malaria-specific immune responses:

    a. To evaluate different statistical and machine learning methods for investigating multiplex malaria-specific antibody response data and predicting exposure to malaria;

    b. To categorize recent and historical exposure to malaria using antimalarial antibody response data, and formulate a prediction method of classification for use in cohort sampling;

    c. To develop a study protocol for a longitudinal cohort study in the Philippines for characterizing and comparing the longevity, and the Pf-specific memory T cell, B cell and antibody responses of malaria-exposed individuals in areas of varying levels of malaria endemicity.

The use of mouse models allowed for an in-depth analysis of mechanisms of the seemingly defective immune memory responses against *Plasmodium* infection. The malaria-specific CD4$^+$ T cell memory is not as well-studied as other aspects of the response, despite its established crucial role during blood-stage infection. This then became the main focus of the study in the mouse model, along with investigating how chronic stimulation and specific immune regulators influence immunological memory. In particular, the cytokine IL-27 was previously observed to affect the generation and maintenance of CD4$^+$ T cell memory during malaria, and its regulatory role in this immunological context was elucidated through examining PbT-II cells during the course of malaria infection.

For the approach using human studies, the antibody responses to malaria in sites with varying transmission intensities was examined through available multiplex serology data from a cross-sectional study in the Philippines. Immunological studies in humans are limited to studying immune cells in the peripheral blood. Despite this limitation, studies from the recent decade have provided better insights on the mechanisms of naturally acquired immunity to malaria. However, the concept of immunological memory to malaria for both T and B cells are still not well understood. The simultaneous analyses of malaria-specific antigens have been used in serological surveys using serum or dried blood spot samples to measure antibody levels as proxy for malaria transmission intensity. Through the analysis of available antibody response data from a previous field study in the Philippines, I evaluated different methods of analyzing antibody responses to predict the status of malaria exposure of individuals – whether they are historically exposed, recently exposed, or not exposed. Finally, I also developed a protocol for the planned human cohort study, which aims to characterize the maintenance of the memory cells in malaria-infected and historically exposed individuals. The developed method of analysis that can identify historically exposed individuals will be employed for the participant recruitment of this planned human cohort study in the Philippines, which will provide insights on cellular and humoral aspects of immunological memory to malaria in an elimination context.

This comprehensive study of memory responses aims to generate entirely novel data relating to the longevity of immunological memory to malaria at the cellular level that can potentially contribute to developing novel vaccine approaches and interventions for targeting elimination.

Chapter 3 : Characterization of malaria-specific memory responses in the *Plasmodium chabaudi* mouse model

Chapter overview

This chapter focuses on the mouse studies, wherein I characterized the *Plasmodium*-specific immune memory responses in the chronic *Pcc* infection model. It has been recognized that the cellular aspect of the immune response, particularly of CD4$^+$ T cells, is a gap that needs to be addressed for a better understanding of the memory responses against chronic malaria infection. Presented here are important findings that provide better insights on the complex nature of the immunological memory against malaria.

This chapter is divided into 2 sections. Chapter 3.1 provides a background on how I arrived at the main research focus for the mouse studies. It is further divided into 3 subsections, wherein I presented the initial experiments conducted to observe the immune memory responses to malaria. Chapter 3.2 showed the main results of the study in a manuscript format. It mainly described how IL-27 cytokine signaling affects antimalarial memory responses, using experimental designs optimized in Chapter 3.1.

Chapter 3.1: Preliminary experiments for mouse study

Background

It has been previously shown that CD4$^+$ T cells play a vital role in the elimination of parasites during the blood-stage of malaria infection, and various factors affect its induction and maintenance (Soon and Haque, 2018). One such factor is the action of various cytokines, which have context-dependent functions in shaping immune responses. *Plasmodium* infections are known to induce pro-inflammatory and immunomodulatory cytokines (as described in Section 1.6) that are required to be balanced for a better clinical outcome; however, there is still much to learn about the complex mechanisms of the host immune response against malaria, including immunological memory.

This aspect of the study wished to determine how the mouse model can be used to characterize the nature of the immune memory response during chronic malaria infection. As it aimed to characterize malaria-specific CD4$^+$ T cell responses using the PbT-II system (Fernandez-Ruiz *et al*, 2017, Enders *et al*, 2021), it also gave focus on a cytokine of interest, IL-27, which had been shown to modulate the Th1 development of CD4$^+$ T cells in the malaria context (Findlay *et al*, 2010); moreover, with its pleiotropic nature it could potentially influence other aspects of the innate and adaptive immune responses. IL-27 is a cytokine consisting of two subunits, EBI3 and p28, forming a heterodimer. It is produced by various immune cells,

primarily APCs such as dendritic cells and macrophages, in response to various stimuli (Yoshida and Hunter, 2015). IL-27 engages a receptor complex composed of 2 subunits: WSX1 (IL-27Rα), which is the main subunit responsible for binding IL-27, and gp130, which serves as a signal transducer common to other cytokine receptors (e.g., IL-6). The co-expression of WSX1 and gp130 have been observed in various cell types, including T cells, B cells, natural killer (NK) cells, dendritic cells (DCs), macrophages, and endothelial cells, which underscores this cytokine's broad immunomodulatory effect (Pflanz et al, 2004, Hunter and Kastelein, 2012). IL-27 signaling involves the activation of the JAK/STAT pathway, and the diversity of JAK and STAT present in immune cell subsets dictate its mediated function (Jafarzadeh et al, 2020). Its context-dependent effect has been highlighted in particular in CD4$^+$ T cells, where its signaling has been shown to activate the STAT1 and Tbet transcriptional pathway and promote Th1 differentiation, but also, in the opposite manner, induce STAT3-dependent IL-21 production, thereby sustaining IL-10 expression and suppressing T cell function (Batten et al, 2010, Yoshida and Hunter, 2015).

Based on previous findings in the NU Immunology Laboratory, it has been observed that $WSX-1^{-/-}$ mice, which lack IL-27 signaling, had increased memory responses of CD4$^+$ T cells after chronic Pcc infection (Sukhbaatar et al, 2020), suggesting its role in the development and maintenance of CD4$^+$ T cell memory. The preliminary experiments described in this section were conducted to explore these concepts further. The findings from these experiments established the assays used for the main experiments detailed in this chapter.

3.1.1 Flow cytometry analysis of immune cells during malaria infection

As listed in Table 3.1, known phenotypic markers of CD4+ T, CD8+ T, B cells, and other immune cells, as well as markers of T cell activation, differentiation, proliferation, memory, exhaustion and transcription factors, were analyzed using flow cytometry for characterizing the immune response after malaria infection, with a particular focus on the persisting populations of memory CD4+ T cells (Okada *et al*, 2008, Mahnke *et al*, 2013, Ndungu *et al*, 2013). Through CD4+ T cell adoptive transfer experiments, *Plasmodium*-specific CD4+ T cell responses were characterized during the course of the malaria infection from acute to memory phase using the PbT-II cells (Fernandez-Ruiz *et al*, 2017), while other immune cells were also observed. PbT-II cells are backcrossed to CD45.1 and thus, can be distinguished from the CD45.2+ recipient mice by cell surface staining. Production of IFN-γ, TNF, IL-10, and IL-2 in response to PbT-II peptides or PMA and ionomycin stimulation were also examined by intracellular cytokine assay. Aside from wild-type B6 mice, mouse models lacking IL-27, namely *Il-27*-/- (*p28*-/-) and *Ebi3*-/- mice, were also used as recipients.

Table 3.1. Surface and intracellular molecules used as markers for phenotype and functional analysis

Phenotype/function	Mouse model	Human
T cells	CD3 TCRβ CD4 CD8	CD3 TCRα/β CD4 CD8
B cells	B220 CD19 GL7 CD95	CD19 CD21
Activation markers, Proliferation	CD11a CD44 CD49d Ki67	CD11a CD45RO Ki67
Memory / effector markers, Differentiation	IL-7Rα (CD127) CD62L KLRG1	CD45RA IL-7Rα (CD127)* CD62L* KLRG1*
Inhibitory markers	PD1 LAG3	PD1 LAG3
Th1 / Tfh subset markers, transcription factors	CXCR5 Ly6C CXCR3 CXCR6 TCF1 Tbet Bcl6 Egr2	CXCR5 Ly6C CXCR3 CXCR6 TCF1 Tbet Bcl6 Egr2
Intracellular cytokines	IFN-γ TNF IL-2 IL-10	IFN-γ TNF IL-2 IL-10

*Although the same surface molecules are found in humans, their utility as markers have not been well-studied.

For the optimization of the flow cytometry panels, the phenotype and function of the immune cells in PB and spleen were determined using the markers described above at specific time points during the immune response – specifically, after T cell activation (day 7 pi), and during the chronic and memory phase (day 14 onwards). Gating strategies and representative flow cytometry plots showing typical characteristics of host CD4+ T cells and PbT-II cells are in Fig 3.1.1.
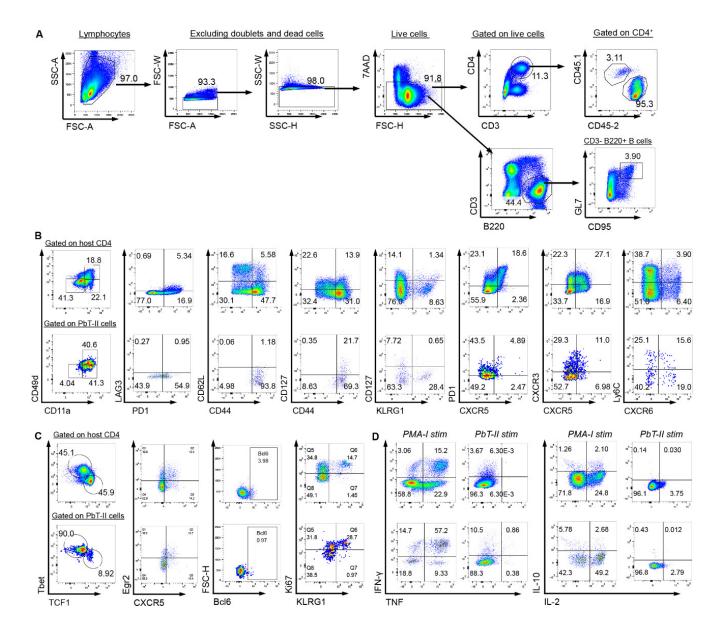
Figure 3.1.1 *Plasmodium*-specific PbT-II cells had expectedly distinct phenotype from polyclonal CD4+ T cells.

A. Gating strategy for the analysis of PbT-II cells and other immune cells. in PB and splenocyte samples. Cells were gated on lymphocytes, excluded doublets and dead cells, then gated on CD4+ T cells, which were gated on the congenically marked PbT-II cells (CD45.1+) and host CD4 (CD45.2+). Both host CD4 and PbT-II cells were gated on different phenotypic markers, transcription factors, and cytokines. CD3, B220, CD95 and GL7 expressions were used to analyze populations of CD3-B220+CD95+GL7+ splenic germinal center (GC) B cells.

B. Representative flow cytometry profiles showing expressions of surface markers of interest in host CD4 and PbT-II cells: CD11a, CD49d, PD1, LAG3, CD44, CD62L, CD127, KLRG1, CXCR5, CXCR3, CXCR6 and Ly6C.

C. Representative flow cytometry profiles showing expressions of transcription factors of interest in host CD4 and PbT-II cells: Tbet, TCF1, Egr2, Bcl6 and Ki67, in relation to surface markers (e.g. CXCR5 and KLRG1).

D. Representative flow cytometry profiles showing intracellular cytokine staining for IL-2, IL-10, IFNγ and TNF production of host CD4 and PbT-II cells.
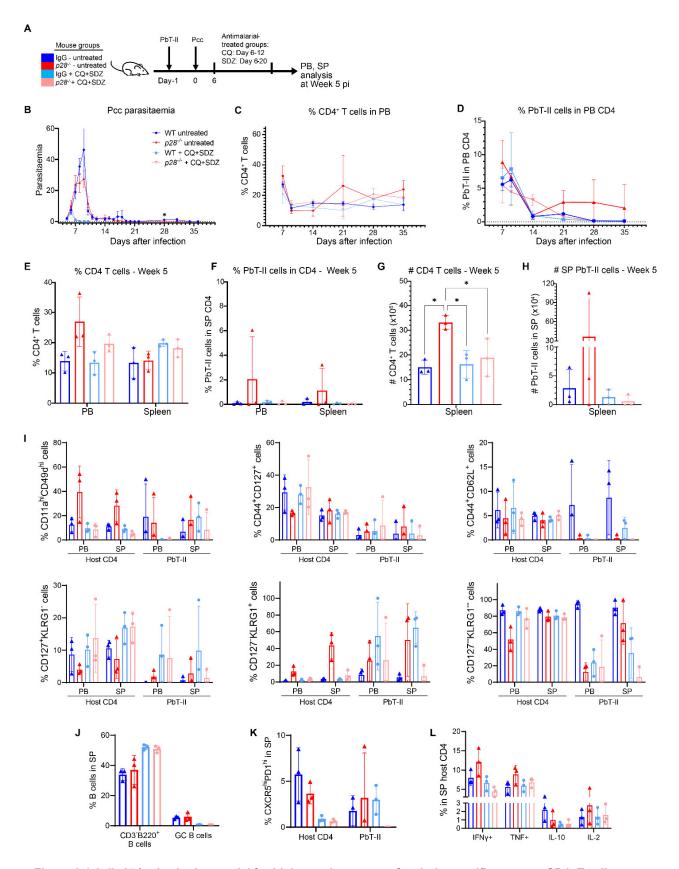
Figure 3.1.2. IL-27^{-/-} mice had potential for higher maintenance of malaria-specific memory CD4⁺ T cells.

A. Experimental scheme. PbT-II cells (1 x 10^6) were transferred via iv injection to WT and *Il27$^{-/-}$* (*p28$^{-/-}$*) mice before Pcc infection, and half (n=3) of the mice were treated with CQ+SDZ starting Day 6 pi. Peripheral blood was collected for monitoring of PbT-II cell proportions on Days 7, 9, 14, 21, 28, and 35 (final PB and spleen analysis). Phenotype of PbT-II cells and other immune cell types were analyzed using antibody fluorescence staining of surface markers, as well as intracellular cytokine staining after PMA-ionomycin and PbT-II peptide stimulation.

B. Parasitaemia monitoring of Pcc-infected mice, showing chronic stimulation of untreated WT and *Il27$^{-/-}$* mice and parasite clearance in treated mice.

C-D. CD4$^+$ T cell (C) and PbT-II cell (D) proportions in peripheral blood (PB).

E-F. Summary graphs of CD4$^+$ T cell (E) and PbT-II cell (F) proportions in the PB and spleen at Week 5 (final day of analyses).

G-H. Summary graphs of total counts of CD4$^+$ T cells (G) and PbT-II (H) cells in spleen samples.

I. Proportions of indicated phenotypes of host CD4 and PbT-II cells in peripheral blood (PB) and spleen (SP) at Week 5 pi.

J-K. CD3$^-$B220$^+$ total B cells and CD3$^-$B220$^+$CD95$^+$GL7$^+$ germinal center (GC) B cell profiles in the spleen (J), as well as GC Tfh CD4$^+$ T cell profiles (K) for each treatment group at Week 5 pi.

L. Cytokine production after PMA-ionomycin stimulation of host CD4 and PbT-II cells at Week 5 pi.

Data is representative of 2 experiments of similar experimental set-ups with 3 mice per treatment group, but with different PbT-II cell numbers transferred (5 x 10^5 or 1 x 10^6).

The use of antimalarial treatment allowed for the observation of the effect of acute versus chronic Pcc infection on the PbT-II cells (Fig 3.1.2). Kinetics of CD4$^+$ T cells and PbT-II cells in peripheral blood (Fig 3.1.2C, D) showed a trend of higher proportions for antimalarial-untreated *Il27$^{-/-}$* mice from Day 21 onwards, while antimalarial-treated *Il27$^{-/-}$* mice along with the treated and untreated B6 mice just showed a maintained low level of CD4$^+$ T and PbT-II cells in the blood from Day 14 onwards. When comparing the proportions of total CD4$^+$ T cells and PbT-II cells at Week 5 pi, no significant differences were observed; however, total CD4$^+$ T cell counts in the spleen was highest in untreated *Il27$^{-/-}$* mice, and there was also a trend of higher maintenance of malaria-specific PbT-II cells in these mice, suggesting that the maintenance of malaria-specific CD4$^+$ T cells is being inhibited by IL-27, and this occurrence required chronic stimulation. The next set of experiments then focused on antimalarial-untreated groups to further determine the effect of the absence of IL-27 during a chronic infection.
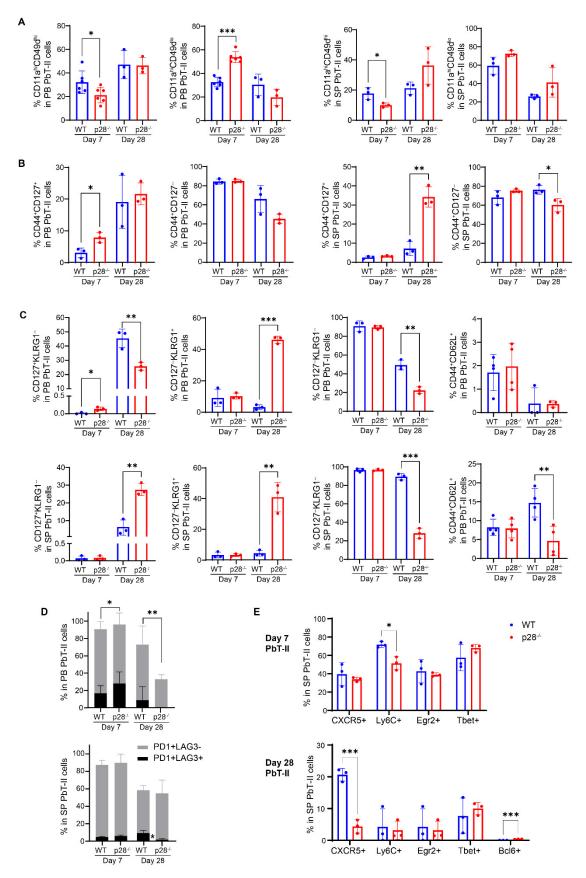
Figure 3.1.3. IL-27 affects shift towards Th1 differentiation during chronic *Plasmodium* infection.

A-B. Phenotypic profiles (CD11a^{hi}CD49d^{hi} and CD11a^{hi}CD49d^{lo} in A, and CD44+CD127^{+} and CD44+CD127- in B) of PbT-II cells at Days 7 and 28 pi in PB (left) and SP (right) of WT B6 and IL27$^{-/-}$ mice.

C. CD127/KLRG1 and CD44/CD62L profiles of PbT-II cells in PB (top graphs) and SP (bottom) at Days 7 and 28 pi for WT and IL27^{-/-} mice.

D. Proportions of PD1 and LAG3 co-expressions in PbT-II cells at Day 28 pi.

E. Expressions of indicated surface markers and transcription factors at Day 7 and 28 pi.

Data are representative of 2 experiments with 3-4 mice each per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with significance assessed by Student's t test per time point. Error bars represent SD.
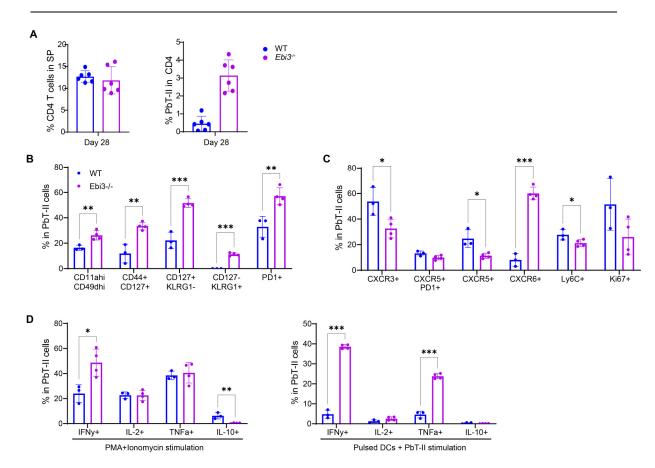
Figure 3.1.4. CD4+ T cells in EBI3^{-/-} mice exhibit shift towards Th1 differentiation during chronic _Plasmodium_ infection.

A. Proportions of CD4+ T cell in SP and PbT-II of WT B6 and Ebi3^{-/-} mice at Day 28 pi, with data pooled from 2 experiments.

B-C. Phenotypic profiles of WT B6 and Ebi3^{-/-} for activation and memory markers (B), and differentiation and proliferation markers (C) at Day 28 pi.

D. Expression of cytokines IFNy, IL-2, TNF and IL-10 of Day 28 PbT-II from WT B6 and Ebi3^{-/-} mice after a 4-hour stimulation with PMA-ionomycin (left) and pulsed DCs with PbT-II peptide (right).

Data are representative of 2 experiments with 3-4 mice each per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with significance assessed by Student's t test per time point. Error bars represent SD.

In comparing antimalarial-treated and untreated mice, it was expectedly observed that untreated mice had lower proportions of CD44$^+$CD127$^+$ memory CD4$^+$ T cells, which was lower still for PB than spleen. CD44+CD62L+ central memory CD4$^+$ T cells (Tcm) had comparable levels in all groups and as previous studies also observed that CD44+CD62L-effector memory cells are predominantly maintained in the Pcc chronic infection model rather than Tcm (Stephens and Langhorne, 2010, Opata *et al*, 2015), this was not investigated further. With the trend of even higher proportions of CD11a^{hi}CD49d^{hi} and CD127$^-$KLRG1$^+$ subsets in both host CD4 and PbT-II cells for untreated *Il27$^{-/-}$* mice than untreated B6 mice, the PbT-II cell phenotypes were characterized at Day 7 and 28 for *Il27$^{-/-}$* (Fig 3.1.3) and *Ebi3$^{-/-}$* mice (Fig 3.1.4).

At Day 7 pi, CD11a^{hi}CD49d^{hi} PbT-II cells were significantly higher for WT B6 mice, while at Day 28 pi, the trend becomes the opposite and was higher for *Il27$^{-/-}$* mice. On the other hand, CD11a^{hi}CD49d^{hi} PbT-II proportions were higher in both time points in *Ebi3$^{-/-}$* mice. As a previous study in the laboratory observed that CD49d marks the Th1 population in this infection model (Jian *et al*, 2021), these results suggest that IL-27 regulates the Th1 subset during the infection, consistent with previous reports (Findlay *et al*, 2010). Day 7 PbT-II profiles of IL-27-deficient mice also had higher proportions of CD44+CD127$^+$ and KLRG1-CD127$^+$ memory precursor effector cells (MPECs), suggesting higher memory potential. At Day 28, *Il27$^{-/-}$* and *Ebi3$^{-/-}$* mice had higher frequencies of activated PbT-II cells, MPECs, as well as KLRG1$^+$CD127- short-lived effector cells (SLECs). Since KLRG1 is considered a marker of short-lived cells, it is interesting that these cells are maintained at high levels in the absence of IL-27. These SLEC and MPEC phenotype CD4$^+$ T cells were further characterized in the next chapter to investigate the underlying mechanisms.

The utility of differentiation markers was assessed for use in the main experiments. In particular, CXCR3, CXCR6 and Ly6C are considered to be markers of Th1 differentiation; however, their expressions were not similar, with WT B6 mice having higher proportions of CXCR3+ and Ly6C+ PbT-II cells, while CXCR6+ PbT-II cells observed to be higher for Ebi3$^{-/-}$ mice. If the IFNy expression is to be considered, the CXCR6 expression then provides a better representation of Th1 differentiation in the PbT-II cells, and this was then used in the succeeding experiments, along with CXCR5 to mark Tfh cells, along with Tbet, TCF1, Bcl6, and IFNy. For cytokine expression, stimulated PbT-II cells have shown trends of higher proportions for IFNy and TNF for *Il27$^{-/-}$* mice (significantly higher proportions in *Ebi3$^{-/-}$* mice), suggesting the maintenance of more functional *Plasmodium*-specific CD4$^+$ T cells 5 weeks

after chronic infection (Fig 3.1.2L, 3.1.4D). Higher proportions of IFNy-producing cells also confirm increased proportions of Th1 phenotype.

3.1.2 Effect of IL-27 on protective recall responses
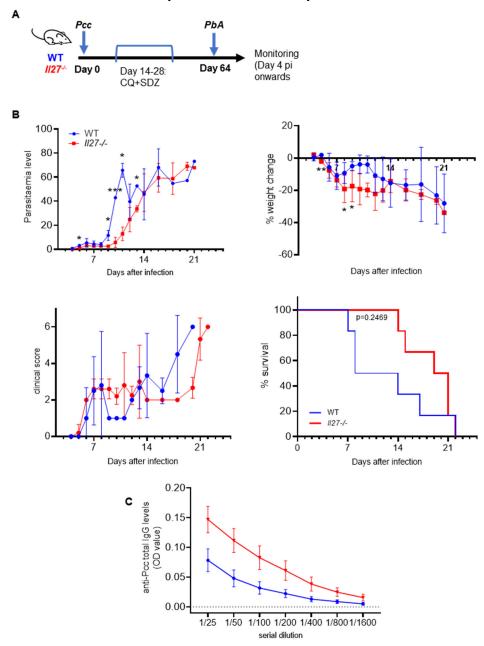
Figure 3.1.5. *Il27⁻ᐟ⁻* mice had better parasite control but had increased immunopathology after heterologous challenge.

A. Experimental scheme. Pcc-infected WT and Il27⁻ᐟ⁻ mice were treated with antimalarials at Day 14-28, and later rechallenged with the lethal *P. berghei* ANKA (PbA) at Day 64, and monitored during the course of PbA infection.

B. Results of parasitaemia (% in blood), weight change (baseline weight at Day 0 pre-PbA infection), clinical

score for ECM symptoms, and Kaplan-Meier survival curves showing % survival for each group.

C. Levels of anti-*Pcc* total IgG in the serum of WT and Il27^{-/-} mice at Day 64 pi.

Data are pooled from 2 experiments with 3 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with significance assessed by Student's t test per time point. Error bars represent SD.

For heterologous challenge experiments, B6 and *Il-27*^{-/-} mice were infected by intraperitoneal injection of 5×10^4 Pcc-infected red blood cells (iRBCs), and treated starting Day 14 pi with chloroquine (CQ) for 7 days and sulfadiazine (SDZ) in drinking water for 14 days (Fig 3.1.5). Mice were challenged with PbA at Day 64 after initial infection. Parasitaemia, weight change, clinical scores for experimental cerebral malaria (ECM) symptoms, and survival were monitored every other day from 4 days after PbA infection. *Plasmodium*-specific antibody levels were measured using ELISA to be able to relate the humoral responses to the observed characteristics of CD4^{+} T cell-mediated responses.

It was observed that although both groups were able to keep parasitaemia low until Day 8, IL-27^{-/-} mice were able to better control parasitaemia from Day 9 onwards, when the WT mice started to succumb to the infection. This is during the critical period when ECM is expected to occur (from Day 5 to 12 during a primary PbA infection), and at this point IL-27^{-/-} mice had significantly lower parasitaemia, higher weight loss, but higher survival rates (although not significant), suggesting that there was increase in immunopathology that was able to control parasitaemia and promoted survival in these IL-27 deficient mice. Nonetheless, all mice eventually succumbed to the infection, suggesting that the mounted memory response was not enough to eliminate the heterologous challenge.

3.1.3 Requirement of specific environment for memory cell maintenance

Memory CD4^{+} T cell transfer experiments were performed to determine if the environment affects the maintenance of memory populations. WT and Il27^{-/-} mice were transferred with PbT-II cells, infected with Pcc, and CD4^{+} T cells were purified from spleen samples using anti-CD4 IMag beads at 21 or 28 dpi. Naïve or infection-matched *Tcrb*^{-/-} recipient mice were adoptively transferred with total CD4^{+} T cells, which included <10% of persisting PbT-II cells. CD4^{+} T cells from 3-4 mice per group were purified and pooled, and equally transferred to recipients. The transferred CD4^{+} T cells with PbT-II cells were monitored in the peripheral blood of *Tcrb*^{-/-} mice from Day 1 post-transfer, and the spleens, bone marrow and/or liver samples were analyzed at 8-14 days post-transfer.
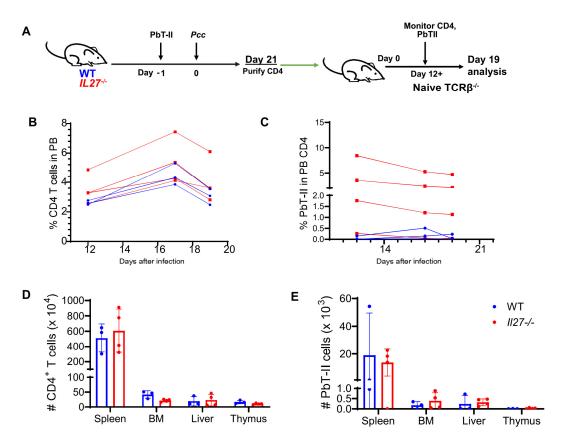
Figure 3.1.6. PbT-II cells can persist but not proliferate in non-inflammatory environment

A. Experimental scheme for memory CD4+ T cell transfer to naïve *Tcrb-/-* mice. WT B6 and Il27-/- mice were transferred with PbT-II cells a day before Pcc infection. Total CD4+ T cells were purified at Day 21 post-infection, and adoptively transferred to *Tcrb-/-* mice. Transferred CD4+, including memory PbT-II cells, were monitored until final analysis in the blood and spleen at Day 20 post-transfer.

B-C. Proportion of CD4+ T cells (B) and PbT-II cells (C) within CD4+ in the peripheral blood in *TCRb-/-* recipients.

D-E. Total counts of CD4+ T cells (D) and PbT-II cells (E) in the indicated tissues of *TCRb-/-* recipients.

Data are representative of 2 experiments with 2-4 mice/group. Pairwise comparisons were assessed by Student's t test. Error bars represent SD. WT = wild-type B6 mice, BM = bone marrow.

In the representative data in Fig 3.1.6, purified splenic CD4+ T cells from WT mice (n=3) had 0.98% PbT-II, while Il27-/- mice (n=4) had 9.18% PbT-II. Analysis was performed from Day 12 post-transfer to allow for homeostatic proliferation, and monitoring of CD4+ T cells and PbT-II in the blood showed that there was a steady decrease of PbT-II proportion in the blood from Day 12 until the day of final analysis (Day 19). Spleens had the greatest number of CD4+ T cells and PbT-II cells as expected, and both cell subsets were maintained
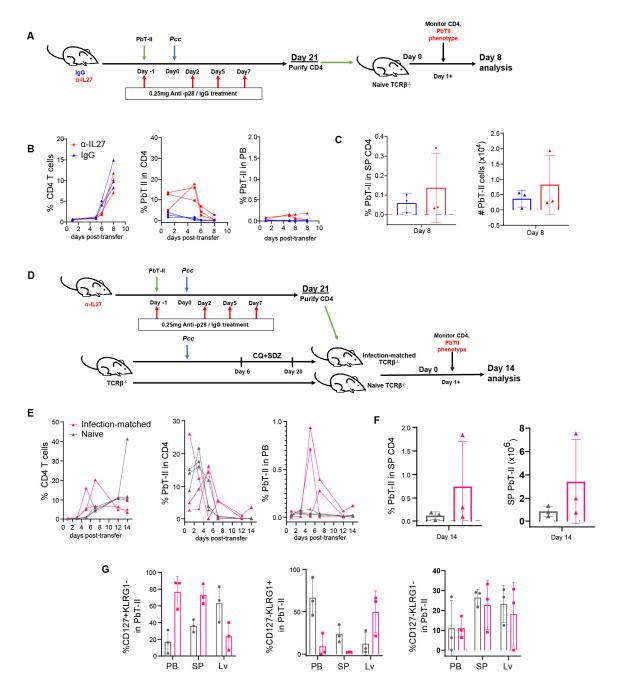
Figure 3.1.7. Maintenance of PbT-II cells require specific inflammatory environment.

A. Experimental scheme for memory CD4+ T cell transfer to naïve *Tcrb-/-* mice. B6 mice were transferred with PbT-II cells a day before Pcc infection and treated with either IgG control or anti-IL-27 mAb between -1 and 7 days post-Pcc infection. Total CD4+ T cells were purified at Day 21 post-infection, and adoptively transferred to *Tcrb-/-* mice. Transferred CD4+, including memory PbT-II cells, were monitored until final analysis in the blood and spleen at Day 8 post-transfer.

B. Monitoring of CD4+ proportions within the blood, and PbT-II proportions within CD4+ population and relative to the blood from Day 1 to 8 post-transfer. Each line represents 1 mouse.

C. Proportion (left) and total count (right) of PbT-II cells in the spleen 8 days after transfer to recipient naïve *Tcrb-/-* mice.

D. Experimental scheme for memory CD4$^+$ T cell transfer to naïve and infection-matched *Tcrb$^{-/-}$* mice. PbT-II-transferred B6 mice that were treated with anti-IL-27 mAb, as well as a group of *Tcrb$^{-/-}$* mice were infected with Pcc. Pcc-infected *Tcrb$^{-/-}$* mice.were treated with antimalarials 6 days post-infection. CD4$^+$ T cells were purified from anti-IL-27 mAb mice at Day 21 and transferred to recipient naïve and infection-matched *Tcrb$^{-/-}$* mice. Transferred CD4$^+$, including memory PbT-II cells, were monitored until final analysis in the blood, spleen and liver at Day 14 post-transfer.

E. Monitoring of CD4$^+$ proportions within the blood, and PbT-II proportions within CD4$^+$ population and relative to the blood from Day 1 to 14 post-transfer. Each line represents 1 mouse.

F. Proportion (left) and total count (right) of PbT-II cells in the spleen 14 days post-transfer.

G. Proportions for indicated PbT-II phenotype as 14 days pos-transfer to recipient naïve and infection-matched *Tcrb$^{-/-}$* mice.

Data are representative of 3 experiments for A-C and 2 experiments for D-G with 3-4 mice/group. Pairwise comparisons were assessed by Student's t test. Error bars represent SD.

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at similar numbers in bone marrow, liver and thymus, despite Il27$^{-/-}$ recipients with more PbT-II cells transferred. These results of the adoptive transfer experiments to naïve lymphopenic mice suggests that memory PbT-II cells can be maintained in a non-inflammatory environment such as in naïve Tcrb$^{-/-}$ mice, although its proliferation was not observed.

A similar experimental scheme was performed in IL-27 neutralization experiments as described in Fig 3.1.7. This experiment showed how the inflammatory environment in infection-matched recipients allowed for better maintenance of transferred PbT-II cells, which is consistent with the observation in the antimalarial treatment experiments.

RESEARCH PAPER COVER SHEET

Please note that a cover sheet must be completed <u>for each</u> research paper included within a thesis.

SECTION A – Student Details

Student ID Number	1901756	Title	Ms
First Name(s)	Maria Lourdes		
Surname/Family Name	Macalinao		
Thesis Title	Investigating the generation and maintenance of immunological memory to malaria infection		
Primary Supervisor	Katsuyuki Yui (NU), Julius Hafalla (LSHTM)		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	EMBO Molecular Medicine
Please list the paper's authors in the intended authorship order:	Maria Lourdes Macalinao, Shin-Ichi Inoue, Sanjaadorj Tsogtsaikhan, Hirotaka Matsumoto, Ganchimeg Bayarsaikhan, Jiun-Yu Jian, Kazumi Kimura, Yoshiaki Yasumizu, Tsuyoshi Inoue, Hiroki Yoshida, Julius Hafalla, Daisuke Kimura, Katsuyuki Yui

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	Conception and design of study, supervision of the data and sample collection and conduct of molecular and serological assays for the project, analysis and interpretation of data, writing and revising of the manuscript.

SECTION E

Student Signature	Maria Lourdes Macalinao
Date	20 Sept 2023

Supervisor Signature	Katsuyuki Yui / Julius Hafalla
Date	21 Sept, 2023

Chapter 3.2: IL-27 produced during acute malaria infection regulates *Plasmodium*-specific memory CD4+ T cells

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The Paper Explained

Problem：Immunity to malaria tends to decline along with the reduction in parasite exposure. It is not clear how the generation and maintenance of immunological memory is regulated during malaria infection.

Results：In this paper, we elucidated the role of the cytokine IL-27 in modulating CD4+ T cell memory during malaria infection. Transient inhibition of IL-27 during acute malaria expanded unique Th1-type memory CD4+ T cells that are maintained during chronic infection independent of active infection. Moreover, IL-27-neutralized mice exhibited enhanced immune responses and protection.

Impact：Our results suggest that transient IL-27 neutralization enhanced cellular and humoral immune responses against chronic malaria infection, which contributed to better protective immunity, thus demonstrating its potential application in vaccine development and other interventions.

Abstract

Malaria infection elicits both protective and pathogenic immune responses, and IL-27 is a critical cytokine that regulates effector responses during infection. Here, we identified a critical window of CD4$^+$ T cell responses that is targeted by IL-27. Neutralization of IL-27 during acute infection with *Plasmodium chabaudi* expanded specific CD4$^+$ T cells, which were maintained at high levels thereafter. In the chronic phase, *Plasmodium*-specific CD4$^+$ T cells in IL-27-neutralized mice consisted mainly of CD127$^+$KLRG1$^-$ and CD127$^-$KLRG1$^+$ subpopulations that displayed distinct cytokine production, proliferative capacity and are maintained in a manner independent of active infection. Single cell RNA-seq analysis revealed that these CD4$^+$ T cell subsets formed independent clusters that express unique Th1-type genes. These IL-27-neutralized mice exhibited enhanced cellular and humoral immune responses and protection. These findings demonstrate that IL-27, which is produced during the acute phase of malaria infection, inhibits the development of unique Th1 memory precursor CD4$^+$ T cells, suggesting potential implications for the development of vaccines and other strategic interventions.

Keywords

Introduction

Protective immunity to malaria develops after repeated infections over time in individuals living in endemic regions (Koch, 1900, Langhorne *et al*, 2008, Doolan *et al*, 2009, Hafalla *et al*, 2011, Crompton *et al*, 2014). In the initial phase of infection with *Plasmodium* parasites, specific T and B cells clonally expand upon recognition of the antigen and differentiate into effector lymphocytes playing crucial roles for the control of infection and disease development. In mouse models, *Plasmodium* infection results in the differentiation of specific CD4+ T cells into T helper 1 (Th1) and follicular T helper (Tfh) cells, which have important functions for the control of *Plasmodium* infection during blood-stage infection (Perez-Mazliah and Langhorne, 2014, Lönnberg *et al*, 2017). Th1 cells enhance the activation of phagocytic cells that participate in clearing parasites and infected RBCs (iRBCs), while Tfh cells support the differentiation of B cells to generate long-lived high affinity antibody responses (Hansen *et al*, 2017, Kurup *et al*, 2019, Chan *et al*, 2020, Kumar *et al*, 2020). Immunity to malaria is, however, partial in reducing the parasites, and sterile immunity is hardly achieved by natural human infection with *P. falciparum* (Langhorne *et al*, 2008). Individuals acquire resistance to infection and disease after repeated infection over time through the development of host immune responses that can control parasitemia at a low density, and chronic infection status is established. Epidemiological studies in endemic areas suggest that chronic infection offer protection against newly inoculated malaria parasite infection (Smith *et al*, 1999). Experimental studies using *P. chabaudi chabaudi* AS (*Pcc*) infection, a murine model of chronic infection, demonstrated that persistent infection can maintain effector and memory CD4+ T cells, which effectively control re-infection, and that the loss of protective immunity against blood-stage infection correlate with the progressive decline in memory T cell responses (Freitas do Rosario *et al*, 2008, Stephens and Langhorne, 2010, Opata *et al*, 2018). Furthermore, continuous antigenic stimulation in chronic infection was suggested to enhance Th1 effector function and protective capacity of memory T cells in malaria in a model of TCR transgenic murine CD4+ T cells specific for MSP1 (Stephens and Langhorne, 2010). However, memory CD4+ T cells in chronically *Plasmodium*-infected mice show altered function in which they produced cytokines but proliferated poorly upon re-infection with homologous parasites (Opata and Stephens, 2017). It remains unclear how the generation and maintenance of immunological memory is regulated during chronic malaria infection as observed in endemic areas with stable transmission (Struik and Riley, 2004).

The induction and maintenance of memory T cells is regulated by various cytokines (Raeber *et al*, 2018). IL-7 and IL-15 are two major cytokines that are essential for survival and

homeostatic proliferation of memory CD8+ and CD4+ T cells. IL-2 signal during priming of CD8+ T cells is indispensable for their robust recall response upon secondary challenge (Williams *et al*, 2006). Type I interferons that are produced by inflammatory responses act on memory CD8+ T cells in the secondary response and enhance their ability to lyse target cells (Kohlmeier *et al*, 2010). IL-10 and IL-21 act together via STAT3 signaling pathway to promote memory CD8+ T cell differentiation and functional maturation during LCMV infection (Cui *et al*, 2011). IL-27 is a heterodimeric cytokine of the IL-12 family composed of p28 and EBI3 subunits and plays critical roles in the regulation of T cell responses (Pflanz *et al*, 2002, Hunter and Kastelein, 2012, Yoshida and Hunter, 2015). It is mainly secreted by dendritic cells and macrophages, while CD4+ T cells also produce IL-27 during chronic infection such as malaria and tuberculosis (Xia *et al*, 2014, Yoshida and Hunter, 2015, Kimura *et al*, 2016). IL-27 is inhibitory for IL-2 production by CD4+ T cells, suppress development of Th17 cells, and promote effector CD4+ T cells to produce IL-10, and is regulatory for the immune responses (Hunter and Kastelein, 2012). Along with this line, *IL-27ra-/-* mice exhibited exacerbated Th1-mediated immune response in an acute model of *P. berghei* infection and were susceptible to infection due to liver pathology despite efficient parasite clearance, indicating that IL-27 regulates Th1 response during acute infection (Findlay *et al*, 2010, Villegas-Mendez *et al*, 2013). Furthermore, *IL-27ra-/-* mice that were infected with *P. berghei* NK65 and cured displayed improved parasite control during secondary infection with the homologous parasites, suggesting its role in the memory response (Gwyer Findlay *et al*, 2014). It is, however, unclear how IL-27 regulates the generation and maintenance of memory immune responses.

We previously showed that *IL-27ra-/-* mice that were infected with *Pcc* displayed reduced parasitemia during chronic phase and enhanced CD4+ T cell responses following rechallenge with heterologous *P. berghei* ANKA parasites (Sukhbaatar *et al*, 2020). In this study, we investigated the role of IL-27 in the development of immunological memory to malaria during chronic *Plasmodium* infection using MHC-II restricted TCR transgenic mouse, PbT-II (Fernandez-Ruiz *et al*, 2017, Enders *et al*, 2021). Our study suggests that IL-27 produced during acute malaria infection inhibits the development of memory precursor CD4+ T cell subsets that are committed to Th1 memory CD4+ T cells. Inhibition of IL-27 during acute infection allowed maintenance of higher levels of memory CD4+ T cells and antibody, contributing to better protection upon reinfection, which depended on persistence of the infection. Our findings indicate that IL-27 is a key cytokine that regulate the generation of memory CD4+ T cells during chronic malaria infection.

Materials and Methods

Mice

Il-27$^{-/-}$ and *Ebi3$^{-/-}$* mice were described previously (Igawa *et al*, 2009, Kimura *et al*, 2016). PbT-II mice (Fernandez-Ruiz *et al*, 2017) were generously provided by Dr. W.R. Heath and crossed with B6.SJL-Ptprc mice (CD45.1^{+}) to generate CD45.1^{+} PbT-II mice. C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan) and B6.SJL mice were bred in-house and maintained at controlled pathogen-free conditions in the Laboratory Animal Center for Animal Research at Nagasaki University. Mice were used at 8-12 weeks of age, and were age and gender matched for each experiment. All mice were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University. Animal experiments were performed with approval from the Institutional Animal Care and Use Committee of Nagasaki University (#2003091602), and were conducted according to the guidelines for Animal Experimentation at Nagasaki University.

Plasmodium Parasites and antimalarial drug treatment

Plasmodium chabaudi chabaudi AS (Pcc) is a cloned parasite which is originally from Dr. Richard Carter and David Walliker's rodent malaria parasite collection at the University of Edinburgh (Stephens *et al*, 2012), and was obtained from Dr. R Culleton (Ehime University, Ehime, Japan). *P. berghei* ANKA (PbA) was originally obtained from Dr. RE Sinden (Imperial College London, UK) and kindly provided by Dr. M Yuda (Mie University, Mie, Japan). Cryopreserved Pcc and PbA parasites were passaged through B6 mice before use in infecting experimental mice. Mice were infected with infected red blood cells (iRBC)(5×10^{4}) intraperitoneally (i.p.), and parasitemia was monitored from 4 days post-infection (dpi) by observing thin blood smears stained with a diff-quick procedure (Sysmex, Kobe, Japan).

For antimalarial drug treatment, chloroquine (10 μg/g body weight; Sigma) was administered i.p. from 6 or 21 dpi for 7 days, and sulfadiazine in drinking water (30 mg/L; Sigma) starting on the same day for 14 days unless otherwise specified in the experimental schemes.

Neutralization of IL-27 *in vivo*

For IL-27 neutralization, anti-IL-27p28 mAb (250 ug/mouse; Clone: MM27.7B1; Bio X Cell) was injected i.p. 1 day before and 2, 5, 7 days after Pcc infection, unless otherwise specified in the experimental schemes. Rat IgG (250 ug/mouse; Sigma-Aldrich) was administered to

control groups following the same dose intervals as experimental groups.

Flow cytometry

Peripheral blood (PB) was collected from the tail vein of mice during monitoring, and by cardiac puncture when mice were sacrificed. Single cell suspensions were prepared from spleen, and red blood cells in PB and spleen were lysed using Gey's solution. After incubation with anti-Fcγ receptor mAb (purified anti-mouse CD16/32; Clone: 93; Biolegend), cells (3×10^6) were stained for surface markers for 30 min at 4°C. Isotype control antibodies were used to assess staining of specific markers. Antibodies used for multiple panels of flow cytometry analysis are listed in the Reagents and Tools table. 7-amino-actinomycin D (7AAD; Cayan Chemical Company) was used to excluded dead cells from analysis.

For intracellular staining of transcription factors (TF), splenocytes were treated with anti-Fcγ receptor mAb (Clone: 93; Biolegend), stained for surface markers, fixed, and permeabilized using a Foxp3/Transcription Factor Staining Buffer set (eBioscience) following the manufacturer's instructions. The TF antibodies used were anti-mouse Ki-67 PE/Cy7 (Clone: 16A8; Biolegend), anti-T-bet PE/Cy7 (Clone: 4B10; Biolegend), anti-TCF-7/TCF-1 PE (Clone: S33-966; BD Pharmingen™), anti-human/mouse Bcl-6 PE (Clone: 7D1; Biolegend). Fixed/permeabilized cells were incubated with antibodies specific for T-bet and TCF1 in permeabilization buffer for 1hr at 4°C, and for Bcl6 and Ki67 for 1hr at room temperature.

For analysis of cytokine production, cells were cultured in R10 medium (RPMI 1640 with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin/streptomycin, 2-mercaptoethanol (5×10^{-5} M), 0.1 mM non-essential amino acids and 1 mM sodium pyruvate) in the presence of brefeldin A (10ug/ml; Cayan Chemical Company). Splenocytes (2×10^6) were stimulated with PMA (25ng/ml; Cayan Chemical Company) and ionomycin (1ug/ml; Cayan Chemical Company) for 4hr at 37°C in a humidified atmosphere of 5% CO_2. For stimulation with antigenic peptide, DCs were prepared from splenocytes of naïve B6 using anti-CD11c magnetic-activated cell sorting (MACS) microbeads (Miltenyi Biotech) and AutoMACS cell separator (Miltenyi Biotech), and were pulsed with PbT-II peptide (2uM; SIGMA Genosys) (Enders *et al.*, 2021) for 1hr. The peptide was custom synthesized by SIGMA Genosys (Sigma-Aldrich Japan, Tokyo, Japan). CD4+ T cells were purified from splenocytes by magnetic separation using anti-CD4 IMag beads (BD Biosciences) and were cultured with peptide-pulsed DCs for 4hr at 37°C in a humidified atmosphere of 5% CO_2. After culture, cells were surface stained, fixed for 20 min using Cytofix/Cytoperm™ (BD Biosciences), and stained with

anti-cytokine mAbs in permeabilization buffer. Analysis of the stained cells were performed using a LSRFortessa X-20 cell analyzer (BD Biosciences) and FlowJo software.

scRNA-seq sample and library generation

B6 mice were transferred with PbT-II cells, infected with Pcc, and were treated with control IgG or anti-IL-27 mAb between -1 and 7 days after infection with 1 mouse for each time point. Spleen cells were prepared 7 and 28 days after infection for IgG control and 7, 14, and 28 days after infection for anti-IL-27 mAb-treated groups. CD4$^+$ T cells were prepared from spleen using mouse CD4$^+$ T cell Isolation Kit (Miltenyi Biotech) and AutoMACS cell separator (Miltenyi Biotech) according to manufacturer's instructions. Enriched CD4$^+$ T cells, which included PbT-II cells, were incubated with a panel of TotalSeq^{TM} -C antibodies (BioLegend) and surface antibody markers (BioLegend) in PBS + 1% BSA: TotalSeq™-C0250 anti-mouse/human KLRG1 (MAFA) (Clone: 2F1/KLRG1), TotalSeq™-C0198 anti-mouse CD127 (IL-7Rα) (Clone: A7R34), TotalSeq™-C0078 anti-mouse CD49d (Clone: R1-2), and TotalSeq™-C0238 Rat IgG2a (Clone: RTK2758), as well as anti-mouse CD4 APC/Cy7 (Clone: GK1.5), anti-mouse TCR β chain FITC (Clone: H57-597), anti-mouse CD45.1 APC (Clone: A20), and 7AAD (Cayan Chemical Company). Stained CD4$^+$ T cells were washed using the recommended Cell Wash Protocol 1 in preparation for 10X Single Cell RNA sequencing (Chromium Next GEM Single Cell 5' v2 Reagent kits (Dual Index); 10X Genomics). PbT-II cells (CD4$^+$CD45.1$^+$TCR$^+$) were sorted using FACSAria II cell sorter (BD Biosciences). FACS-sorted cells were collected in PBS + 1%BSA after washing, and resulted in >99% purity and >98% viability in all samples.

Single-cell RNA-seq libraries were prepared using the Chromium Next GEM Single Cell 5' Reagent Kits v2 (Dual Index; 10X Genomics), SPRI reagent (Beckman Coulter Genomics), and High Sensitivity DNA Analysis Kits (Agilent Technologies), according to the manufacturer's instructions. Briefly, sorted PbT-II cells suspended in PBS + 1%BSA (900-1,200 cells/μL). For GEM generation and barcoding, cells were mixed with master mix and loaded with the gel beads and run on the Chromium Controller (10X). After reverse transcription and barcoding in droplets, cDNA was purified from GEMs, amplified, and were used for generating the gene expression libraries and cell surface protein libraries. Libraries were quantified using Bioanalyzer High Sensitivity Chip (Agilent). Gene expression libraries and cell surface protein libraries were pooled for library construction and sequencing was performed using MGISeq-2000RS at Research Institute for Microbial Disease, Osaka University (Osaka, Japan).

Cell culture

CD4$^+$ T cells were purified (>95%) from the spleen using anti-CD4 IMag (Miltenyi Biotech) according to the manufacturer's instructions. Dendritic cells were prepared from spleen of uninfected B6 mice using anti-CD11c magnetic activated cell sorting (MACS) microbeads (Miltenyi Biotech) and autoMACS (Miltenyi Biotech) following manufacturer's instructions. CD4$^+$ T cells (2 x 10^5) were suspended in culture medium and cultured in a 96-well round-bottomed plate in the presence of dendritic cells (1 x 10^4) with or without Pcc crude antigen (freeze-thaw lysate lysate of 5 x 10^6 infected RBCs) for 48 hours at 37∘C in a humidified atmosphere of 5% CO_2.

ELISA

Serum antibody levels against crude Pcc or PbA antigen (prepared in the lab) were determined using ELISA as previously described (Nakamae *et al*, 2019). Briefly, ELISA plates were coated with crude Pcc or PbA freeze-thaw lysate (5 x 10^6 infected RBC per well) in PBS overnight and washed thrice with PBS-T washing buffer (PBS with 0.2% Tween 20) before blocking with PBS with 10% FCS and 0.2% Tween 20 for 1 h. After washing, serum samples (1:1000 dilution, 50uL) were added, incubated at 4°C overnight, washed, and anti-mouse IgG HRP antibody (Southern Biotech; 1:1000 dilution) was added. After another washing, TMB substrate solution (100uL) (Thermo Fisher Scientific) was added in each well, developed for at most 10 min, before adding stop solution (10% phosphoric acid, 50uL) and read at 595/450 nm dual wavelength using an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). For the detection of IgM and IgG isotypes, biotin-conjugated rabbit anti-mouse IgG2c (Bethyl Laboratories, Montgomery, TX, USA), IgM, IgG1 or IgG2b (ZyMED, San Francisco, CA, USA) antibodies were added, incubated for 1 h, washed, incubated with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA), washed, and NPP (4-Nitrophenyl phosphate disodium salt hexahydrate) solution was added. Plates were read at 405nm absorbance.

The levels of IFN-γ in culture supernatant were determined with sandwich ELISA using anti-IFN-γ (R4-6A2) and biotin-anti-IFN-γ (XMG1.2) mAbs as described previously (Nakamae *et al*, 2019).

Statistical analysis

All statistical analyses were performed using Graphpad Prism 9 (https://www.graphpad.com;

GraphPad). Flow cytometry data were analyzed using Flowjo software (TreeStar). Data were assessed for normality using the Shapiro–Wilk test. For determining statistical significance of results, two-tailed unpaired Student's t-tests were used for pairwise comparisons for normally distributed data (parametric), while Mann-Whitney U test (non-parametric) was used for non-normally distributed data. For comparing more than 2 groups, one-way analysis of variance (ANOVA) with Tukey HSD post hoc tests (parametric) or Kruskal-Wallis test with Dunn's *post hoc* tests (non-parametric) were performed where applicable. Survival curves were assessed using Log-rank (Mantel-Cox) test. Comparisons with p values <0.05 were considered statistically significant.

scRNA-seq and CITE-seq data analysis

Sequence of the pooled libraries was mapped to the reference mouse genome using Cell Ranger 6.0.0.at Next-Generation Sequencing Core Facility, Research Institute for Microbial Disease, Osaka University (Osaka, Japan). The fastq files of five PbT-II samples (IgG-treated Days 7 and 28, and anti-IL-27-treated Days 7, 14, and 28) were processed using the Cell Ranger v.6.0.0 (10x Genomics) count pipeline against the mm10-2020-A mouse reference sequence. We used Seurat v.4.2.0 (Stuart *et al*, 2019) for detailed analysis. Cells with < 1,500, < 1,000, <1,500, <1,000 and <1,000, or > 5,000, >4,000, >5,000, >4,000 and >3,000 detected genes were also excluded for each dataset (IgG-treated Days 7 and 28, and anti-IL-27-treated Days 7, 14, and 28), respectively. The RNA data was subsequently normalized using the "NormalizeData" function, searched for 5,000 highly variable genes using the "FindVariableFeatures" function, and the Protein data was subsequently normalized using the "NormalizeData" and "ScaleData" function. For each analysis, we integrated groups using the "FindIntegrationAnchors" and "IntegrateData" functions and visualized the integrated data using UMAP (Becht *et al*, 2018) with adjustments for resolution parameters to limit cluster numbers depending on the analysis (*i.e.*, 0.15 resolution for the comparison of day 7 IgG and anti-IL-27 mAb-treated groups in Fig 3.2.4A, 0.25 resolution for the comparison of day 28 IgG and anti-IL-27 mAb-treated groups in Fig 3.2.5A and 0.3 resolution for the analysis of PbT-II in anti-IL-27 mAb-treated mice shown in Fig 3.2.5F). The cell cycle phase of each cell in the datasets was predicted using the CellCycleScoring function with genes converted from "cc.genes$s.gens" and "cc.genes$g2m.genes". The Th1, Tfh, Tmem, and Tcmp signatures are defined by the average expression of Th1, Tfh, Tmem, and Tcmp genes based on Ciucci et al. (2019) for each cell. To identify genes that define each cluster, differential expression analysis was performed using the FindConservedMarkers function to identify

genes that are conserved across independent samples (different treatments or timepoints) in each cluster. Cluster gene expression patterns were compared for the different cluster analyses done (Fig 3.2.4A, Fig 3.2.5A, Fig 3.2.5F), and cluster labels were modified to reflect the observed similarities in gene expression patterns accordingly. Moreover, FindMarkers function was also performed to compare 2 cell subsets of interest. Gene ontology analysis of the differentially expressed genes between cell subsets was performed using Metascape (http://metascape.org) (Zhou *et al*, 2019).

We analyzed our PbT-II data on days 7 and 28 in anti-IL-27 mAb-treated mouse together with IgG-treated counterparts, which were integrated as described above, and the cluster distributions were compared. A reference-based approach of analysis was also carried out by mapping the scRNA-seq data to a reference CD4^{+} T cell atlas (Andreatta *et al*, 2022) using the ProjecTILs R package (version 3.0.0) for classifying and comparing the distribution of T cell states. We compared our PbT-II from anti-IL-27 mAb-treated and IgG control on days 7 and 28, by first downsizing each dataset to 1,000 single-cell data points per sample before projecting onto the reference atlas using default parameters.

Data availability

Single-cell RNA-seq data have been deposited at GEO under the accession number GSE225556 and will become publicly available as of the date of publication.

Acknowledgements

Disclosure Statement and Competing Interests

The authors declare no competing interests.

Results

IL-27 regulates the maintenance of memory PbT-II cells

We used CD4$^+$ T cells from malaria antigen-specific TCR transgenic mice, PbT-II, to monitor antigen-specific T cell response during infection with *Pcc* (Figure 3.2.1A, Expanded View (EV) Figure 1A). Mice lacking IL-27 due to a defect in p28 (*Il-27*$^{-/-}$) or EBI3 (*Ebi3*$^{-/-}$) received PbT-II cells and were infected with *P. chabaudi*. The proportions of CD4$^+$ T cells in peripheral blood (PB) were significantly higher at day 7 and 28 compared to wild-type (WT) for *Ebi3*$^{-/-}$ mice and at day 21 for *Il-27*$^{-/-}$ mice, although parasitemia levels were comparable (Fig 3.2.1B, C). The difference between *Il-27*$^{-/-}$ and *Ebi3*$^{-/-}$ may be due to the lack of IL-35, heterodimer cytokine of EBI3 and p35, in *Ebi3*$^{-/-}$ mice (Yoshida & Hunter, 2015). The proportions of PbT-II cells in PB CD4$^+$ T cells peaked on day 7, declined by day 14 and were maintained at low levels in WT mice. A substantial increase in the number of PbT-II cells was observed on day 21 in both *Il-27*$^{-/-}$ and *Ebi3*$^{-/-}$ mice, which allowed for higher proportions to be maintained by day 28. In the spleen of *Il-27*$^{-/-}$ mice, PbT-II cells had consistently higher proportions and total counts on day 14-28 post infection (Fig 3.2.1D).

To establish whether neutralization of IL-27 can lead to enhanced memory CD4$^+$ T cell responses as observed in gene-knock-out mice, we administered anti-IL-27p28 neutralizing antibody in mice infected with *Pcc*. In initial experiments, we administered anti-IL-27 mAb from day -1 to day 19 (8 doses) of *Pcc* infection, covering the entire period in which the PbT-II proportion increases, and found that PbT-II cells increased comparably to those in gene knock-out mice (Fig 3.2.1E, F). Since the proportion of PbT-II cells increased in the absence of IL-27 in two phases: early (day 0-7; 4 doses) and late (day 7-14; 4 doses) phases (Fig 3.2.1B, C), we administered anti-IL-27 mAb at different timepoints to determine the critical window of IL-27 action on the kinetics of PbT-II cells (Fig 3.2.1E). Mice treated with anti-IL-27 mAb in the early phase of the infection had higher proportions of PB PbT-II cells comparable to continuously treated mice; those treated during late phase did not (Fig 3.2.1F). Further analysis showed that a 2-dose treatment (days -1 and 2 post-infection) was sufficient to observe the effect of IL-27 neutralization, although the enhancing effect was reduced when compared with 4-dose treatment (Fig EV1B). Therefore, the 4-dose treatment (day -1, 2, 5, 7) was used for further experiments. Analysis of spleen cells in the treated mice showed consistently higher PbT-II proportions and counts at day 14-28 post-infection comparable to IL-27 deficient mice (Fig 3.2.1G). Altogether, these results indicate that transient IL-27
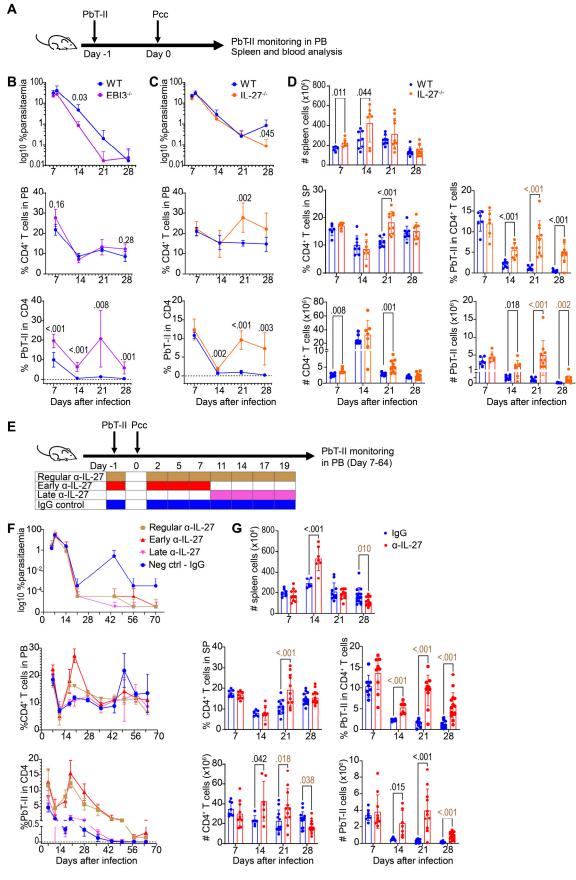
Figure 3.2.1. IL-27 signaling regulates induction and maintenance of *Plasmodium*-specific CD4+ T cells during chronic malaria infection

(A-D) B6, *Ebi3*^{-/-}, and *Il27*^{-/-} mice were transferred with PbT-II cells a day before Pcc infection. Levels of parasitemia, proportion of PbT-II cells in CD4$^+$ T cells in peripheral blood (PB), as well as spleen (SP) profiles were monitored. Gating strategy for CD4$^+$ T and PbT-II cell analysis is described in Fig EV1A.

(A) Experimental scheme for adoptive transfer of PbT-II to B6, *Ebi3*^{-/-}, and *Il27*^{-/-} mice.

(B, C) Parasitemia levels (log10-transformed) and proportions of CD4$^+$ T and PbT-II cells in PB of B6 (WT) vs *Ebi3*^{-/-} mice (B; n = 4 mice/group), and B6 (WT) vs *Il27*^{-/-} mice (C; n = 4 mice/ group). Representative data of 2 independent experiments are shown.

(D) CD4$^+$ T and PbT-II cell profile in the spleen of B6 (WT) vs *Il27*^{-/-} mice (n = 6, 8, 9, 9 for WT and n=6, 7, 10, 10 for *Il27*^{-/-} mice at day 7, 14, 21, 28 post infection (pi), respectively). Data are pooled from 2, 2, 3 and 3 independent experiments for day 7, 14, 21, and 28 timepoint, respectively.

(E, F) Adoptive transfer of PbT-II cells to B6 mice later infected with *Pcc*. Anti-IL-27 mAb was administered for regular, early and late αIL-27 groups at -1 to 19, -1 to 7, and 11 to 19 dpi, respectively, and IgG control group received IgG between -1 to 19 dpi.

(E) Experimental scheme of IL-27 neutralization with anti-IL-27 mAb.

(F) Parasitemia levels (log10-transformed), and proportions of CD4$^+$ T and PbT-II cells in PB during weekly monitoring (n = 3 mice/group). Dose effect results were replicated in Fig EV1 (B-D).

(G) Adoptive transfer of PbT-II to Pcc-infected B6 mice administered with either IgG or anti-IL-27 mAb during the early phase (between -1 and 7 days after infection). Total number of cells, proportion, and number of CD4$^+$ T and PbT-II cells in the spleen were monitored (n = 9, 6, 11, 15 for IgG-treated and n=9, 7, 11, 15 mice for anti-IL-27 mAb-treated mice at day 7, 14, 21, 28 days pi, respectively). Data are pooled from 2, 2, 3 and 4 independent experiments for day 7, 14, 21, and 28 timepoint, respectively.

Data information: Statistical significance in B, C, D, and G was assessed by unpaired two-tailed Student's *t*-tests or Mann-Whitney *U* test for comparing WT to EBI3^{-/-}/Il27^{-/-} mice or IgG- to anti-IL-27 mAb-treated mice per timepoint, depending on normality assessment. Significant *p* values (< 0.05) are indicated as black text for Student's *t*-tests and brown text for Mann-Whitney *U* tests performed. Error bars represent SD in all graphs.

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neutralization during the acute phase of *Pcc* infection enhances the levels of *Plasmodium*-specific CD4$^+$ T cells during chronic phase of *Pcc* infection, with comparable kinetics and magnitude to mice genetically deficient in IL-27.

Distinct subsets of memory CD4$^+$ T cells were induced during chronic infection by the IL-27 neutralization

To evaluate how IL-27 signaling affects the development of PbT-II cells during *Pcc* infection, we characterized the phenotype of these cells (Fig 3.2.2, Fig EV2). The proportions of antigen-experienced CD11a^{hi}CD49d^{hi} PbT-II cells in the spleen were higher in anti-IL-27 mAb-treated mice on days 14 and 21, and the numbers were higher on days 21 and 28 (Fig 3.2.2A). The CD127$^+$KLRG1$^-$ and CD127$^-$KLRG1$^+$ phenotypes are markers of long-lived memory precursor and short-lived effector CD8$^+$ T cells, respectively (Kaech and Cui, 2012). Most PbT-II cells in the control mice were CD127$^-$KLRG1$^-$ from day 7 to day 28. In anti-IL-27 mAb-treated mice,

the proportion of CD127+KLRG1- PbT-II cells was higher when compared with those in control on day 7. On day 28 of infection, CD127+KLRG1- and CD127-KLRG1+ PbT-II cells were dominant and reached 39.0 ± 1.4 % and 39.9 ± 6.5 %, respectively (Fig 3.2.2B). The
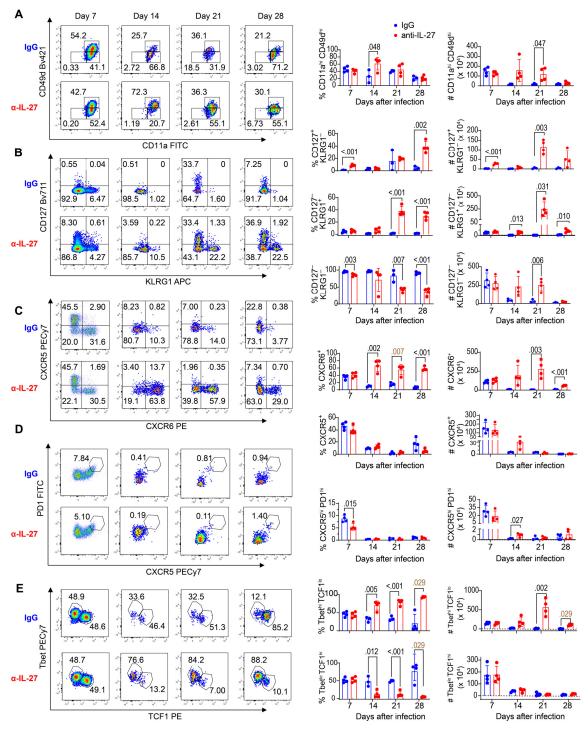
Figure 3.2.2. Inhibition of IL-27 signaling affects the phenotype of *Plasmodium*-specific CD4+ T cells during chronic infection.1

B6 mice were transferred with PbT-II cells and treated with either control (IgG, blue) or anti-IL-27 mAb (α-

IL-27, red) between -1 and 7 days post-*Pcc* infection. Splenic PbT-II (CD45.1$^+$CD45.2$^-$TCRβ$^+$CD4$^+$) cells were analyzed by flow cytometry at 7, 14, 21, and 28 dpi.

(A-D) Representative flow cytometry profiles (left) of cell surface expression of CD11a/CD49d (A), CD127/KLRG1 (B), CXCR5/CXCR6 (C), and PD-1/CXCR5 (D) on PbT-II cells from control (blue) and α-IL-27 (red) mouse groups, and corresponding summary of the frequencies and total numbers of PbT-II cell subpopulations for mouse groups (right).

(E) Representative flow cytometry profiles (left) of expression of T-bet and TCF1 in PbT-II cells, and corresponding summary of the frequencies and total numbers of PbT-II cell subpopulations (right).

Data information: Numbers in flow cytometry profiles indicate PbT-II proportions (%) within each area. n = 4, 3, 3, 4 for IgG-treated and n=4, 4, 4, 4 mice for anti-IL-27 mAb-treated mice at day 7, 14, 21, 28 days pi, respectively. Data for each timepoint are representative of 2, 2, 3 and 4 independent experiments for 7, 14, 21, 28 dpi, respectively. Statistical significance assessed by Student's *t* test or Mann-Whitney *U* test per time point, depending on normality assessment. Significant *p* values (< 0.05) are indicated as black text for Student's *t*-tests and brown text for Mann-Whitney *U* tests performed. Error bars represent SD.

proportions of CXCR5$^-$CXCR6$^+$ and CXCR5$^+$CXCR6$^-$ PbT-II cells, putative Th1 and Tfh cells respectively (Kim *et al*, 2001, Crotty, 2011), were comparable on day 7 in anti-IL-27 mAb-treated and control mice (Fig 3.2.2C). The CXCR5$^-$CXCR6$^+$ PbT-II cells increased and were maintained at higher levels through day 28 in anti-IL-27 mAb-treated mice, while they decreased after day 14 in control mice. The proportions of CXCR5$^+$ PbT-II cells were maintained at low levels after day 14 in both groups as were the proportion of CXCR5^{hi}PD1^{hi} germinal center (GC) Tfh PbT-II cells. The difference in expression of these surface markers were also observed in PB PbT-II subsets (Appendix Fig S1). PbT-II cells expressing transcription factors associated with Th1 and Tfh/memory, T-bet and TCF-1, respectively, were not significantly different on day 7, while the proportion of T-bet^{hi}TCF-1^{lo} cells increased in anti-IL-27 mAb-treated mice from day 14 onwards reaching 93.4 ± 1.9 % on day 28; however, total numbers of Tbet^{lo}TCF-1^{hi} PbT-II cells in the spleen were comparable (Fig 3.2.2E). The expression of Tfh-associated transcription factor Bcl6 was also comparable at days 7 and 28, further suggesting that Tfh subset was not affected by the IL-27 neutralization (Appendix Fig S1).

Additionally, we also analyzed phenotypes of CD11a^{hi}CD49d^{hi} and CD11a^{hi}CD49d^{lo} PbT-II subpopulations 7 and 28 days after infection to determine whether they represent Th1 and Tfh cells as we previously reported (Jian *et al*, 2021) (Fig EV2). On day 7 after infection, majority of CD11a^{hi}CD49d^{hi} PbT-II cells were Th1 type, while Tfh type cells were dominant in CD11a^{hi}CD49d^{lo} cells in both anti-IL-27 mAb- and IgG-treated mice (Fig EV2A, C). On day 28, however, PbT-II cells in anti-IL-27 mAb-treated mice showed strong Th1 dominance in which the majority of CD49d^{hi}CD49^{hi} and CD49d^{hi}CD49^{lo} cells were KLRG1$^+$T-bet^{hi} and CXCR6$^+$T-bet^{hi}, respectively, while both CD49d^{hi}CD49^{hi} and CD49d^{hi}CD49^{lo} PbT-II cells were dominated
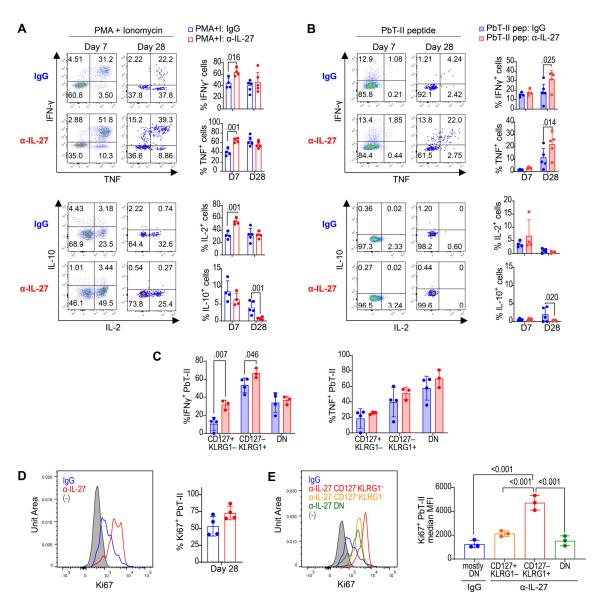
Figure 3.2.3. *Plasmodium*-specific memory CD4⁺ T cells induced under IL-27 neutralization exhibit enhanced cytokine production.

Cytokine production was analyzed for PbT-II cells transferred to B6 mice infected with *Pcc*, and were treated with control (IgG, blue) or anti-IL-27 mAb (α-IL-27, red) between -1 and 7 days after infection.

(A-B) Splenocytes from IgG and α-IL-27-treated mice collected 7 and 28 days after infection were stimulated with PMA and ionomycin for 4 h (A), and splenic CD4⁺ T cells were cultured with PbT-II peptide-pulsed dendritic cells for 4h (B), stained for surface markers, fixed, permeabilized and stained for IFN-γ, TNF, IL-10, and IL-2. Representative flow cytometry profiles (left) of cytokine expression of PbT-II cells (CD45.1⁺CD45.2⁻TCRβ⁺CD4⁺) and frequencies of cytokine-producing PbT-II cells (right) are shown for control (blue) and anti-IL-27 mAb (red)-treated mice at day 7 (n = 4 mice/group) and day 28 (n = 5 mice/group) days pi. Representative data of 2 independent experiments are shown.

(C) Proportions of IFN-γ or TNF-producing cells in response to PbT-II peptide within indicated subpopulations of control (n = 4) and anti-IL-27 mAb-treated (n = 3) mice.

(D, E) Splenic cells were prepared 28 days after Pcc infection and stained for TCRβ, CD4, CD45.1, CD127, and KLRG1, fixed, permeabilized and stained for Ki67 to analyze expression in PbT-II cell subpopulations.

(D) Representative histogram plots (left) and summary graphs (right) of Ki67 expression in PbT-II cells, and

isotype control of IgG group (gray) at 28 days pi (n = 4 mice/each group). Data are representative of 3 independent experiments.

(E) Representative histograms (left) and summary graph (right) of median fluorescence intensity (MFI) levels of Ki67 expression of total PbT-II cells from IgG, and CD127$^+$KLRG1$^-$ (orange), CD127$^-$KLRG1$^+$ (red), CD127$^-$KLRG1$^-$ (green) PbT-II subpopulations from anti-IL-27 mAb-treated mice (n = 3 mice/each group). Data are representative of 3 independent experiments.

Data information: Statistical significance was assessed by Student's t test for comparing IgG and anti-IL-27 mAb-treated mouse groups in (A), (B), and (D), and per subpopulation in (C), and one-way ANOVA followed by Tukey's multiple comparison test in (E). p values (< 0.05) are shown. Error bars represent SD in all graphs.

by T-bet^{lo}TCF1^{hi} phenotype in IgG-treated mice (Fig EV2B, D).

To examine the functional activity of PbT-II cells, we analyzed the cytokines produced as well as their proliferative phenotype (Fig 3.2.3). The proportions of PbT-II cells producing IFN-γ and TNF in response to PMA and ionomycin were higher in anti-IL-27 mAb-treated mice on day 7, while those in response to antigenic peptide was not significantly different (Fig 3.2.3A, B). On day 28, however, PbT-II cells exhibited significantly higher production of IFN-γ and TNF in response to the peptide in anti-IL-27 mAb-treated mice, consistent with the higher proportion of Th1 type PbT-II cells on day 28 (Fig 3.2.3B). Production of IL-2 and IL-10 was mostly comparable, except for the higher IL-2 production in response to PMA and ionomycin on day 7 and reduced IL-10 production in response to peptide in anti-IL-27 mAb-treated mice on day 28. Within PbT-II subpopulations on day 28, the proportion of IFN-γ-producing cells was highest in CD127$^-$KLRG1$^+$ cells while production of TNF was the highest in CD127$^-$KLRG1$^-$ PbT-II cells (Fig 3.2.3C). When compared with IgG treated mice, CD127$^+$KLRG1$^-$ and CD127$^-$KLRG1$^+$ subsets of PbT-II cells in anti-IL-27 mAb-treated mice produced more IFN-γ. Proliferation was enhanced in anti-IL-27 mAb-treated mice compared to control on day 28 as evaluated by Ki67 expression (Fig 3.2.3D). Among PbT-II subpopulations, CD127$^-$KLRG1$^+$ cells showed highest Ki67 expression followed by CD127$^+$KLRG1$^-$ and CD127$^-$KLRG1$^-$ cells in anti-IL-27 mAb-treated mice (Fig 3.2.3E). Altogether, these results show that neutralization of IL-27 in the acute phase of the infection profoundly affected PbT-II cell phenotype and function during chronic phase, although PbT-II cells from anti-IL-27 mAb-treated mice were phenotypically indistinguishable from control group during acute phase except for a slight but significant increase in CD127$^+$ cells in anti-IL-27 mAb-treated mice.

Transcriptome analysis revealed distinct subsets of PbT-II cells were induced by IL-27 neutralization during malaria

The effect of IL-27-neutralization suggested that the development of memory precursor subpopulations could be inhibited by IL-27. To uncover the responding CD4$^+$ T cell subsets

at the transcriptional level, we performed single cell RNA-seq (scRNA-seq) and compared PbT-II cells from anti-IL-27 mAb- and control IgG-treated mice on day 7 and 28 after infection (Fig 3.2.4, 3.2.5, Fig EV3). Dimensional reduction and clustering of the cells on day 7 of infection based on their gene expression profiles identified 5 clusters: *Tbx21⁺Id2⁺Ifng⁺Cxcr6⁺Cxcr3⁺* cells (Cluster 1) with high Th1 as well as T central memory precursor (Tcmp) signature scores, *Bcl6⁺ Id3⁺Il21⁺Cxcr5⁺* cells (Cluster 2) with high Tfh signature scores, *Tcf7⁺Klf2⁺Sell⁺Ccr7⁺* cells (Cluster 3) with high memory T cell (Tmem)
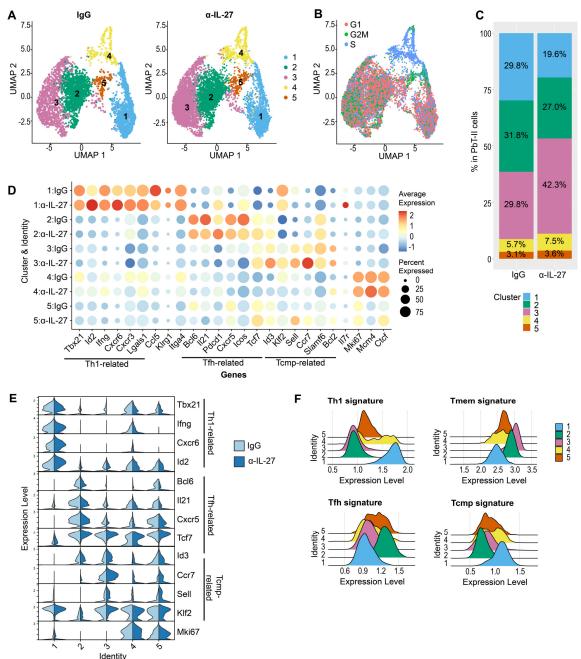
Figure 3.2.4. Early IL-27 neutralization affects memory signature during acute phase of Pcc infection

B6 mice were transferred with PbT-II cells, infected with Pcc, and treated with control (IgG; n = 1 biological

replicate) or anti-IL-27 mAb (α-IL-27; n = 1 biological replicate) between -1 and 5 days of infection. PbT-II cells were purified from these mice 7 days after infection and single-cell RNA sequencing (scRNA-seq) analysis was performed. Details of the experiments are shown in Fig EV3 A-C.

(A) UMAP plots of PbT-II cells from IgG control (n=4,030) and anti-IL-27 mAb-treated mice (n=7476) after unsupervised clustering of pooled single cell data from the 2 groups, with clusters colored by gene expression profiles.

(B) UMAP clustering of PbT-II cells colored by cell cycle profiles.

(C) Summary graph of proportions of PbT-II cells in each cluster for IgG and anti-IL-27 mAb-treated mice in (A).

(D) Dot plots showing the expression of Th1-, Tfh-, Tcmp-related genes (Ciucci *et al.*, 2019), and other genes of interest in each UMAP cluster of PbT-II cells from IgG and anti-IL-27 mAb-treated mice. Dot colors represent the intensity of expression, while dot size represents the proportion of cells with the corresponding expression.

(E) Violin plots showing the expression of Th1-, Tfh-, Tcmp-, and proliferation-associated genes in PbT-II cells from IgG (light blue) and anti-IL-27 mAb (blue) treated mice.

(F) Ridge plots showing the expression of published Th1, Tfh, Tmem and Tcmp CD4$^+$ T cell signatures in each of the UMAP clusters in (A) based on (Ciucci *et al.*, 2019).

signature scores, *Mki67$^+$Mcm4$^+$Ctcf$^+$* cells (Cluster 4) in the S phase of the cell cycle, and cells with low expression in most of the analyzed genes, including those in G2/M phase (Cluster 5) (Fig 3.2.4A, B, D-F). While CD127$^+$ cells were slightly increased in anti-IL-27 mAb-treated mouse, this population (*Il7r$^+$*) belongs to cluster 1 (Th1) (Fig 3.2.2B, 4D). There were variations in the expression among individual genes in each cluster by anti-IL-27-mAb treatment such as increase in *Id2* and *Il7r* in cluster 1 and *Id3* and *Ccr7* in cluster 3, as well as increase in *Bcl2* in minor clusters 4 and 5 (Fig 3.2.4D, E). These differences were observed despite comparable parasitaemia levels for the IgG and anti-IL-27-mAb samples (25-27%), as well as comparable surface marker phenotypes (Fig EV3C). Still, there was agreement between the transcriptome and flow cytometry results, showing slightly higher Th1 proportions for the IgG sample (Fig 3.2.4C, EV3C). We also observed a shift in the proportions of cells in cluster 1 and 3 between the 2 groups (Fig 3.2.4C) and higher Tcm population in anti-IL-27 mAb-treated mouse in the reference mapping approach (Fig EV3D), suggesting an effect of anti-IL-27 mAb-treatment at day 7 after *Pcc* infection.

We next analyzed the scRNA-seq data of PbT-II cells from anti-IL-27 mAb- and IgG-treated mice on day 28 after infection (Fig 3.2.5, Fig EV3A-C, E). Dimensional reduction and clustering of the cells based on their gene expression profiles identified 7 clusters and their surface markers were visualized by CITE-seq analysis (Fig 3.2.5A-G). For consistency, we numbered
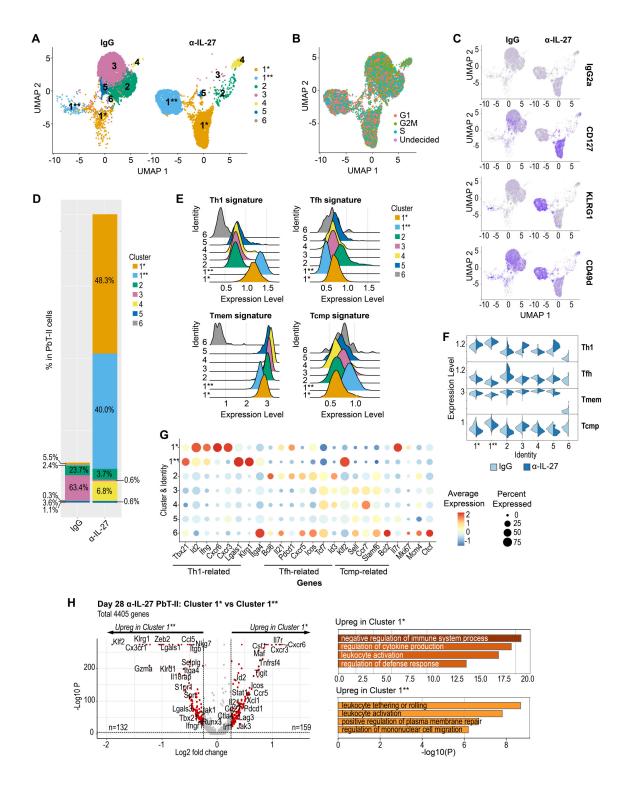
Figure 3.2.5. Transcriptome analysis reveals distinct T cell subsets during chronic infection induced by IL-27 neutralization

B6 mice were transferred with PbT-II cells, infected with Pcc, and were treated with either IgG or anti-IL-27 mAb between -1 and 7 days after infection (n = 1 biological replicate per timepoint). PbT-II cells were prepared from spleen at day 28 pi, stained for CD4/TCR/CD45.1 and for CD127, KLRG1, and CD49d with TotalSeq antibodies, sort purified, and processed for scRNA-seq and CITE-Seq analysis. Details of the

experiment are found in Fig EV3 A-C.

(A-G) Comparative analysis of scRNA-seq data from IgG and anti-IL-27 mAb-treated PbT-II cells. (A) UMAP plot colored of day 28 PbT-II cells from IgG control (n=7,491) and anti-IL-27 mAb-treated mice (n = 4,944) after unsupervised clustering of pooled single cell data from the 2 groups, with clusters colored by gene expression profiles. Cluster labels were harmonized to reflect similar gene expression patterns in the clusters at day 7 pi (Fig 3.2.4) and anti-IL27 mAb day 7-28 PbT-II analysis (Fig EV4).

(B) UMAP clustering of PbT-II cells colored by cell cycle profiles.

(C) CITE-seq analysis of PbT-II cells for IgG2a (isotype control), CD127, KLRG1 and CD49d, shown in the same UMAP clustering as (A).

(D) Proportions (%) of each cluster within PbT-II cells, with bar graph sizes shown relative to the total number of PbT-II cells in IgG (36.8 x 10^4) and anti-IL-27 mAb treated (265.7 x 10^4) mice.

(E) Ridge plots of PbT-II cells showing the expression of published CD4$^+$ T cell signature genes (Ciucci *et al.*, 2019).

(F) Violin plots comparing the expression of the CD4$^+$ T cell signature genes.

(G) Dot plots showing the expression of Th1-, Tfh-, Tcmp-, and proliferation-associated genes in each cluster. Dot colors represent the intensity of expression, while dot size represents the proportion of cells with the corresponding expression.

(H) Volcano plot of differentially expressed genes between major clusters 1* and 1** within PbT-II cells from anti-IL-27-treated mice, and corresponding Gene Ontology enrichment analysis for the upregulated genes in each group using Metascape.

these clusters to correspond with the same-numbered clusters on day 7 that exhibit similar gene signature (Fig 3.2.5A, C, E). Two major clusters exhibiting high Th1 signature scores in anti-IL-27-treated mice were named cluster 1* and cluster 1** based on their relation to day 7's cluster 1, although these cells were rare in IgG control on day 28. Cluster 1* cells were CD127$^+$KLRG1$^-$ based on CITE-Seq, and showed high *Id2, Ifng, Cxcr6* and *Cxcr3*; Cluster 1** cells were CD127$^-$KLRG1$^+$ and expressed *Tbx21*, *Lgals1*, *Itga4* and *Klf2* at high levels and exhibited high Tcmp signature in addition to high Th1 score, suggesting their memory potential (Fig 3.2.5E-G). Comparison of the 2 clusters showed that cluster 1* cells are distinguished by their expression of co-inhibitory genes (*Tigit, Lag3* and *Pdcd1*) and chemokine receptors (*Cxcr6*, *Cxcr3* and Ccr5) while cluster 1** express genes related to leukocyte migration (*Cx3cr1, Ccl5, and S1pr1*), killer cell lectin-like receptors (*Klrg1* and *Klrd1*) and transcription factors (*Klf2*, *Zeb2*, *Tbx2*) suggesting their differential function (Fig 3.2.5H). Cluster 2-6 were CD127^{lo}KLRG1^{lo} (Fig 3.2.5A, C). Cluster 2 cells, although a minority in anti-IL-27 mAb-treated mice, were present in similar absolute numbers in anti-IL-27 mAb- and IgG-treated mice, exhibited a high Tfh signature score and expressed genes including *Bcl6*, *IL21* and *Cxcr5* (Fig 3.2.5A, D-G). Cluster 3 cells comprised the largest cluster in IgG-treated mice, exhibited high Tmem score and expressed memory phenotype genes including *Tcf7, Sell, Ccr7* and *Bcl2* (Fig 3.2.5A, D-G). Clusters 4 was the third largest population in anti-

IL-27 mAb-treated mice exhibiting highest Tmem score (Fig 3.2.5A, D, E). Interestingly, the expression levels of Th1 signature genes in cluster 3 and 5 cells in anti-IL-27-treated mice were higher than those in IgG-treated mice (Fig 3.2.5F). Cluster 6 was detected in IgG control alone. Mapping to the CD4+ T cell reference atlas (Andreatta *et al.* 2022) showed that the major populations in PbT-II cells in the anti-IL-27 mAb-treated mouse had Th1 effector and Th1 memory subsets, while those in the IgG-treated mouse were Tcm and Tfh memory subsets (Fig EV4E). These features indicate the expansion of unique Th1-type PbT-II cells in *Pcc*-infected mice when IL-27 is neutralized during acute infection. Taken together, PbT-II cells in anti-IL-27 mAb-treated and IgG-treated mice were comprised of clearly distinct subpopulations on day 28 of infection. The increase in the maintained PbT-II populations in anti-IL-27 mAb-treated mice at day 28 was due to two major populations of Th1-like clusters 1* and 1**, and Tmem-like cluster 4 as well as a minor Tfh cluster.

We next compared scRNA-seq data of PbT-II cells in anti-IL-27 mAb-treated mice in transition from acute to chronic infection (Fig EV4). The scRNA-seq data of PbT-II cells from day 7, 14 and 28 after infection were dimensionally reduced and their clustering analysis revealed a total of 7 clusters, which were labeled with the same numbering applied to previous cluster analyses (Fig EV4A). Cluster 1* and 1**, both corresponding to Th1 cluster on day 7, increase with time and each occupied >40% on day 28 (Fig EV4B). Cluster 2, corresponding to the Tfh cluster on day 7, was reduced on day 14 and became a minor cluster (<5%) by day 28. Cluster 3, which corresponds to the Tmem-like cells on day 7, were maintained through day 14 but became a minor population on day 28. These features suggested a gradual shift in the phenotypes of PbT-II cells during transition from acute to chronic Pcc-infection.

Active infection is not essential for the maintenance of Th1-type memory PbT-II cells

To determine whether live parasites are required for the induction and maintenance of memory CD4+ T cells, *Pcc*-infected mice with and without anti-IL-27 mAb were treated with antimalarial drugs (Fig 3.2.6). Antimalarial drug treatment starting 6 days post-infection did not have significant impact on the proportions of PbT-II cells in control IgG group on day 28. In anti-IL-27 mAb-treated mice, however, the increase in PbT-II cells on day 21 after infection was not observed in mice also treated with antimalarial drugs, suggesting its dependence on active infection. Despite this lack of increase, the total number of PbT-II cells remained higher in antimalarial-treated anti-IL-27 mAb-treated mice when compared with its IgG-treated counterpart in both PB and spleen after 28 days of infection (Fig 3.2.6B, C). Phenotypically,

PbT-II cells in anti-IL-27 mAb-treated mice exhibited higher proportions of CD127$^+$KLRG1$^-$, CD127$^-$KLRG1$^+$, CXCR6$^+$, and T-bet^{hi}TCF-1^{lo} cells than those in control IgG-treated mice in both antimalarial drug-treated and untreated mice (Fig 3.2.6D, E). These results suggest that the increase of PbT-II cells between 14 and 21 days of infection is dependent on active infection, while phenotypical differentiation of PbT-II cells during memory phase is independent of the presence of live parasites during chronic infection.

Next, we treated mice with antimalarial drug starting at day 21 post-infection (Fig EV5). The proportion of PbT-II cells in PB and the number of PbT-II cells in spleen did not decrease, although the total number of CD4$^+$ T cells decreased 14 days after infection (Fig EV5B, C). The proportions of PbT-II cells expressing CD127, KLRG1, CXCR5, CXCR6, T-bet, and TCF-1 were not significantly different between antimalarial treated and untreated mice (Fig EV5D, E). In sum, these results imply that once memory CD4$^+$ T cells are induced, their maintenance does not require live parasite infection for more than 14 days.
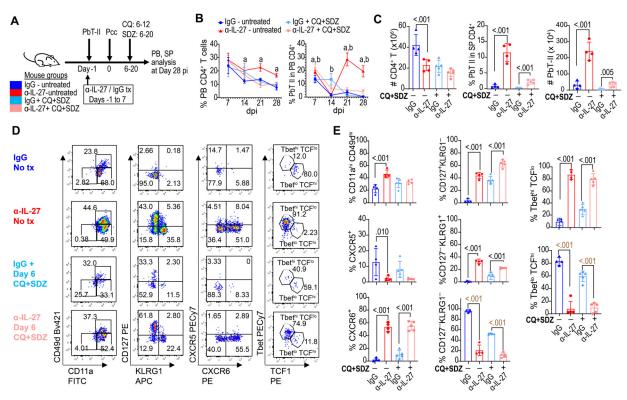
Figure 3.2.6. Enhanced memory PbT-II cells induced by transient IL-27 neutralization are maintained without live parasites during chronic phase.

B6 mice were transferred with PbT-II cells, treated with IgG (blue, light blue) or anti-IL-27 mAb (red, pink), and treated (light, blue pink) or not treated (blue, red) with antimalarial drugs starting 6 days after Pcc infection.

(A) Experimental scheme for antimalarial treatment experiment (n = 4 for antimalarial untreated groups, n =

5 mice for antimalarial-treated groups).

(B) Kinetics of the proportion of CD4$^+$ T cells in PB and of PbT-II cells in PB CD4$^+$ T cells during the course of Pcc infection. Small letters indicate significant differences ($p < 0.05$) between IgG vs anti-IL-27 in antimalarial-untreated (a), and -treated (b) mice.

(C) Total number of CD4$^+$ T cells in the spleen and proportions of PbT-II cells in CD4$^+$ T cells and their total numbers in spleen on day 28 pi.

(D) Representative flow cytometry profiles of splenic PbT-II cells on day 28 pi.

(E) Proportions of PbT-II cells with the indicated phenotype on day 28 pi.

Data information: Data are representative of 4 independent experiments. Statistical significance was assessed by Student's t test per time point for (B), comparing IgG vs anti-IL-27 in antimalarial-untreated, and -treated mice. For (C)(E), depending on normality assessment, one-way ANOVA followed by Tukey's multiple comparison test or Kruskal-Wallis test with Dunn's *post hoc* tests were performed, with significant p values (< 0.05) indicated as black text or brown text, respectively. In B, 'a' corresponds to the p-values of .002, <.001, and <.001 in the left graph, and .012, <.001, and <.001 in the right graph for days 14, 21, and 28, respectively, while 'b' corresponds to p-values .001, .024, .002, and <.001 for days 7, 14, 21, and 28, respectively. Error bars represent SD.

Memory CD4 T cells induced by IL-27 neutralization contribute to protective immunity

Finally, we examined whether the immunity induced under IL-27-neutralizaion exhibit enhanced recall response against challenge infection. Mice treated with anti-IL-27 mAb or control IgG were infected with *P. chabaudi,* and treated and not treated with antimalarial drugs without transfer of PbT-II cells (Fig 3.2.7A, Appendix Fig S1). Mice were sacrificed 63 days after infection and splenocytes were examined. Phenotypical analysis did not show significant skewing in lymphocyte composition and CD4$^+$ T cell subpopulations between anti-IL-27 mAb-treated and control mice except the increase of CD11a^{hi}CD49d^{hi} CD4$^+$ T cells in antimalarial treated mice (Fig 3.2.7B, Appendix Fig S1A, B). However, production of IFN-γ by CD4$^+$ T cells in response to crude *Pcc* antigens in antimalarial untreated mice was higher than IgG control, suggesting that memory CD4$^+$ T cells were maintained at higher levels in anti-IL-27 mAb-treated mice (Fig 3.2.7C). In the non-lymphoid compartment, there was a reduction in the proportion of CD11b$^+$Ly6C^{hi} inflammatory monocytes observed in anti-IL-27 mAb-treated antimalarial untreated mice, suggesting that IL-27 neutralization also has long term effect on the non-lymphoid compartment (Appendix Fig S1A, C). Levels of anti-*Plasmodium* IgM and IgG were highest in anti-IL-27 mAb-treated mice without antimalarial therapy (Fig 3.2.7D). Among IgG isotypes we observed a significant difference only in IgG1 levels (Fig 3.2.7E). IgG cross reactive to *P. berghei* ANKA (*Pb*A) antigens was also higher in anti-IL-27 mAb-treated mice (Fig 3.2.7F). Since *Pcc*-primed mice exhibit strong protective immunity against re-challenge with homologous parasites, we determined whether the protective ability of Pcc-
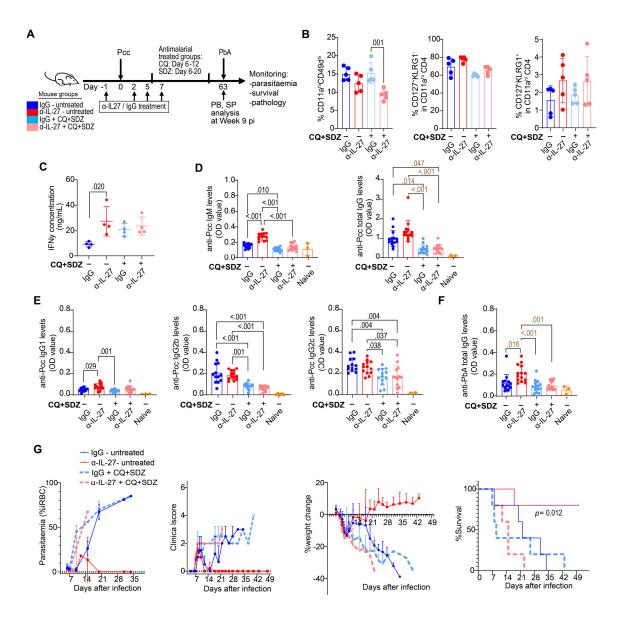
Figure 3.2.7. Transient IL-27 neutralization induces long-lasting protective immunity against challenge infection with heterologous parasites

B6 mice were treated with IgG (blue, light blue) or anti-IL-27 mAb (red, pink), infected with *Pcc*, and treated (light blue, pink) or not (blue, red) with antimalarial drugs. PB profiles were monitored weekly. Splenocytes were examined at 63 dpi (B-F), or mice were challenged with *Pb*A at 63 days post-*Pcc* infection and monitored post-*Pb*A infection (G).

(A) Experimental scheme.

(B-F) Splenocytes and serum were examined 63 days after infection.

(B) Summary of the proportions of CD11a^hiCD49d^hi cells in splenic CD4^+ T cells, and the proportions of CD127^+KLRG1^- and CD127^-KLRG1^+ cells in CD11a^hiCD4^+ T cells (n = 4 mice/group).

(C) IFN-γ production as determined by ELISA of splenic CD4^+ T cells purified and cultured in the presence of dendritic cells and crude Pcc antigens for 48 hrs (n = 4 mice/group).

(D-F) Levels of anti-*Pcc* IgM and IgG (D), IgG subclasses (E), and anti-*Pb*A cross-reactive IgG (F) in the serum (n = 13 mice/group). Pooled data from 3 experiments (4-5 mice/ group) are shown. Serum from uninfected

mice (naïve, n = 3 mice) was used as negative control.

(G) Parasitemia, clinical scores, body weight, and survival were monitored after challenge infection with *Pb*A at day 63 post-*Pcc* pi (4 mice/group). Survival was assessed using log-rank test.

Data information: Representative data of 2 independent experiments are shown before (B, C) and after challenge infection (G). Statistical significance assessed by one-way ANOVA followed by Tukey's multiple comparison test or Kruskal-Wallis test with Dunn's *post hoc* tests, depending on normality assessment. Significant *p* values (< 0.05) are indicated as black text for one-way ANOVA followed by Tukey's multiple comparison test and brown text for Kruskal-Wallis test with Dunn's *post hoc* tests performed. Error bars represent SD.

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primed mice was improved in anti-IL-27 mAb-treated mice using a previously established rechallenge infection model with heterologous virulent parasites, *Pb*A (Nakamae *et al*, 2019). Mice were challenged with *Pb*A 63 days after *Pcc* infection and the protective ability was evaluated. In anti-IL-27 mAb-treated antimalarial-untreated mice, the levels of parasitemia reduced and clinical score recovered after 10 days of infection, and mice survived the infection, while parasitemia levels continued to increase, clinical scores continued to increase, and body weights reduced in all other 3 groups, with mice eventually succumbing to death (Fig 3.2.7G). Taken together, these results denote that neutralization of IL-27 during acute infection induced and maintained cellular and humoral immune responses that are protective against recall challenge infection, although chronic infection is required for the maintenance of the protective ability.

Discussion

This study shows that IL-27 produced during the acute phase of malaria infection quantitatively and qualitatively modulates the induction and maintenance of memory CD4+ T cells. Monitoring of the *Plasmodium* specific-CD4+ T cells, PbT-II, showed that they exhibit biphasic expansion in the absence of IL-27. The first phase derived from clonal expansion of activated CD4+ T cells resulting in the generation of Th1 and Tfh effector cells as well as memory precursor-like cells, followed by their contraction. The second expansion phase came between 2 and 3 weeks after infection, dominated by the expansion of Th1-type memory cells, which remained for a long time thereafter during chronic infection. In a classical model of *P. chabaudi* infection, it was proposed that CD4+ T cells undergo biphasic activation; IFN-γ producing Th1-type CD4+ T cells, followed by antibody-helper-type response which were earlier proposed to be Th2 and later described as predominantly Tfh cells (Perez-Mazliah and Langhorne, 2014, Soon and Haque, 2018). We found that IL-27 is critical regulator that switches Th1 dominance to Tfh type response during *P. chabaudi* infection.

Our study also shows that this second expansion phase of malaria-specific CD4+ T cells is not a direct effect of IL-27 inhibition, since inhibition with anti-IL-27 Ab starting 11 days of infection had no such effect. Rather, inhibition of IL-27 during the initial 7 days of infection was critical, suggesting that IL-27 inhibits the generation of memory precursor CD4+ T cells that were destined towards differentiation to Th1-type. Several studies suggest that memory CD4+ T cell fate is determined early during infection with *Pcc*. Memory precursor CD4+ T cells are detected among those with effector differentiation during early activation of lymphocytic choriomeningites virus (LCMV) infection and these cells exhibited a gene signature that distinguishes memory precursors (Tcmp) from effectors supporting for an early decision of memory precursors (Harrington *et al*, 2008, Marshall *et al*, 2011, Pepper and Jenkins, 2011, Ciucci *et al*, 2019). In *Plasmodium* infection model, the decision of Th1-Tfh fate is suggested to be made early after activation by studies on the endogenous TCRαβ sequences in PbT-II cells and by cell transfer experiments (Soon *et al*, 2020). Our study indicates that the critical time span of the effective IL-27 neutralization is limited to a short-period during early activation of specific CD4+ T cells, revealing the critical window of T cell activation for fate determination towards memory generation. IL-27 is produced mainly by myeloid cells including macrophages and dendritic cells in response to Toll-like receptor-dependent signaling as well as immune stimuli such as type I and type II IFNs (Yoshida & Hunter, 2015). IL-27 may directly modulate precursor memory CD4+ T cells as observed in the gene expression patterns during acute infection. Alternatively, early IL-27 neutralization might have long-term effects on the environment of T cell response in a way that predominantly induce high levels of Th1-type memory cells. Further studies will reveal details of molecular mechanisms that underly the modulation of memory CD4+ T cell generation during early activation and their maintenance.

Previous studies of scRNA-seq analysis on PbT-II cells suggested that CD4+ T cells bifurcate to Th1 and Tfh differentiation at a single-cell level during acute *Pcc* infection and these cells transit to memory cells while partially retaining effector phenotypes (Lönnberg *et al*, 2017, Soon *et al*, 2020). However, our study identified PbT-II cells that do not belong to either Th1 or Tfh on day 7 of *Pcc* infection and exhibit high Tmem scores. These may include memory precursor cells that differentiate in parallel to effector cells. Th1 type cells on day 7 also exhibited high Tcmp scores, suggesting their potential as memory precursors (Ciucci *et al*, 2019). PbT-II cells from the IgG-treated mouse had slightly higher Th1 proportions despite having similar parasitaemia to the anti-IL-27 mAb-treated mouse, which we can speculate to be merely a biological variation for a replicate, and an ongoing transitory phase during

effector differentiation. Still, we observed the evidently higher expression levels of Th1-related genes in the Th1 cluster for the anti-IL-27 mAb-treated mouse PbT-II. While adoptive transfer experiments may resolve the relationship between memory precursor and their progenies, the limited ability of CD4$^+$ T cell expansion *in vivo* after adoptive transfer hampered these studies. Nonetheless, during chronic infection, majority of the PbT-II cells exhibited the gene expression signatures of Th1 effector and memory in mice, in which IL-27 was neutralized during acute infection, suggesting the generation of Th1 memory-like characteristics is inhibited by IL-27 produced during acute phase of malaria infection.

Circulating memory CD4$^+$ T cells are categorized into CCR7$^+$CD62L^{hi}CD45RA$^-$ central memory (T$_{CM}$) and CCR7$^-$CD45RA$^-$ effector memory (T$_{EM}$) subsets based on their trafficking potential (Sallusto *et al*, 1999). Studies using *Pcc* infection model demonstrated that T$_{EM}$ cells are the major memory cells during chronic infection, which produce cytokines and participate in the control of parasitemia, while T$_{CM}$ were induced after short-term exposure to parasites with infection followed by the drug treatment (Stephens and Langhorne, 2010, Opata *et al*, 2018). We demonstrated that memory type PbT-II cells induced without IL-27 were phenotypically and functionally distinct from those induced in IL-27 sufficient mice. Furthermore, scRNA-seq analysis clearly demonstrated that largely non-overlapping populations of PbT-II cells are induced in IL-27-mAb-treated and IgG-treated mice. We identified 3 populations of PbT-II cells in IL-27 neutralized mice: CD127$^+$KLRG1$^-$, CD127$^-$KLRG1$^+$, and CD127$^-$KLRG1$^-$ PbT-II cells. While both CD127$^+$KLRG1$^-$ (cluster 1*) and CD127$^-$KLRG1$^+$ (cluster 1**) populations expressed Th1-like gene signatures, they are distinct in their gene expression and function. CD127$^+$KLRG1$^-$ cells expressed higher levels of *Id2*, *Ifng*, Cxcr6, and *Cxcr3*, genes, produced less IFN-γ and TNF in response to antigenic peptide, and proliferated less *in vivo* when compared with CD127$^-$KLRG1$^+$ cells. These cells also express *Bcl2* at high level suggesting their memory potential. CD127$^-$KLRG1$^+$ cells expressed higher levels of *Tbx21*, *Lgals1*, *Klf2, Cx3cr1, Ccl5* and produce proinflammatory cytokines and proliferate more than CD127$^+$KLRG1$^-$ cells. Recent studies reported KLRG1^{hi}CD127^{lo} effector-like long-lived memory CD8$^+$ T cells in mice with chronic viral infection, that can survive long-term, have robust effector function, and are effective in clearance of pathogens against rechallenge infection, and also sensitive to checkpoint blockade therapy (Olson *et al*, 2013, Hudson *et al*, 2019, Renkema *et al*, 2020). Not only KLRG1 protein expression but the transcriptional profile of CD127$^-$KLRG1$^+$ cells is comparable to those CD8$^+$ T cells; both express high levels of transcription factors (*Tbx21, Klf2, Zeb2),* killer cell lectin-like receptors (*Klrg1*, *Klrd*), and genes related to leukocyte migration (*Cx3cr1, Ccl5, S1pr1*). These features

suggest that a common genetic program may exist among unique CD4$^+$ and CD8$^+$ T cell subsets, which allow their long-term survival and effector function during chronic infection, that is critical for the maintenance of protective immunity in the face of ongoing low-level infection.

To compare PbT-II cells in IL-27-sufficient mice, we performed re-analysis of the gene expression of PbT-II cells in mice 28 days after *Pcc* infection (Soon *et al*, 2020) and found that there exist a subpopulation exhibiting the gene expression comparable to CD127$^+$KLRG1$^-$ cells (cluster 1*) in IL-27-neutralized mice, although these cells did not express CD127. Interestingly, cells comparable to cluster 1** were completely absent in IL-27-sufficient mice, suggesting that IL-27 inhibits the induction of their precursors. Treatment of *Pcc*-infected mice with antimalarial drug starting 6 days after infection prevented second wave of PbT-II expansion induced under IL-27 neutralizing condition. However, the development of CD127$^+$KLRG1$^-$ and CD127$^-$KLRG1$^+$ cells was unaffected by the antimalarial drug treatment implying that their development is independent of their second expansion. Furthermore, both cell types were stably maintained after clearance of *Plasmodium* infection with antimalarial drug treatment starting 21 days after infection, suggesting that they are not short-lived. Therefore, IL-27-sensitive fate decision of PbT-II cell differentiation occurs early after *Pcc* infection and that, once the decision is made, active infection is not required for their maintenance. Among PbT-II subpopulations on day 28 after infection in anti-IL-27 mAb-treated mice, KLRG1$^-$CD127$^-$ cells were a minor population but exhibited the highest Tmem signature. Further studies will be required to determine whether these cells can replenish CD127$^+$KLRG1$^-$ and CD127$^-$KLRG1$^+$ populations during *Pcc* chronic infection.

Mice infected with *Pcc* under IL-27 neutralization condition were resistant against challenge infection with heterologous parasite, *Pb*A. CD4$^+$ T cells from these mice exhibited higher IFNγ production in response to *Plasmodium* antigen and higher anti-*Plasmodium* antibodies in the serum 63 days after infection. However, this protective immune memory was dependent on active infection with *Pcc* since antimalarial treatment abrogated the maintenance of the protective immunity. This is consistent with the previous study showing that T cells from chronically infected mice protected better than those from mice that were treated with antimalarial drug, implying a critical role for persistent infection in the maintenance of protective memory CD4$^+$ T cells (Stephens and Langhorne, 2010). Our study showed that not only cellular immunity, but also humoral immunity, was improved in chronically infected mice after early IL-27 neutralization. The antibody response in malaria appears to be dominated by

long-lasting somatically hypermutated high affinity IgM memory B cells and early secondary response is dominated by IgM responses in both T-dependent and T-independent manner as shown in human study and *Pcc* infection model (Krishnamurty *et al*, 2016). Among Ig isotypes, we showed that anti-*Plasmodium* IgM levels were affected more than IgG responses by IL-27 blockade. We speculate that IL-27 neutralization might have direct effect on B cell compartments in addition to augmenting the maintenance of memory helper T cells.

This study had some limitations. The PbT-II adoptive transfer system is specific to the PbHsp90$_{484-496}$ epitope (Enders *et al*, 2021) for analyzing *Plasmodium*-specific responses, and the experiments were only until 9 weeks of observation, which limits the characterization of the memory CD4$^+$ T cell responses; nonetheless, the same trends in the subset differentiation were observed in experiments focused on polyclonal CD4$^+$ T cells. The scRNAseq analyses were also limited by analyzing only 1 replicate per time point, and to ensure that samples best represented the treatment condition and timepoints, the parasitaemia levels were used as the main criterion in selecting the samples based on results of previous experiments, especially for the Day 7 samples. Additionally, the study did not investigate further the effect of IL-27 neutralization on malaria-induced pathology such as tissue damage and hemozoin accumulation, and the fate trajectories of specific T cell subsets during the memory phase, suggesting avenues for future research. These limitations are further discussed in Chapter 6.

The immune response in malaria has biphasic roles: prevention of the infection and host damage. The regulatory role of IL-27 is critical in preventing host pathology by exacerbated Th1-type immune responses as shown in studies of malaria models using IL-27 receptor-deficient mice (Findlay *et al*, 2010, Villegas-Mendez *et al*, 2013, Yui and Inoue, 2020). Our study identified the critical window of CD4$^+$ T cell activation and differentiation during *Plasmodium* infection that is targeted by IL-27, in which its transient neutralization enhances memory CD4$^+$ T cells and improves protection against challenge infection without exacerbated immune responses. IL-27 levels are increased in individuals infected with *P. falciparum* (Otterdal *et al*, 2020) and this study opens a possibility to improve the host protective immune response while preventing tissue damage due to exacerbated immune responses. Finally, these findings show how the absence of IL-27 enhances memory during malaria, suggesting potential applications in the development of vaccines and other strategic interventions.

Manuscript supplementary figures

Expanded View (EV) figures
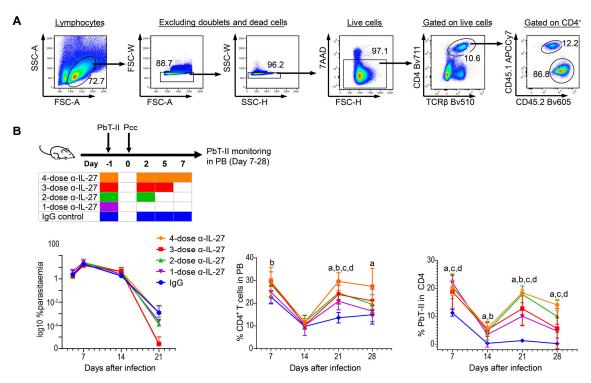
Figure EV1. IL-27 acts on PbT-II cells during the early phase of infection. Related to Figure 3.2.1.

(A) Gating strategy for flow cytometry analysis of PbT-II cells. Samples were stained for CD4, TCRβ, CD45.1 and CD45.2 to identify PbT-II cells. APCCy7 anti-CD45.1 and Bv605 anti-CD45.2 mAbs for the congenic markers, and Bv510 anti-TCRβ mAb were maintained in all panels. For CD4 staining, Bv711 anti-CD4 mAb was used in Figs 3.2.1, 3.2.2A-B, 3.2.6E, and EV2A-B; Pacific Blue-anti-CD4 mAb for Figs 3.2.2C-D, 3.2.3, 3.2.6E and EV2C, and FITC anti-CD4 mAb for Figs 3.2.2E, 3.2.6F and EV2D.

(B) B6 mice transferred with PbT-II cells were administered with anti-IL27 mAb in 4 different conditions or IgG control at timepoints indicated and were infected with Pcc (n = 4 mice/group). Parasitemia levels (log10-transformed), proportion of CD4$^+$ T cells and proportions of PbT-II cells in CD4$^+$ T cells in PB were monitored. Representative data of 2 independent experiments are shown.

Data information: Statistical significance was assessed by one-way ANOVA followed by Tukey's multiple comparison test in (B) and by Student's *t* test in (C). *p* values (< 0.05) are shown. Small letters in (B) indicate significant differences compared to IgG control (a=4-dose, b=3-dose, c=2-dose, d=1-dose treatment), and *p*-values are: day 7, b = 0.010; day 21, a = <0.001, b = 0.015, c = 0.010, d = 0.022; day 28, a = 0.035 (middle graph); day 7, a = 0.031, c = 0.022, d = 0.009; day 14, a = 0.023, b = 0.029; day 21, a = <0.001, b = 0.002, c = <0.001, d = 0.018; day 28, a = 0.001, c = 0.044, d = 0.036 (right graph). Error bars represent SD.
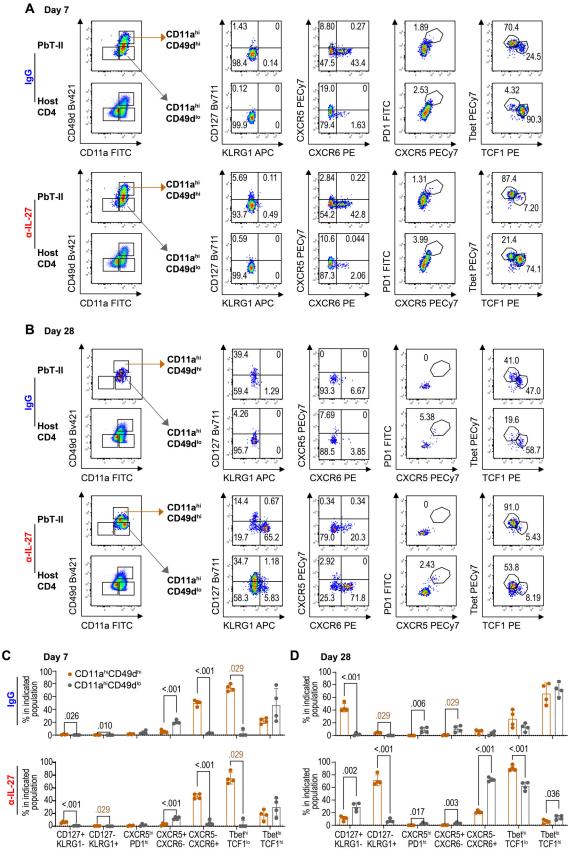
Figure EV2. IL-27 affects development of PbT-II cells during the transition from acute to chronic malaria infection. Related to Figure 3.2.2.

Spleen cells were prepared from mice on day 7 (A, C) and 28 (B, D) pi and were stained with CD11a and CD49d, along with other markers described in Figure 3.2.2 (n=4 mice/group).

(A-B) Representative CD11a/CD49d profiles of CD45.1$^+$CD45.2$^-$ (PbT-II) and CD45.1$^-$CD45.2$^+$ (host CD4$^+$ T) cells (left) and flow cytometry profiles of PbT-II subpopulations of CD11a^{hi}CD49d^{hi} and CD11a^{hi}CD49d^{lo} PbT-II subpopulations are shown (right).

(C, D) Corresponding summary frequencies of CD11a^{hi}CD49d^{hi} and CD11a^{hi}CD49d^{lo} PbT-II subpopulations.

Data information: Numbers in flow cytometry profiles indicate PbT-II proportions (%) within each area. Statistical significance assessed by Student's t test or Mann-Whitney U test per time point, depending on normality assessment. Significant p values (< 0.05) are indicated as black text for Student's t-tests and brown text for Mann-Whitney U tests performed. Error bars represent SD.
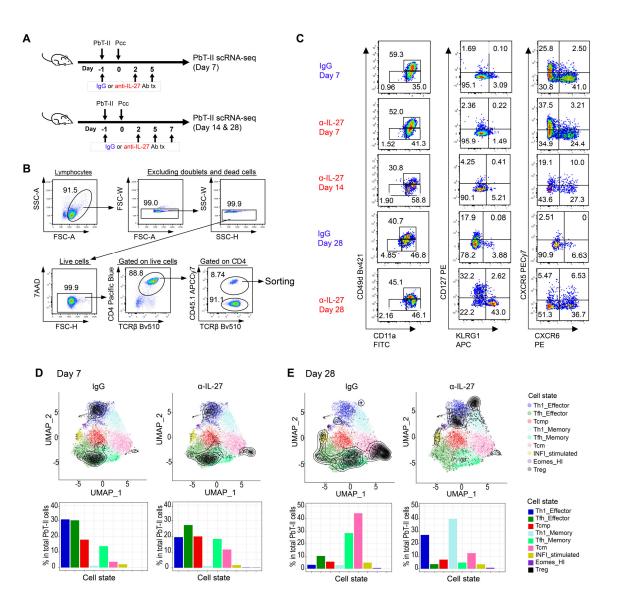
Figure EV3. scRNA-seq analysis shows Th1-biased CD4⁺ T cell development during malaria chronic infection. Related to Figures 3.2.4 and 3.2.5.

B6 mice were transferred with PbT-II cells, treated with IgG or anti-IL-27 mAb on day -1, 2 and 5 for day 7 analysis, while mice were treated with anti-IL-27 mAb on day -1, 2, 5 and 7 for day 14 and 28 analysis (n = 1 biological replicate per timepoint). PbT-II cells were purified and subjected to single cell RNA sequencing (scRNA-seq) and CITE-seq analysis. The ProjecTILs algorithm (Andreatta *et al*, 2021) was used to analyze CD4⁺ T cell states of PbT-II cells based on a published reference atlas (Andreatta *et al.*, 2022).

(A) Experimental scheme.

(B) Gating strategy for the sorting of PbT-II cells for the scRNA-seq experiments: Spleen cells were stained for CD4, TCRβ, and CD45.1 to distinguish PbT-II cells, and for TotalSeq IgG2a, CD127, KLRG1, and CD49d for CITE-seq analysis.

(C) Flow cytometry profiles for each PbT-II sample analyzed for single cell transcriptomics.

(D, E) Predicted distribution of the projected PbT-II cells in IgG and anti-IL-27 mAb-treated mice on day 7 (D) and day 28 (E) after Pcc infection as density contours in a UMAP plot of a CD4⁺ T cell reference map (Andreatta *et al.* 2022). The bar graphs represent the proportions of the PbT-II cells projected in the indicated reference subtype.
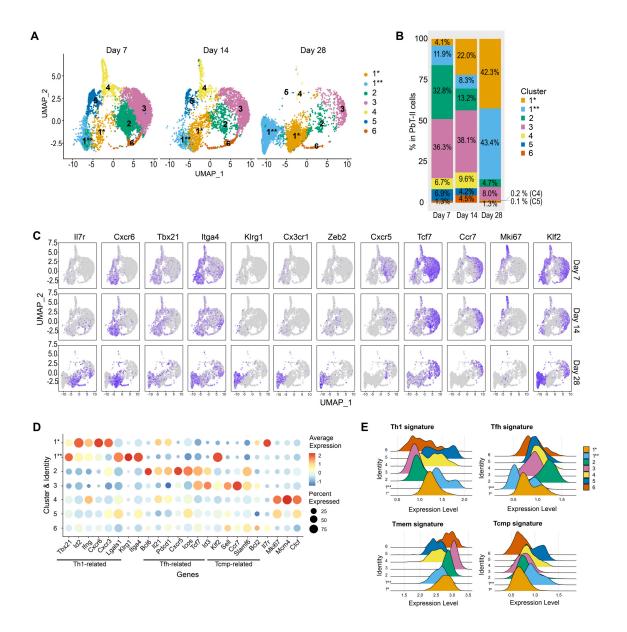
Figure EV4. scRNA-seq analysis suggests transition of CD4⁺ T cell subsets under IL-27-neutralization condition. Related to Figure 3.2.5.

scRNA-seq data of PbT-II cells from Pcc-infected anti-IL-27 mAb-treated mice (day7, 14, and 28) were pooled, and unsupervised clustering was performed.

(A) UMAP plot colored by gene expression clustering.

(B) Proportions (%) of each cluster for each time point.

(C) Feature plots of indicated genes across cell clusters as distributed in UMAP plots. (D) Dot plots showing the expression of Th1-, Tfh-, Tmem-, and proliferation-associated genes in each cluster. Dot colors represent the intensity of expression, while dot size represents the proportion of cells with the corresponding expression.

(E) Ridge plots of PbT-II cell clusters showing the expression of published CD4⁺ T cell signature genes (Ciucci *et al.* 2019).
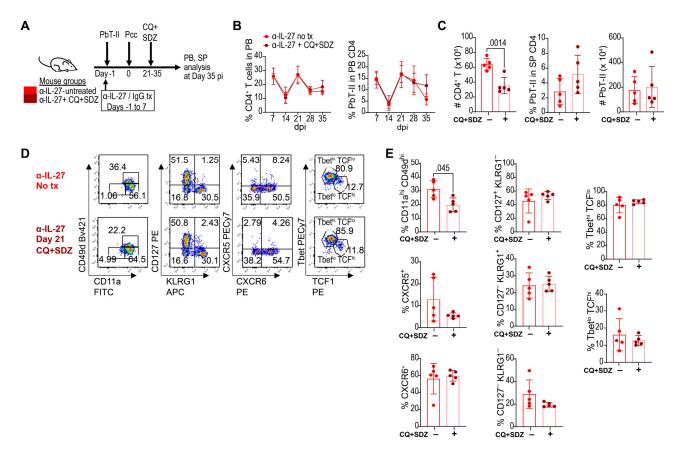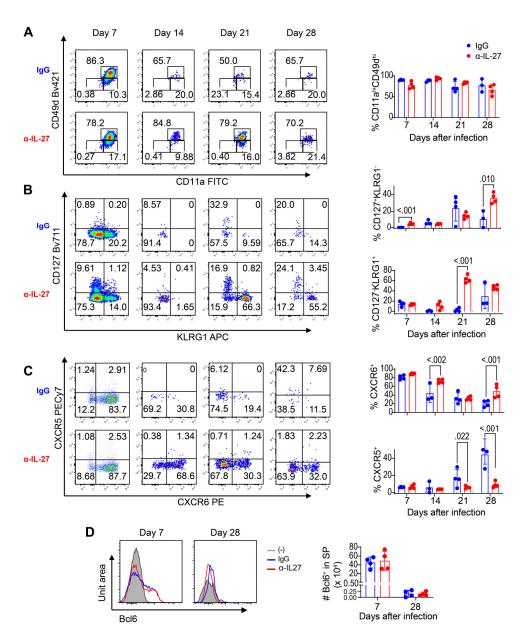
Figure EV5. Antimalarial treatment during chronic phase of Pcc infection does not affect the persistence and phenotype of memory PbT-II cells. Related to Figure 3.2.5.

B6 mice were transferred with PbT-II cells, treated with anti-IL-27 mAb, and treated (dark red) or not treated (red) with antimalarial drugs starting 21 days after Pcc infection (n = 5 biological replicates per treatment group). PB was monitored weekly and PbT-II cells in the spleen were analyzed 35 days post-Pcc infection.

(A) Experimental scheme.

(B) Kinetics of proportions of CD4$^+$ T cells in PB and of PbT-II cells in PB CD4$^+$ T cells.

(C) Total number of CD4$^+$ T cells in spleen, and proportions within CD4$^+$ T cells and total number of PbT-II cells in spleen (n=5 mice/group).

(D) Representative flow cytometry profiles of splenic PbT-II cells on day 35 of Pcc infection.

(E) Proportions of PbT-II cells with the indicated phenotype and those expressing the indicated transcription factors 35 days after Pcc infection (n=5 mice/group).

Data information: Representative data of 2 independent experiments are shown. Statistical significance was assessed by Student's *t* test or Mann-Whitney *U* test, depending on normality assessment. Significant *p* values (< 0.05) for Student's *t* test (black text) are shown. Error bars represent SD.

Appendix supplementary figures

Appendix Figure S1. IL-27 neutralization affects the differentiation of PbT-II profile in peripheral blood. Related to Figure 3.2.2.
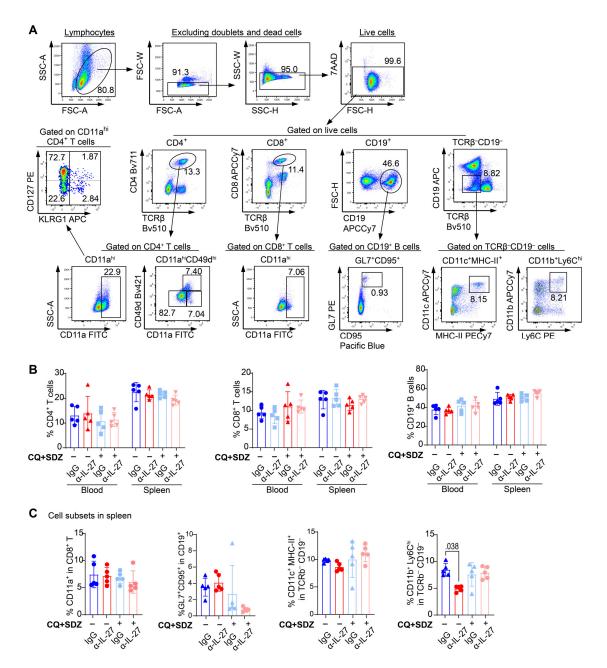
B6 mice were transferred with PbT-II cells and treated with either control (IgG, blue) or anti-IL-27 mAb (α-IL-27, red) between -1 and 7 days post-Pcc infection. PbT-II cells in PB were analyzed by flow cytometry at 7, 14, 21, and 28 dpi.

(A-C) Representative flow cytometry plots (left) depicting cell surface marker expression of CD11a/CD49d (A), CD127/KLRG1 (B), and CXCR5/CXCR6 (C) in PbT-II cells (IgG-treated mice, n=4, 3, 4, 3, and anti-IL-27 mAb-treated mice, n=4, 4, 4, 4 mice for days 7, 14, 21, 28 days pi, respectively), and summary of the frequencies of PbT-II subpopulations indicated (right). Representative data of 2, 2, 3 and 3 independent

experiments for day 7, 14, 21, and 28 pi, respectively.

(D) Representative histograms (left) of Bcl6 expression gated on PbT-II cells in control (blue) and α-IL-27 (red) mouse groups at days 7 and 28 pi (n = 4 mice/group) and their isotype control (gray), with corresponding summary graphs of total numbers of Bcl6^{+} PbT-II cells (right).

Data information: Numbers in flow cytometry profiles indicate the proportions (%) of PbT-II cells within each area. Statistical significance was assessed by Student's *t* test or Mann-Whitney *U* test, depending on normality assessment. *p* values (< 0.05) are shown. Error bars represent SD.

Appendix Figure S2. IL-27 neutralization affects the distribution of inflammatory monocytes during the memory phase after Pcc infection. Related to Figure 3.2.7.

B6 mice were prepared in 4 groups: IgG and no drug (blue), anti-IL-27 mAb and no drug (red), IgG and antimalarial drugs (light blue), and anti-IL-27 mAb and antimalarial drugs (pink) as shown in Fig 3.2.7 (n = 5 biological replicates per treatment group). Cells in PB and splenocytes were stained and analyzed using flow cytometry 63 days after infection with Pcc.

(A) Gating strategy for determining the distribution of CD4^{+} T cells, CD8^{+} T cells, B cells, activated lymphocytes, dendritic cells, and inflammatory monocytes.

(B) Proportions of CD4^{+} T cells, CD8^{+} T cells and CD19^{+} B cells in PB and spleen.

(C) Proportions of activated CD8$^+$ T cells (CD11a$^+$), and populations within TCRb$^-$CD19$^-$ (non-T and B cell), including germinal center B cells (GL7$^+$CD95$^+$), conventional dendritic cells (CD11c$^+$MHC$^+$), and inflammatory monocytes (CD11c$^+$Ly6C^{hi}) in spleen.

Data information: Numbers in flow cytometry profiles indicate the proportions (%) of gated cells within each area. Statistical significance assessed by one-way ANOVA followed by Tukey's multiple comparison test or Kruskal-Wallis test with Dunn's *post hoc* tests, depending on normality assessment. *p* values (< 0.05) are shown. Error bars represent SD.

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Chapter 4 : Analysis of antimalarial antibody response data from the Philippines

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Student ID Number	1901756	**Title**	Ms
First Name(s)	Maria Lourdes		
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Thesis Title	Investigating the generation and maintenance of immunological memory to malaria infection		
Primary Supervisor	Katsuyuki Yui (NU), Julius Hafalla (LSHTM)		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	The Lancet Regional Health – Western Pacific		
When was the work published?	May 2023		
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SECTION E

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Date	20 Sept 2023

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Date	21 Sept, 2023

Analytical approaches for antimalarial antibody responses to confirm historical and recent malaria transmission: an example from the Philippines

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Abstract

Background

Assessing the status of malaria transmission in endemic areas becomes increasingly challenging as countries approach elimination. Serology can provide robust estimates of malaria transmission intensities, and multiplex serological assays allow for simultaneous assessment of markers of recent and historical malaria exposure.

Methods

Here, we evaluated different statistical and machine learning methods for analyzing multiplex malaria-specific antibody response data to classify recent and historical exposure to *Plasmodium falciparum* and *Plasmodium vivax*. To assess these methods, we utilized samples from a health-facility based survey (n = 9132) in the Philippines, where we quantified antibody responses against 8 *P. falciparum* and 6 *P. vivax*-specific antigens from 3 sites with varying transmission intensity.

Findings

Measurements of antibody responses and seroprevalence were consistent with the 3 sites' known endemicity status. Among the models tested, a machine learning (ML) approach (Random Forest model) using 4 serological markers (PfGLURP R2, Etramp5.Ag1, GEXP18, and PfMSP1$_{19}$) gave better predictions for *P. falciparum* recent infection in Palawan (AUC: 0.9591, CI 0.9497–0.9684) than individual antigen seropositivity. Although the ML approach did not improve *P. vivax* infection predictions, ML classifications confirmed the absence of recent exposure to *P. falciparum* and *P. vivax* in both Occidental Mindoro and Bataan. For predicting historical *P. falciparum* and *P. vivax* transmission, seroprevalence and seroconversion rates based on cumulative exposure markers AMA1 and MSP1$_{19}$ showed reliable trends in the 3 sites.

Interpretation

Our study emphasizes the utility of serological markers in predicting recent and historical exposure in a sub-national elimination setting, and also highlights the potential use of machine learning models using multiplex antibody responses to improve assessment of the malaria transmission status of countries aiming for elimination. This work also provides baseline antibody data for monitoring risk in malaria-endemic areas in the Philippines.

Research in context

Evidence before this study

Serology has been shown to provide robust estimates of malaria transmission intensity in populations. Measurement of antibody levels against disease-specific and species-specific antigens through finger prick blood samples has the capacity for sensitive and high throughput monitoring that is further improved with the recent development of multiplex immunoassays. There is, however, a need to evaluate the methods of analysis and interpretation of multiplex serology data for use in classifying disease exposure profiles. We searched PubMed on 08 September 2022 using the terms ("malaria" OR "plasmodium") AND ("IgG" OR "antibody" OR "antigen" OR "markers") AND ("serolog∗") AND ((("quantitative") OR ("multiplex") OR ("multivariate") OR ("model∗")) AND ("analy∗")), which returned 145 articles describing multiplex serology studies that not only focused on antimalarial antibodies, but also included seroprevalence studies for simultaneous assessment of multiple diseases, including SARS-CoV-2 and neglected tropical diseases. Although there have been numerous studies that utilized multiplex assays to assess serological exposure markers, most studies analyzed the markers individually, and only 3 studies explored the use of the quantitative data in multivariate statistical methods such as machine learning to see whether this method will improve classification or exposure predictions. Moreover, a PubMed search of malaria serology studies in the Philippines resulted in only 2 recent studies, both of which did not delve into evaluating serological markers of recent and historical malaria exposure against *Plasmodium falciparum* and *Plasmodium vivax*.

Added value of this study

Antibody data is usually analyzed individually through seropositivity cutoffs to produce binary outcomes, or estimation of seroconversion rates through reverse catalytic models fitted using maximum likelihood methods. Our study advances on previous work on serological markers with our assessment of the different analytical approaches for interpreting multiplex antibody response data. By comparing the classical approaches to the more dynamic application of multiplex quantitative data in machine learning and ensemble learning, we are able to show that utilizing a combination of antibody measurements and available training data, we can potentially improve predictions for exposure to specific pathogens, may it be recent or historical. In our panel of *P. falciparum* markers, we identified a combination of 4 antigens that was able to accurately predict recent *P. falciparum* infection using a machine learning model. Our alternative approach in multiplex analysis was also able to confirm the absence of recent transmission of both falciparum and vivax malaria in our Philippine sites, which have not reported local cases in recent years. This is also the first study to describe the malaria exposure levels of areas of varying endemicity in the Philippines based on multiple *P. falciparum* and *P. vivax* serological markers, and can then provide the baseline antibody data for future malaria serosurveillance studies in the country.

Implications of all the available evidence

In this study we presented alternative approaches in analyzing multiplex serological data to make full use of the quantitative antimalarial antibody measurements for improved predictions of malaria exposure levels and transmission intensities. Although supervised classification algorithms would rely on the availability of training data, we have shown and validated that trained models can potentially be useful in areas with likely similar transmission patterns (*e.g.*, Philippines and Malaysia), while its application in other settings needs to be further evaluated. Our study highlights the utility of quantitative antibody data from previously reported recent and historical malaria exposure markers in providing a sensitive assessment of the malaria situation of varied endemic settings. These recent advances in multiplex immunoassays allow for opportunities for an integrated disease surveillance.

Introduction

In malaria elimination areas, where reporting of local cases continually declines, assessing and differentiating areas with residual transmission becomes increasingly challenging. In the case of the Philippines, which is aiming to eliminate malaria by 2030 following a sub-national elimination approach (World Health Organization, 2014), the country saw >70% decrease in malaria cases in the past decade, with only 2 of 81 provinces currently reporting local cases in 2021, and 19 in elimination phase. *Plasmodium falciparum (*Pf) contributes >80% to the total malaria cases, *P. vivax* (Pv) is at >20%, while the other species *P. malariae, P. ovale* and *P. knowlesi* make up <1% of the cases (World Health Organization, 2019). Innovative tools that are capable of detecting both past and present infections can potentially be used to confirm the presence or absence of malaria transmission in this kind of setting that employs subnational specific control approaches (Drakeley *et al*, 2005, Cotter *et al*, 2013, malERA Refresh Consultative Panel, 2017, World Health Organization, 2017a).

Several studies have utilized serology and malaria-specific antibody responses to estimate malaria transmission intensities (Fowkes *et al*, 2010, Folegatti *et al*, 2017), showing that these represent a viable additional metric of both historical and recent exposure (van den Hoogen *et al*, 2015, Kerkhof *et al*, 2016, Pothin *et al*, 2016, Idris *et al*, 2017b, Ssewanyana *et al*, 2017, Wu *et al*, 2020b). Antibody prevalence alone and as age-adjusted seroconversion rates correlate with entomological and parasitological measures used in estimating malaria transmission (Corran *et al*, 2007, Stanisic *et al*, 2015, Niass *et al*, 2017). Many of the original studies examined single antigen platforms and antigens associated with cumulative exposure to infection such as Pf apical membrane antigen-1 (PfAMA1) and the 19KDa fragment of Pf merozoite protein 1 (PfMSP1$_{19}$). More recent advances in array and bead-based assay platforms allow for simultaneous analysis of antibody responses to multiple antigens (Fouda *et al*, 2006, Ondigo *et al*, 2012, Perraut *et al*, 2014, Koffi *et al*, 2015, Kerkhof *et al*, 2016, Wu *et al*, 2019). These approaches allow the inclusion of multiple targets that can represent diversity in the parasite and variation in individual immune response. Recent multi-antigen studies have identified markers associated with antibody responses describing recent and historical *P. falciparum* and *P. vivax* exposure (Helb *et al*, 2015, van den Hoogen *et al*, 2015, Longley *et al*, 2020, Wu *et al*, 2020a). To fully realize the additional information provided by examining multiple antigenic targets, more advanced statistical approaches and algorithms such as machine learning can be employed to predict optimal combinations of antibody responses for the outcome of interest.

The overall aim of this study was to evaluate known malaria-specific *P. falciparum* and *P. vivax* serological markers for their predictive capacity to distinguish recent infections from historically exposed individuals. Specifically, we sought to evaluate different approaches to determining seropositivity for estimating malaria transmission intensities and exposure in areas of varying endemicity, and apply this using serological data from the Philippines. To achieve this, we: 1) evaluated analysis methods to determine seropositivity using malaria-specific antibody responses to single and multiple antigens, utilizing multiplex serological data from health facility surveys in 3 sites in the Philippines, and 2) analyzed antibody responses and estimated transmission intensities in relation to the supposed immune status of these populations.

Methods

Ethical approval

This study was reviewed and approved by the Research Institute for Tropical Medicine – Institutional Review Board in Manila, Philippines (RITM IRB 2016-04) and LSHTM Research Ethics Committee in London, United Kingdom (11597).

Data Source: Study Sites and Samples

The study was conducted in 3 municipalities in 3 provinces in the Philippines, representing areas of varying malaria endemicity: Rizal, Palawan, currently the most endemic municipality, and reported >60% of the total cases, with an annual parasite index (API) of 5.7 per 1,000 risk population in 2018; Abra de Ilog, Occidental Mindoro, a municipality reporting sporadic local cases and with declining transmission (API of 0.38 in 2018); and Morong, Bataan with a last reported indigenous case in 2011 and declared malaria-free in 2019
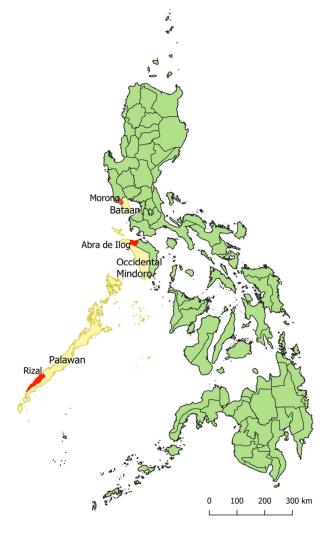
Figure 4.1. Map showing the study sites, with red areas as the focused municipalities within the provinces marked yellow.

(Figure 4.1).

Participants were recruited from June 2016 to June 2018 in a health facility-based rolling cross-sectional survey detailed in Reyes et al (2021). Briefly, all health facility attendees were invited as study participants, who provided a finger-pricked blood sample for malaria diagnosis through microscopy (blood smear), rapid diagnostic test (RDT), and a dried blood spot sample (DBS) on Whatman 3MM CHR filter paper for malaria diagnosis by PCR and serological analysis. DBS samples were stored with desiccant at −20°C.

Multiplex bead-based assay of malaria-specific antibodies

Serological analysis was conducted using a multiplex bead-based assay as previously described (Helb *et al*, 2015, Coutts *et al*, 2017, van den Hoogen *et al*, 2020b), with an antigen panel that included 8 *P. falciparum*-specific and 6 *P. vivax*-specific recombinant antigens coupled to Magplex beads (Luminex Corp, Austin, TX, USA). The antigens were PfAMA1 (apical membrane antigen 1), PfMSP1$_{19}$ (merozoite surface protein), and their *P. vivax* homologues PvAMA1 and PvMSP1$_{19}$; PfGLURP R2 (glutamate rich protein), Etramp5.Ag1 (early transcribed membrane protein 5), PfSEA1 (schizont egress antigen), GEXP18 (Gametocyte exported protein 18), MSP2 CH150/9 (CH150/9 allele of MSP2), MSP 2 Dd2 (Dd2 allele of MSP2), PvEBP (erythrocyte binding protein), PvRBP1a (reticulocyte binding protein 1a), PvRII and PvDBPRII (region II, Duffy binding protein). Antigen characteristics are shown in Table 4.1.

The assay was conducted as described in Wu et al (2019). Briefly, serum was eluted from DBS samples (~1uL from 3mm punch; 1:400 final dilution). Approximately 1000 beads per antigen were added per well in 96-well flat bottom plates, which are incubated for 1.5 hours on a shaker with 50uL sample and controls (2 positive controls for *P. falciparum* and *P. vivax* – pooled plasma from adults in hyperendemic malaria setting; 1 negative control – European malaria-naïve blood donors). After washing, samples are incubated for 1.5 hours with 50uL 1:200 goat anti-human Fcγ-fragment-specific IgG (Jackson Immuno 109-116-098: conjugated to R-PE). Samples were washed, suspended in PBS, and read using a Luminex 200 or Magpix machine to measure IgG antibody levels reported as net median fluorescence intensity (MFI) values. During initial testing, strong correlation and comparable values were observed for samples assayed in both Luminex platforms. Plate-specific adjustments were performed based on the outcome of standard control curves generated from positive control pools included on each plate.

Table 4.1. List of antigens in the multiplex bead-based assay panel

Antigen	Description	Gene ID	Reference
Pf-specific			
PfAMA1	apical membrane antigen 1; cumulative Pf exposure marker	PF3D7_1133400	Collins et al., 2007
PfMSP1$_{19}$	19 kDa fragment of merozoite surface protein (MSP) 1; cumulative Pf exposure marker	PF3D7_0930300	Burghaus and Holder, 1994
PfGlurp R2	glutamate rich protein R2; cumulative/recent Pf exposure marker	PF3D7_1035300	Theisen et al., 1995; van den Hoogen et al., 2019
Etramp5.Ag1	early transcribed membrane protein 5; Pf recent exposure marker	PF3D7_0532100	van den Hoogen et al., 2019 ; Spielmann et al., 2003
PfSEA1	schizont egress antigen; Pf exposure marker	PF3D7_1021800	Raj et al., 2014
GEXP18	Gametocyte exported protein 18; Pf recent exposure marker	PF3D7_0402400	Helb et al., 2015
MSP2 CH150	CH150/9 allele of MSP2; Pf exposure marker	PF3D7_0206800	Polley et al., 2006
MSP2 Dd2	Dd2 allele of MSP2; Pf exposure marker	PF3D7_0206800	Taylor et al., 1995
Pv-specific			
PvAMA1	apical membrane antigen 1; cumulative Pv exposure marker	PVX_092275	Chuquiyauri et al., 2015
PvMSP1$_{19}$	merozoite surface protein; cumulative/recent Pv exposure marker	PVX_099980	França et al., 2016b; Longley et al, 2020
PvRII	region II, Duffy binding protein; Pv exposure marker	PVX_110810	França et al., 2016a
PvDBP RII	region II, Duffy binding protein; Pv exposure marker	PVX_110810	Ntumngia et al., 2012
PvEBP	erythrocyte binding protein; Pv exposure marker	PVX_110835	Hester et al., 2013 ; Menard et al., 2013
PvRBP 1a	reticulocyte binding protein 1a; Pv exposure marker	PVX_098585	França et al., 2016a

Data analysis

Statistical analyses were performed using R version 3.6.3 and Graphpad Prism 8. IgG antibody responses recorded as net MFI values were analyzed using different methods. Quantitative continuous antibody response data (reported as log$_{10}$ MFI values) were compared for different groups (*i.e.*, by age group, study site or recent malaria infection) using

Table 4.2. Training and validation data used in the classification models.

Training data used	N	Validation dataset [a]	Negative population model [b]	SuperLearner / Random Forest: Recent Pf exposure	SuperLearner / Random Forest: Recent Pv exposure	SuperLearner / Random Forest: Historical Pf exposure	Random Forest Historical Pf exposure	Random Forest Historical Pv exposure
European naive controls	179	**neg**	−	−	−	−	−	−
Positive controls								
African *P. falciparum*-positive	4	**Pf+**		+				
African *P. vivax*-positive	10	**Pv+**			+			
This study								
P. falciparum-positive subset	568	**Pf+**		+				
P. vivax microscopy-positive subset	46	**Pv+**			+			
Below 10 y.o. from Bataan	202	**neg**	−	−	−	−	−	−
Historical positives aged ≥50y.o. from Occidental Mindoro and Palawan	711			−		+		
PfAMA1 and PfMSP1$_{19}$ seropositive subset	512						+	
PvAMA1 and PvMSP1$_{19}$ seropositive subset	324							+
Randomly selected malaria-negatives from Palawan aged <50y.o.	550			−	−			
Bataan study								
Below 10 y.o. from Bataan	73	**neg**	−	−	−	−	−	−
Malaysia study								
P. falciparum-positive subset	17	**Pf+**		+				
P. vivax-positive subset	37	**Pv+**			+			
Historical positives aged ≥50y.o. from Malaysia	1581					+		
PfAMA1 and PfMSP1$_{19}$ seropositive subset	1479			−			+	
PvAMA1 and PvMSP1$_{19}$ seropositive subset	873			−				+

[a] Finite mixture models (FMM) were derived from the entire ENSURE dataset (n = 9132), and indicated validation dataset was used to determine receiver operating characteristics (ROC) curves for both FMM and Negative population models. Seropositivity classifications from single antigens presented in Supplementary Tables S1 and S2 were assessed using combined Pf+ and neg datasets, and Pv+ and neg datasets, respectively. [b] For the supervised classification models (negative population, Super Learner, and Random Forest models), +means it was classified as positive for the model, and − means it is considered for negative classification. Abbreviations: Pf- *P. falciparum*, Pv- *P. vivax*, neg - malaria-negative; y.o.- years old.

Student's t-test or one-way ANOVA Kruskal-Wallis test with Wilcoxon test for pairwise comparisons. Correlations between the levels of antibody responses as well as age and malaria positivity were analyzed using Spearman's rank correlation.

Determining seropositivity rates using single antigen responses

Binary outcomes for seropositivity to each antigen were determined through the computation of cut-off values in 2 ways: 1) a 2-component finite mixture model (FMM); and 2) a reference negative population (NegPop) model. FMM was computed using the mixEM function in R mixtools package. For the NegPop model, European naïve samples were used as reference population. For both models, samples were considered seropositive for specific antigens if MFI values are higher than the antigen cutoff values (mean MFI plus 3 standard deviations). To evaluate the accuracy of these classifications, we used samples from known exposed and unexposed populations as validation data (Table 2), comprising *P. falciparum* (Pf+) and *P. vivax*-positive (Pv+) datasets, and a malaria-negative (neg) dataset collated from previous studies and this study. Sensitivity and specificity for identifying recent *P. falciparum* and *P. vivax* infection from this study and the validation dataset, and receiver operating characteristics (ROC) curves, were determined for each serological marker (Supplementary Tables S1 and S2).

Applying machine learning techniques for multiplex analysis of antigen responses

We next utilized machine learning (ML) techniques on multiplex serological data for determining recent and historical malaria exposure. The training data used for these models are in Table 4.2. Focusing on continuous covariates, the 8 Pf-specific antigens along with age were the covariates evaluated for *P. falciparum* exposure, while for *P. vivax* exposure, only the 6 Pv-specific antigens were analyzed (age data not available for the Pv-positive training datasets used for Pv exposure predictions). To account for the age-dependent cumulative increase in antibodies, the negative dataset for classifying recent infection included historical positives—survey participants aged >50 years from Malaysia and Philippines (Hubbard *et al*, 2013, Fornace *et al*, 2019, van den Hoogen *et al*, 2020a) assumed to have had historical exposure, as well as a random selection of Pf or Pv-negatives from all age groups from this current study, all of which confirmed to be PCR-negative in addition to negative microscopy/RDT diagnosis. For classifying historical exposure, the training dataset included the historical positives as positives, and the same negatives from the validation dataset (Table 4.2).

The R package Super Learner (SL) optimized with AUC (Area under the ROC Curve) was used as an ensemble modelling algorithm to allow for evaluating multiple models simultaneously (Hubbard *et al*, 2013, Helb *et al*, 2015, Arnold *et al*, 2017), namely Random Forest (RF), k-Nearest Neighbor (kNN), generalized boosted models (GBM), Support Vector Machine (SVM), and GLM with Lasso (glmnet). Feature selection with corP, that screens for univariate correlation, was also included for some component models (GBM, RF). The SL model gives a prediction value that ranges from 0 to 1, and this can be used to obtain binary classification (*i.e.*, those with prediction values higher than 0.5 were considered positive). A 20-fold cross-validation was performed for internal validation to evaluate the performance of the fitted SL models using a withheld training dataset, and the whole training dataset. ROC curves were used to evaluate the outcome of predicted values for the samples and were then compared to single antigen performances. The performance of each ML model, or base learner, in the SL ensemble was also assessed. For additional assessment, external validation was performed for the classification models through an independently collated dataset (Fornace *et al*, 2018a), which used the same panel of *P. falciparum* antigens (not available for *P. vivax* panel).

Evaluating model performance in identifying recent Pf and Pv infections in this study

Using this study's malaria-positive samples, we assessed the capability of the serological markers in identifying falciparum and vivax infections (Supplementary Table S1). The single antigen performances were compared with SL predictions, where models included 3 to 9 covariates combined based on the variable importance outcomes. The AUC values from the ROC analysis based on training data and ENSURE malaria-positives (test data) were then computed. With Super Learner giving weights to multiple ML models simultaneously, we also evaluated the individual ML models that were given the most weight or importance, namely RF and GBM.

Estimating seroconversion rates and historical exposure

Seropositivity of the cumulative exposure markers (AMA1, $MSP1_{19}$ for both *P. falciparum* and *P. vivax*) were used to estimate seroconversion rates (SCR) and seroreversion rates (SRR), by fitting the age-specific prevalence for each study site into reverse catalytic models using likelihood ratio methods, as previously described (Drakeley *et al*, 2005, Sepulveda *et al*, 2015). The predicted time of change in transmission is analyzed. In

addition to the AMA1 and MSP1$_{19}$ seroprevalence based on FMM models, RF classifications using the combined AMA1 and MSP1$_{19}$ data for both *P. falciparum* (Pf-RF:2-covar) and *P. vivax* (Pv-RF:2-covar) were also used to generate seroprevalence curves. The positive training data used for these RF models are subsets of the historical positives that were seropositive to both markers for each species based on FMM cutoff values (Table 2). The Super Learner approach was also used to predict Pf and Pv historical exposure using multiplex antibody data.

Role of the funding source

The funders of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. MLM, CD, and KF had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Heterogeneity of antibody responses from the 3 collection sites

A total of 9132 DBS samples (6572 for Palawan, 1683 for Occidental Mindoro, and 877 for Bataan) were available for serological evaluation. Females comprised >60% of survey participants, while all age groups were well-represented (Table 3). Malaria infections were detected only in Palawan, with 51.8% and 48.2% of infected males and females, respectively, aged under 10 years old. Of the 889 *Plasmodium*-positive samples confirmed through either microscopy, RDT and/or PCR, 58.0% had *P. falciparum*, 12.4% had *P. vivax*, 6.7% had mixed Pf + Pv infections, 6.1% had *P. malariae*, *P. ovale*, or *P. knowlesi*, while species identification could not be confirmed for 16.8% of the PCR-positive samples. From the Pf and Pv malaria-positive individuals (n = 707, including mixed infections), 388 (54.8%) were symptomatic (had fever before or during the health facility visit), 62.1% of which were ≤10 years old. Conversely, 63.1% of the asymptomatic malaria infections were >10 years old.

Firstly, we compared the magnitude of antibody responses to *P. falciparum* and *P. vivax* antigens from the 3 collection sites. Palawan consistently had the highest antibody levels to all antigens and in all age groups, followed by individuals from Occidental Mindoro then Bataan (Fig 4.2a and Supplementary Fig S1). Expectedly, cumulative exposure markers PfAMA1, PfMSP1$_{19}$, PfGLURP R2, PvAMA1, and PvMSP1$_{19}$ had moderate associations with age (Spearman's coefficient ≥0.47, $p < 0.0001$), while recent exposure markers Etramp5.Ag1 and GEXP18 were less moderately associated (Spearman's

Table 4.3. Characteristics of study population by site

	Morong, Bataan (Malaria-eliminated)		Abra de Ilog, Occidental Mindoro (Near elimination)		Rizal, Palawan (Ongoing transmission)	
Number of analyzed samples	877		1683		6572	
Female, %	628	(71·6%)	1101	(65·4%)	3734	(56·8%)
Age, median (IQR)	26	(11-39)	28	(15-43)	13	(5-31)
number (% in site)						
0 to 5	150	(17·1%)	150	(8·9%)	1900	(28·9%)
6 to 10	63	(7·2%)	154	(9·2%)	1055	(16·1%)
11 to 25	215	(24·5%)	445	(26·4%)	1488	(22·6%)
26 & up	449	(51·2%)	934	(55·5%)	2129	(32·4%)
Plasmodium-positive by microscopy/RDT/PCR (% in site) [a]	0	(0·0%)	0	(0·0%)	889	(13·5%)
P. falciparum-positive (by microscopy/RDT/PCR) [a]	0	(0·0%)	0	(0·0%)	595	(9·1%)
P. vivax-positive (by microscopy/RDT/PCR) [a]	0	(0·0%)	0	(0·0%)	172	(2·6%)
PCR-confirmed species ID, n (% in site) [b]						
P. falciparum, Pf mono-infection	0	(0·0%)	0	(0·0%)	516	(7·9%)
P. vivax, Pv mono-infection	0	(0·0%)	0	(0·0%)	110	(1·7%)
Pf + Pv mixed infection	0	(0·0%)	0	(0·0%)	60	(0·9%)
Other species (mono- or mixed infections)	0	(0·0%)	0	(0·0%)	55	(0·8%)
With fever or history of fever (% in site) [c]	69	(7·9%)	172	(10·2%)	2727	(41·5%)
Symptomatic Pf and Pv infections [a, c]	0	(0·0%)	0	(0·0%)	388	(5·9%)
Asymptomatic infections (no fever, *Plasmodium*-positive) [a, c]	0	(0·0%)	0	(0·0%)	428	(6·5%)
Asymptomatic microscopy-positive Pf infections	0	(0·0%)	0	(0·0%)	132	(2·0%)
Asymptomatic microscopy-positive Pv infections	0	(0·0%)	0	(0·0%)	35	(0·5%)

[a] Numbers include all *Plasmodium* infections detected by microscopy, RDT and/or PCR. Some samples did not have enough material for further species identification by PCR, for which results were based on microscopy and/or RDT diagnosis, while some (n = 149) were confirmed *Plasmodium*-positive only. Pf and Pv numbers reported include mixed infections, which were specified in the PCR-confirmed species ID breakdown. [b] All 5 *Plasmodium* species, including *P. malariae* (n = 50), *P. ovale* (n = 4) and *P. knowlesi* (n = 1), were detected in Palawan, with some as co-infections with Pf and/or Pv. [c] Presence of fever and/or history of fever (within the past 3 days of health facility visit) in malaria-positive participants was used to classify symptomatic infections.
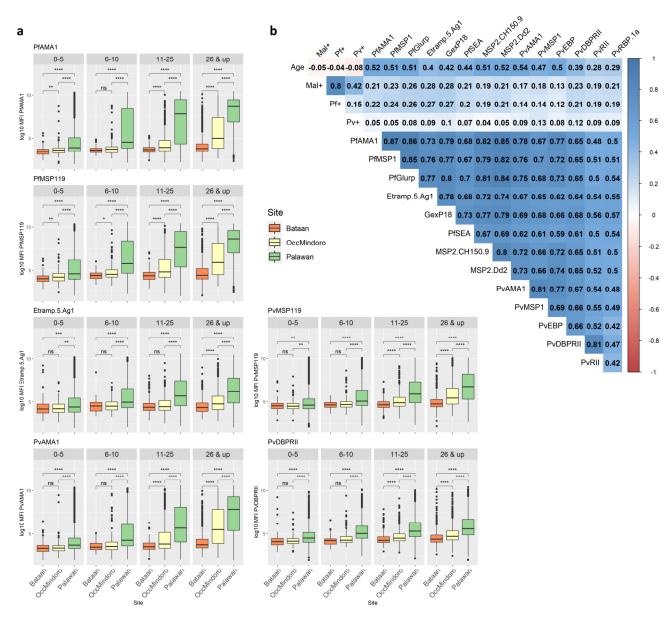
Figure 4.2. Antibody responses to serological markers of *P. falciparum* and *P. vivax* correlate with malaria incidence.

a) Antibody levels (reported as log10 MFI) in response to *P. falciparum* cumulative and recent exposure markers PfAMA1, PfMSP1$_{19}$, and Etramp5.Ag1, and *P. vivax* serological markers PvAMA1, PvMSP1$_{19}$, and PvDBP.RII by study site and age group. Statistical difference of overall antibody responses among study sites within age groups were determined using Kruskall–Wallis test and Wilcoxon test for pairwise comparisons (∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001). Data for the rest of the antigens in the panel are shown in Supplementary Figure S1. **b)** Spearman's correlation coefficients for age, malaria diagnosis (Mal+: *Plasmodium*-positive, Pf+: *P. falciparum*-positive, Pv+: *P. vivax*-positive), and antibody responses to the 14 antigens in the panel for Palawan samples (n = 6572).
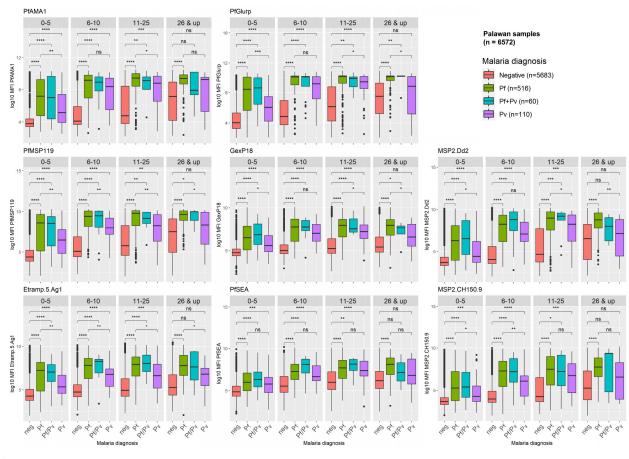
coefficient of 0.39 and 0.42, respectively, p < 0.0001). PvMSP1$_{19}$ and PvDBPRII showed weak correlation (Spearman's coefficient of 0.13 and 0.12, respectively, p < 0.0001) with recent *P. vivax* infection (Fig 4.2b). Similarly, mean antibody levels were higher in a species-specific manner (Fig 4.3). Taken together, our results confirm the applicability of these serological markers in differentiating areas of varying malaria endemicity, and there were species-specific associations with recent infection.

Identifying markers of recent falciparum and vivax malaria exposure and recent infection

Next, we applied different analytical approaches to ascertain whether the serological markers can be used to predict recent or historical exposure. The resulting seropositivity cutoff values were comparable for FMM (unsupervised) and Negpop (supervised) classification models, with AUC values from the validation data ranging from 0.812 to 0.932 for the 8 *P. falciparum* markers, and 0.534 to 0.943 for the 6 *P. vivax* markers (Fig 4.4a and b and Supplementary Tables S1 and S2). When applied to classifying study samples, some of the markers showed low predictive ability, with AUC values ranging from 0.497 to 0.756 for *P. falciparum* markers, and 0.513 to 0.731 for *P. vivax* markers. Etramp5.Ag1 and GEXP18 for *P. falciparum,* and PvMSP1$_{19}$ for *P. vivax,* had significantly higher AUC values (>0.735, p < 0.002) compared to the rest of the markers analyzed (Supplementary Tables S1 and S2). Density plots of antibody responses also show overlap of malaria-negative and positive distributions, contributing to the lower AUCs (Supplementary Fig S2). Seroprevalence rates for all antigens were consistently low in Bataan in all age groups (Fig 4.4a and b), with recent exposure markers (hollow circles) estimating lower rates.

We next applied ML methods for analyzing multiplex antibody response data. The Super Learner (SL) ensemble machine learning algorithm was used to simultaneously evaluate classifications from ML models, which included Random Forest (RF), k-Nearest Neighbor (kNN), generalized boosted models (GBM), Support Vector Machine (SVM), and GLM with Lasso (glmnet), and weights are applied to each learner after cross-validation. Covariates were assessed for their relative importance for the model, and different combinations in a model were assessed using the ROC curves of cross-validated predictions (Fig 4.4c and d and Supplementary Tables S1 and S2). The 9-covariate model (SL: 9-covar; 8 antigens and age as covariates) had the highest AUC among all SL models, with a value of 0.9898 (95% CI 0.9874–0.9921) and 0.9197 (95% CI 0.9093–0.9302) for the binary classification predictions for the training data and test data, respectively, and removing the age as covariate (SL:8-
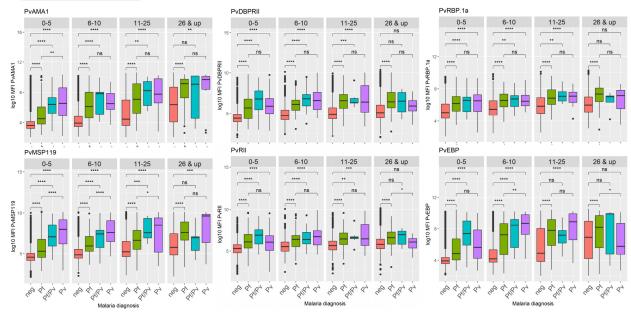
Figure 4.3. Serological markers exhibit species-specific association with recent infection in varying levels.

a and b) Comparison of antibody titers of Palawan samples (n = 6572) for *Plasmodium*-positive (Pf: *P. falciparum*-positive, Pf/Pv: Pf and Pv mixed infection, Pv: *P. vivax*-positive) and negative (neg) samples by age group in each *P. falciparum*-specific and *P. vivax*-specific antigens.

covar model) decreased the prediction capacity (Supplementary Table S1). For predicting *P. vivax* recent exposure, the model incorporating the 6 Pv antigens as covariates (SL:6-covar) had a resulting AUC of 0.8857 (95% CI 0.8429–0.9284) and 0.6332 (0.5979–0.6686) for validation data and test data, respectively (Supplementary Table S2).

After a 20-fold nested cross-validation, generalized boosted models with or without feature selection (GBM and GBM_corP) were given the highest weights in the SL ensemble models for both species' recent exposure predictions (Supplementary Fig S3a and 4.3b). However, upon closer inspection of the classification accuracy of the individual learners for predicting *P. falciparum* recent infections, the Random Forest (RF) models were found to have the highest AUCs in both the test data (Supplementary Fig S3c) and validation data (Supplementary Fig S3d). Computing for the AUCs of resulting binary classifications (Fig 4.4c) also showed RF models with highly improved AUCs compared to FMM-based single antigen classifications and even the SL models, suggesting that RF is the best performing model in distinguishing recent falciparum malaria infections. On the other hand, vivax infection classification models had comparable AUCs ranging from 0.701 to 0.8 for test data (Supplementary Fig S3e), and also for binary classifications for SL, RF, and GBM models (Fig 4.4d and Supplementary Table S2).

Variable importance in the RF model for *P. falciparum* positivity (Fig 4e) shows that responses to Etramp5.Ag1, PfGLURP R2, GEXP18 and PfMSP1$_{19}$ had the highest influence in the predictions, while PvMSP1$_{19}$ and PvAMA1 were the most predictive *P. vivax* antigens (Fig 4.4f). RF.covar4 gave an AUC of 0.9983 for the training data and 0.9591 for the test data (Fig 4.4c and Supplementary Table S1), and had comparable AUC with the RF 8-covariate model (RF.covar8), showing that analysis using the RF model combining just the 4 predictive Pf antigens was sufficient for predicting recent *P. falciparum* infection or exposure.

The predictive performance of the resulting SL models was further compared with RF (both species) and GBM (for Pv) predictions (Fig 4.4g and h). The rates of predicted recent *P. falciparum* infection based on SL and RF models all consistently gave 0%–0.2% prediction for Occidental Mindoro and Bataan, where no *P. falciparum* malaria cases were reported in recent years, thus accurately predicting the absence of recent falciparum malaria exposure and infection (Fig 4.4g). SL and RF models from *P. vivax* antigens also had <1% prediction rate for Bataan and <5% for Occidental Mindoro, also indicative of a predicted absence of recent *P. vivax* infections in these sites (Fig 4.4h and Supplementary Table S2).
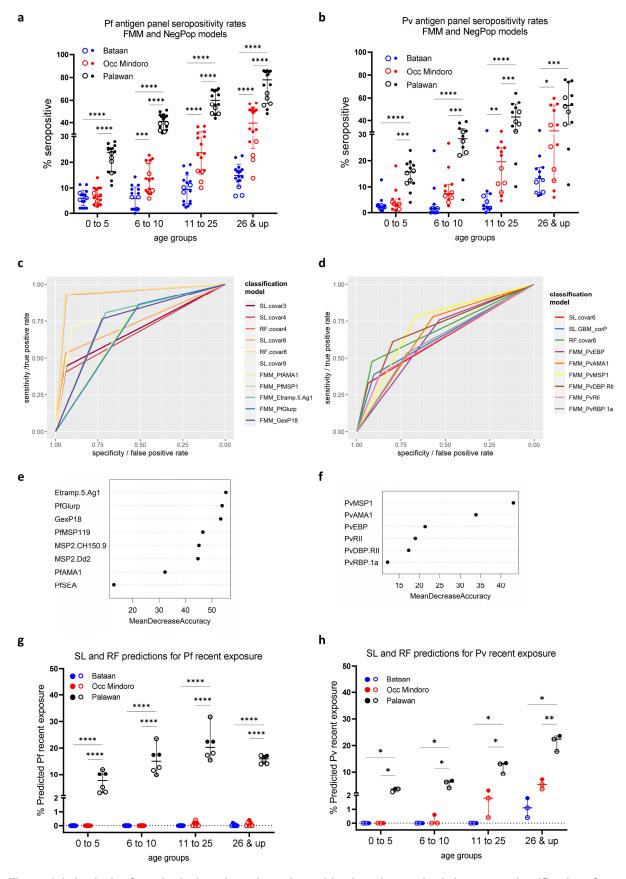
Figure 4.4. Analysis of serological markers through machine learning methods improves classifications for recent *P. falciparum* and *P. vivax* exposure or recent infection.

Cont'd. Figure 4.4: **a and b)** Seropositivity rates of sample population by site and age group based on cutoff values from finite mixture models (FMM) and negative population model (NegPop) for Pf and Pv antigens (detailed in Supplementary Tables S1 and S2). Hollow dots represent the FMM and Negpop seropositivity rates for the two reported recent exposure markers Etramp5.Ag1 and GEXP18 for Pf **(a)**, and PvMSP1$_{19}$ and PvDBP.RII for Pv **(b)** panel. Lines with error bars represent median with 95% CI. **c and d)** Receiver operating characteristic (ROC) curves for the antibody responses to single antigens for individual antigens, and for the SL models (shown in both as binary outcomes of seropositivity/prediction values). **e and f)** Variable importance of the 8 Pf-specific **(e)** and 6 Pv-specific **(f)** antigens based on the Random Forest model. **g)** Predicted rates of recent Pf exposure based on analysis of the continuous antibody responses of the 8-antigen panel using Super Learner. Each hollow dot represents differences in the number of covariates used for the model (3, 4, 8, 9), as well as 2 showing rates from prediction values of the Random Forests (RF) component model (RF.covar4 and RF.covar8 in solid dots). **h)** Predicted rates of recent Pv exposure based on analysis of the continuous antibody responses of the 6-antigen panel using Super Learner. Dots represent the positivity rates from the prediction values of the SL model, and the individual predictions from the 2 most weighted base learners in the resulting model—RF and GBM. RF predictions (RF.covar6) are shown as solid dots (SL: super learner, RF: random Forest, covar#: number of covariates included in the model, FMM: finite mixture models; ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001 with significance assessed by one-way ANOVA followed by Tukey's multiple comparison).

Model validation using external datasets

To further evaluate the performance of the RF:4-covar model for predicting *P. falciparum* recent exposure, external validation was performed using a collated test dataset (Supplementary Table S3) that included samples from a cross-sectional survey in Malaysia (Fornace *et al*, 2018b) (n = 8163; all Pf-negative), a 2017 cross-sectional survey in Bataan (van den Hoogen *et al*, 2020a) (n = 1926; all malaria-negative), PCR-validated malaria cases (Pf = 47, non-Pf = 428), and malaria-naïve or malaria-negative samples (n = 506). The model prediction resulted in an AUC of 0.93 (CI 0.8817–0.9782), and was able to predict 0.47% prevalence of infection for the Malaysia cross-sectional survey, and 0.10% for the Bataan cross-sectional survey. It also correctly identified 41 of 47 (87.2%) of the *P. falciparum* PCR-confirmed cases from the malaria-positive subset.

Estimating *P. falciparum* and *P. vivax* historical exposure

AMA1 and MSP1$_{19}$ responses for *P. falciparum* and *P. vivax* have been widely used to assess historical exposure by estimating seroconversion and seroreversion rates. Using reverse catalytic models and maximum likelihood tests on the age-specific seroprevalence based on the FMM model seropositivity rates for PfAMA1, PfMSP1$_{19}$, PvAMA1, and PvMSP1$_{19}$, we estimated the time of interruption of transmission in our study area (Fig 4.5 and Table 4). The RF algorithm was also employed to generate models that combine both antigens (2-covariate models Pf-RF:2-covar and Pv-RF:2-covar), and the predicted binary outcomes were also used
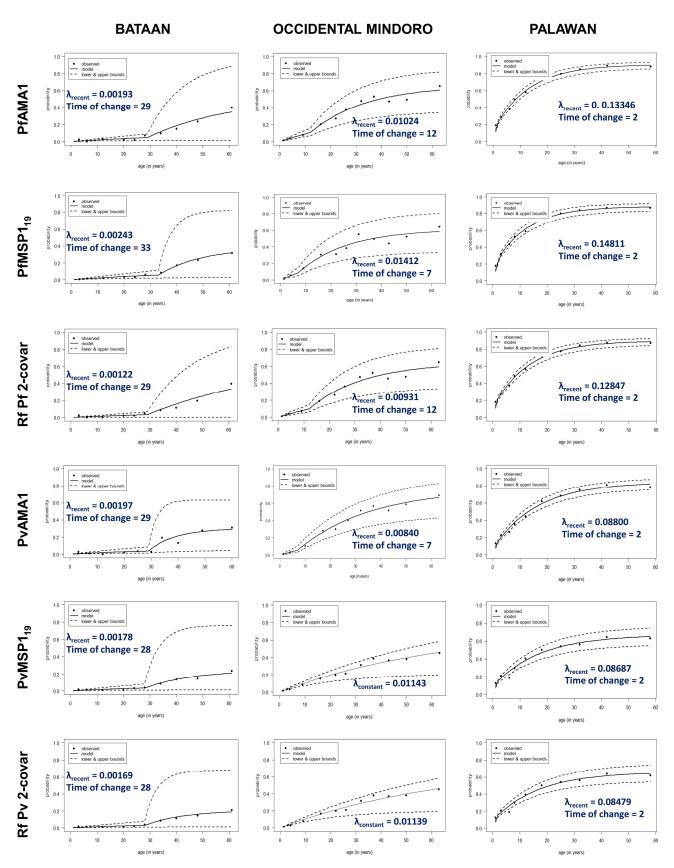
Figure 4.5. Seroconversion curves based on reverse catalytic models using AMA1 and MSP1₁₉ antibody responses provide accurate estimates of historical exposure.

in the reverse catalytic models. Most of the seroconversion curves were best fit with a model assuming a change in transmission based on log likelihood tests ($p < 0.001$), except for PvMSP1$_{19}$ ($p = 0.507$) and the Pv-RF:2-covar ($p = 0.285$) model (**Table 4**). Based on these models, the time of change in transmission in Bataan was estimated at 29–33 years for *P. falciparum* and 28–29 years for *P. vivax*. Occidental Mindoro had varied estimates of the change point (7 and 12 years). For these 2 provinces, the seroconversion rates suggest a decrease in transmission, while an increase of the seroconversion rates for Palawan after the 2-year change point, suggested an increase in transmission in this study site.

We further explored whether the use of SL model can provide improved estimates of historical exposure. The exposure prediction values from the SL model (8 *P. falciparum* markers) were graphed for each sample by site and age (Fig 4.6a and d), and compared with seropositivity rates of PfAMA1 (Fig 4.6c), which is shown to have the highest influence in the RF model prediction (Fig 4.6b). However, comparing the SL-predicted rates with AMA1 and MSP1$_{19}$ seropositivity rates (Fig 4.6d vs 4.6e) show that SL overestimated historical exposure, with higher-than-expected positivity rates in younger age groups from Bataan and Occidental Mindoro, where transmission is reportedly absent. The seropositivity rates based on the 2-covariate RF models with AMA1 and MSP1$_{19}$ seem to provide better estimates of historical exposure to both *P. falciparum* and *P. vivax* based on known malaria prevalence of the sites than the SL models (Fig 4.6e and f).

Discussion

In this study we assessed the utility of multiple antigen-specific antibody responses and different statistical methods to estimate both recent malaria infection and historical transmission in areas of different endemicity in the Philippines. Using a multiplex bead-based assay with a panel of 8 *P. falciparum*-specific and 6 *P. vivax*-specific recombinant antigens on DBS samples, we showed how the use of combined antibody data can improve predictions of recent or historical malaria exposure. In particular, machine learning model predictions of malaria exposure were consistent with reported levels of transmission in our sites, which

Table 4.4. Seroconversion and seroreversion rates from reverse catalytic models in each study site.

		Bataan	Occidental Mindoro	Palawan
PfAMA1	$SCR_{historical}$	0·02102 (0·00599 - 0·07375)	0·03306 (0·02150 - 0·05082)	0·06953 (0·06251 - 0·07736)
	SCR_{recent}	0·00193 (0·00112 - 0·00332)	0·01024 (0·00707 - 0·01483)	0·13346 (0·11802 - 0·15092)
	SRR	0·010002 (0·0010 - 0·10353)	0·01239 (0·00575 - 0·02673)	0·00841 (0·00626 - 0·01129)
	Time of change in transmission (yrs)	29	12	2
$PfMSP1_{19}$	$SCR_{historical}$	0·04042 (0·00836 - 0·19516)	0·03399 (0·02221 - 0·05204)	0·07521 (0·06658 - 0·08495)
	SCR_{recent}	0·00243 (0·00148 - 0·00396)	0·01412 (0·00842 - 0·02367)	0·14811 (0·13109 - 0·16734)
	SRR	0·01959 (0·00564 - 0·06796)	0·01779 (0·00926 - 0·03416)	0·01221 (0·00946 - 0·01575)
	Time of change in transmission (yrs)	33	7	2
RF with PfAMA1 and $PfMSP1_{19}$	$SCR_{historical}$	0·01513 (0·00426 - 0·05370)	0·03338 (0·02172 - 0·05130)	0·06836 (0·06133 - 0·07619)
	SCR_{recent}	0·00122 (0·00064 - 0·00234)	0·00931 (0·00630 - 0·01377)	0·12847 (0·11333 - 0·14564)
	SRR	0·00463 (0·00002 - 0·91264)	0·01327 (0·00639 - 0·02755)	0·00901 (0·00674 - 0·01204)
	Time of change in transmission (yrs)	29	12	2
PvAMA1	$SCR_{historical}$	0·07078 (0·01619 - 0·30943)	0·02760 (0·02094 - 0·03638)	0·05210 (0·04652 - 0·05834)
	SCR_{recent}	0·00197 (0·00102 - 0·00377)	0·00840 (0·00451 - 0·01564)	0·08800 (0·07591 - 0·10202)
	SRR	0·03138 (0·01524 - 0·06460)	0·00715 (0·00269 - 0·01898)	0·01074 (0·00801 - 0·01441)
	Time of change in transmission (yrs)	29	7	2
PvMSP119	$SCR_{historical}$	0·02858 (0·00465 - 0·17570)	0·01143 (0·00921 - 0·01419)	0·03760 (0·03172 - 0·04458)
	SCR_{recent}	0·00178 (0·00092 - 0·00347)		0·08687 (0·07507 - 0·10052)
	SRR	0·02941 (0·00862 - 0·10026)	0·00404 (0·00047 - 0·03486)	0·02146 (0·01599 - 0·02880)
	Time of change in transmission (yrs)	28	-	2
RF with PvAMA1 and PvMSP119	$SCR_{historical}$	0·03387 (0·00559 - 0·20521)	0·01139 (0·00919 - 0·01414)	0·04023 (0·03472 - 0·04661)
	SCR_{recent}	0·00169 (0·00084 - 0·00340)		0·08479 (0·07313 - 0·09833)
	SRR	0·03498 (0·01265 - 0·09673)	0·00388 (0·00042 - 0·03598)	0·02227 (0·01674 - 0·02963)
	Time of change in transmission (yrs)	28	-	2

Seroconversion rates (SCR) and seroreversion rates (SRR) are presented for *P. falciparum* and *P. vivax* based on reverse catalytic models using age-specific seroprevalence from finite mixture models with AMA1 and $MSP1_{19}$ and Random Forest (RF) 2-covariate models. If the best reverse catalytic model to fit the age-adjusted seropositivity data was one with no change point in transmission, only 1 SCR is indicated, while if the best fit is one with a change point, the SCR before the change point is indicated as $SCR_{historical}$ and the SCR after the change point is indicated as SCR_{recent}. The estimated change point in transmission (in years) is also indicated.
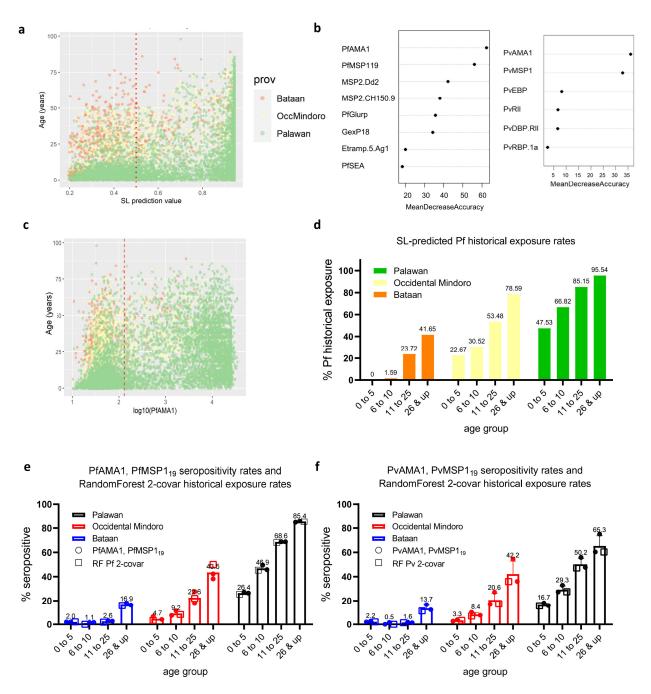
Figure 4.6. Cumulative exposure markers confirm historical *P. falciparum* and *P. vivax* exposure, and heterogeneity of transmission in the 3 sites.

a) Plot of Super Learner prediction values for Pf historical exposure by site and age, using the model with 8 Pf-specific serological markers as covariates. Red dotted line represents positivity cutoff at 0.5. **b)** Variable importance based on the Random Forest model of the 8 Pf-specific antigens and 6 Pv-specific antigens in predicting historical exposure for each species. **c)** Distribution of antibody responses to PfAMA1 by site and age of individuals (n = 9132). Red dashed line represents the seropositivity cutoff value from the FMM model. **d and e)** Summary graphs per age category per site of SL-predicted Pf historical exposure **(d)** and PfAMA1, PfMSP1 seropositivity rates graphed with estimated historical exposure rates using the Random Forest model with PfAMA1, PfMSP1$_{19}$ as covariates **(e)**. **f)** Summary graph of PvAMA1, PvMSP1 seropositivity rates with estimated historical exposure rates using the Random Forest model with PvAMA1, PvMSP1$_{19}$ as covariates per age category per site.

highlight the potential for advanced analysis of multiplex serological data to provide accurate complementary data on incidence levels that could be used by control programs at small spatial scales.

In many countries including the Philippines, malaria-endemic provinces apply for malaria-free status when indigenous cases have not been reported for a set number of years, typically 3 or more. More rapid, subnational demonstration of the absence of exposure could aid in this process. The use of multiple antigens to assess exposure to malaria infection circumvents some issues related to genetic diversity in the parasite and variation in the human immune response to different antigenic targets. The approach also allows application of more advanced statistical analysis to examine optimal combinations of antibody responses in predicting specific outcomes. Here, in addition to the well-studied antigens AMA1 and MSP1$_{19}$, we screened other antigenic markers that have been reported to accurately predict recent exposure in studies in Africa and the Caribbean (Helb *et al*, 2015, van den Hoogen *et al*, 2020c, Wu *et al*, 2020b). PfGLURP R2, Etramp5.Ag1 and GEXP18 have been identified as recent exposure markers for *P. falciparum* (Helb *et al*, 2015, van den Hoogen *et al*, 2020c), while for *P. vivax*, PvMSP1$_{19}$ was one of the eight recent exposure markers in their 8-combination panel (Longley *et al*, 2020) that was included in our assay. Our RF variable importance results also identified these markers to be the most predictive in our panel.

In assessing the individual performances of the serological markers, we show that the antibody responses to all antigens were species-specific, consistent with previous observations (Chotirat *et al*, 2021, Rogier *et al*, 2021). Although we observed differences in antibody levels between genders (Supplementary Figs. S4 and S5) and between patients and companions (Supplementary Fig S6), particularly for Palawan, these factors had little influence overall on recent and historical exposure prediction rates based on the ML model. For some recent exposure markers, we observed <10% seropositivity in younger age groups from malaria-free Bataan, suggesting background seropositivity. When we employed machine learning (Helb *et al*, 2015, Longley *et al*, 2020, Rosado *et al*, 2021), our models were able to confirm the absence of recent infection in Occidental Mindoro and Bataan (although there was lower precision for *P. vivax* exposure prediction), and, using the Random Forest predictions from a 4-covariate model including PfGLURP R2, Etramp5.Ag1, GEXP18, and PfMSP1$_{19}$, accurately identified >92.0% of the *Plasmodium*-positive study samples from this study, whether detected through microscopy, RDT and/or PCR. It also correctly identified 87.2% of *P. falciparum* positives from a Malaysian dataset. The observation that this relatively

simple model, which did not require epidemiological variables such as age, was able to provide accurate predictions for different datasets, shows its potential as a robust indicator for recent infections, in areas with varying levels of transmission.

We expected that the Super Learner model would improve the predictions with its ensemble approach; however, for predicting *P. falciparum* recent infections in particular, RF-based models had the better AUC for both training and test data. This was not the case for the *P. vivax* predictions, which showed similar AUCs (<0.7) for the final SL model and its component models. It is likely that the low predictive ability we observed from the *P. vivax* panel is a limitation of the panel itself rather than the analytical approaches used. Notwithstanding, the SL approach was able to provide the means for evaluating multiple supervised ML models simultaneously, and, for the purpose of accurately distinguishing recent exposure from historical exposure with high sensitivity, the RF model seems to provide accurate estimates.

This study had other limitations. For classifying recent and historical exposure, we had no data on the malaria history of the survey participants, such that we instead focused on predicting for recent infections based on our cross-sectional data. Still, external validation of our prediction algorithm for *P. falciparum* infection confirms its promising performance in accurately predicting malaria infections. Also, since the study analyzed samples from a health facility-based survey, whether or not it accurately represents the populations needs to be assessed. The bias towards sampling health-seeking individuals was partially addressed by also recruiting companions of patients who visited the health facilities (Reyes *et al*, 2021). When we used our RF.covar4 model to predict recent exposure in the 2017 cross-sectional survey also conducted in Morong, Bataan, it also predicted 0.10% recent exposure—similar with our health facility-based data, despite observed differences in antibody levels between the studies (Supplementary Tables S1 and S3 and Fig S7). The 29 to 30-year change point in transmission for Bataan can also be deemed consistent with the reported change point at 22 years in this previous survey (van den Hoogen *et al*, 2020a).

Although we cannot validate the predicted historical exposure, we are able to show that consistent with previous studies (Perraut *et al*, 2014, Rosas-Aguirre *et al*, 2015, Biggs *et al*, 2017, Idris *et al*, 2017b, Wu *et al*, 2020a), AMA1 and MSP1$_{19}$ seropositivity rates (individually or combined) for *P. falciparum* and *P. vivax* seemed to agree with the known malaria situation of our study sites. Changes in SCR over time also reflect this, with Bataan having the lowest,

followed by Occidental Mindoro, and highest for Palawan. Our results are able to confirm the absence of *P. falciparum* and *P. vivax* transmission in malaria-free Bataan for the age groups 10 and below, providing evidence for its malaria elimination status. For Occidental Mindoro, the interruption in transmission from 7 to 12 years ago may be explained by the extensive malaria control activities implemented through the Global Fund for Malaria since the 2000s. Our seroconversion curve results also suggest an increase in malaria transmission in Rizal, Palawan during the time of our survey (2016–2018), and upon checking the records of reported malaria cases, there was indeed a steady rise of the API in this municipality from 25.43 in 2014, 33.97 in 2015, to 46.09 in 2016 and 56.78 in 2018.

Despite the mentioned limitations of the study, our results clearly show the potential use of multiplex antibody responses and applications of machine learning approaches in assessing malaria transmission for countries aiming for malaria elimination. Of interest for countries aiming for elimination is evaluating the impact of a decrease in transmission on the immunity or vulnerability of a population, especially in differing endemic settings (Fowkes *et al*, 2016, World Health Organization, 2017b). Immunological cohort studies are needed to investigate this further. In a sub-national elimination setting such as the Philippines, both recent and historical *P. falciparum* and *P. vivax* exposure metrics were indicative of the absence of recent transmission in Bataan and Occidental Mindoro, and also identified recent infections in Rizal, Palawan, thus showing its ability in assessing the malaria situation in varying areas of endemicity. Our study provides baseline immunological data for monitoring risk populations in the Philippines. This serological surveillance approach can aid in devising control measures by malaria elimination programs, as well as provide evidence of the effectiveness of programs being implemented.

Contributors

CJD, FEJE, JCRH, and MLMM conceived and designed this study. MLMM, KMF, and CJD analyzed and interpreted the data. MLMM wrote the first draft of the manuscript with support from KMF, CJD, JCRH, KY, and FEJE. JSL, RAR, MLMM, and APNB supervised the data and sample collection, and the conduct of assays. KKAT, JHA, CH, and CEC provided the recombinant antigens used for the multiplex bead-based assay. MLMM and RAR performed the serological assays with support from TH and KKAT. All authors read and approved the final manuscript.

Data sharing statement

All data analyzed in this study are available from authors upon reasonable request and with permission of relevant institutional review boards.

Declaration of interests

The authors declare no competing interests.

Acknowledgements

Supplementary data

Figure S1. Antibody levels in response to the rest of the *P. falciparum* and *P. vivax* serological markers in the panel, by study site and age group.

Statistical difference of overall antibody responses among study sites within age groups were determined using Kruskall-Wallis test and Wilcoxon test for pairwise comparisons.
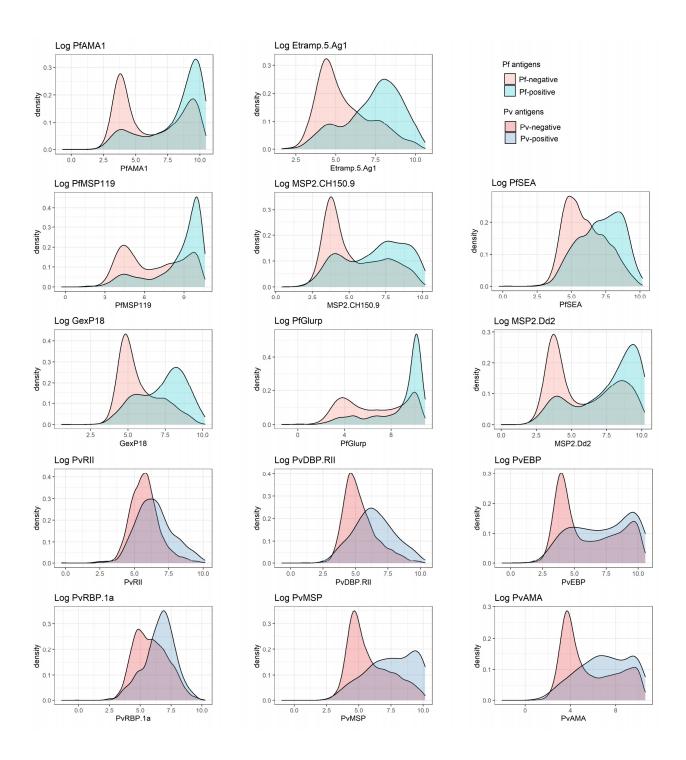
Figure S2. Antibody responses of malaria-negative and malaria-positive populations

(presented as density plots of log10 net MFI values) to the Pf and Pv serological markers in the panel

(Pf and Pv diagnosed by microscopy, RDT and/or PCR. Related to Fig 4.1.
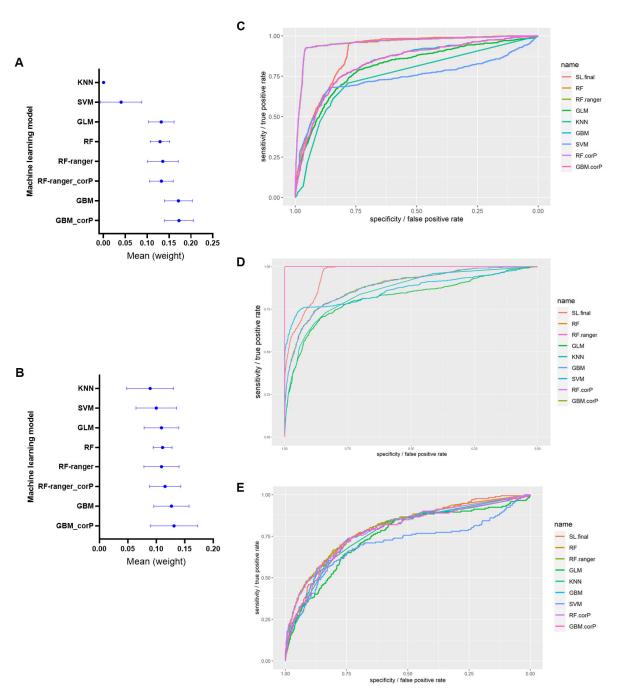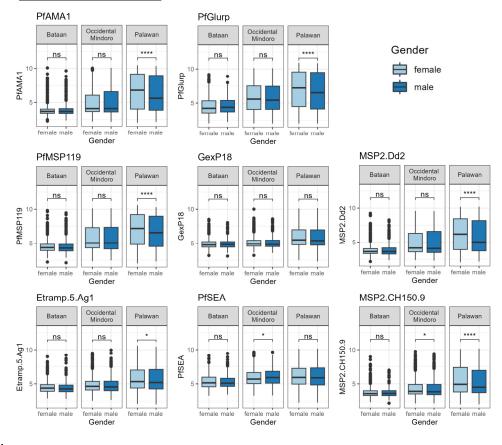
Figure S3. Performances of the individual base learners in the Super Learner ensemble.

a-b) Plots of the assigned weights to each learner included in the Super Learner models for predicting *P. falciparum* (A) and *P. vivax* recent exposure (B), obtained after 20-fold nested cross-validation of the Pf SL-covar8 and Pv SL-covar6 models.

c-e) Receiver operating characteristic (ROC) curves for predicting recent Pf infections using the 8 Pf antigens as covariates (C) for the test dataset (n=9132), and for the validation/training data for Pf recent infection (D; n=3170, with 589 positives, as described in Table 4.2), and predictions for Pv recent infections using the 6 Pv antigens as covariates (E) for the test dataset (n=9132). (SL.final: final Super Learner model, RF: random Forest, RF.ranger: RF from ranger package, kNN: k-Nearest Neighbor, GBM: generalized boosted models (implementation for BRT: boosted regression trees), SVM:Support Vector Machine, and GLM: Generalized linear models; variation of algorithms with "corP" denotes a feature selection that screens for univariate correlation)
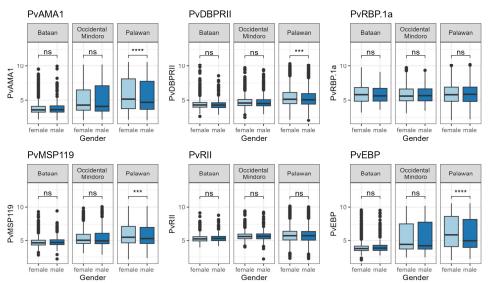
Figure S4. Antibody levels in response to *P. falciparum* and *P. vivax* serological markers in the panel by study site and gender.

a-b) Antibody responses to P. falciparum (a) and P. vivax (b) serological markers presented as box plots of log10 net MFI values by site and gender. Statistical difference of overall antibody responses between males and females were determined using t-test.
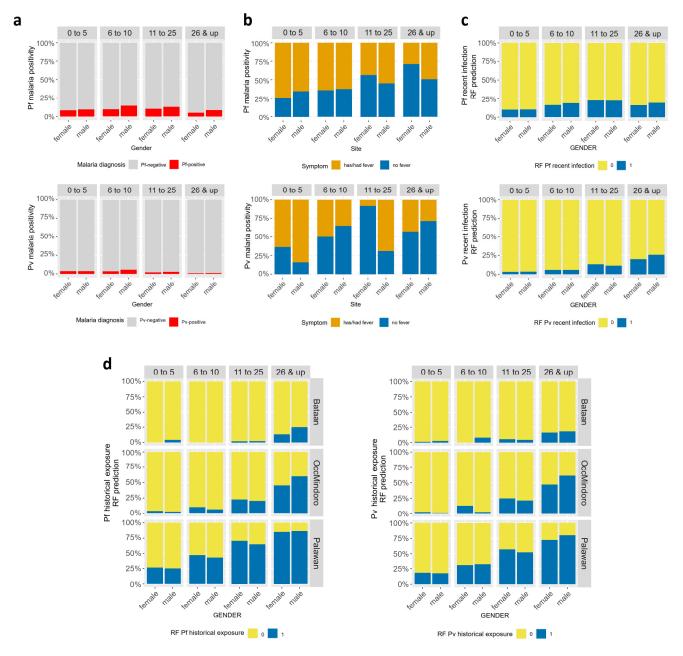
Figure S5. Exploring the effect of gender, age and fever symptoms on recent and historical exposure predictions.

a. Proportions (%) of *P. falciparum* (upper graph) and *P. vivax* (lower) cases in relation to gender and age group for diagnosed by microscopy, RDT and/or PCR in Palawan.

b. Proportions (%) of symptomatic/febrile survey participants within *P. falciparum*-positive (upper graph; n=595) and *P. vivax*-positive (lower graph; n=172) groups in Palawan.

c. Machine learning model predictions by gender and age group of recent exposure to *P. falciparum* based on the RF 4-covariate model (upper graph) and *P. vivax* based on SL 6-covariate model (lower graph) for Palawan study site.

d. Random Forest predictions of historical exposure by gender, age group, and study site, with PfAMA1, PfMSP1$_{19}$ as covariates for *P. falciparum* (left) and PvAMA1, PvMSP1$_{19}$ as covariates for *P. vivax* (right).
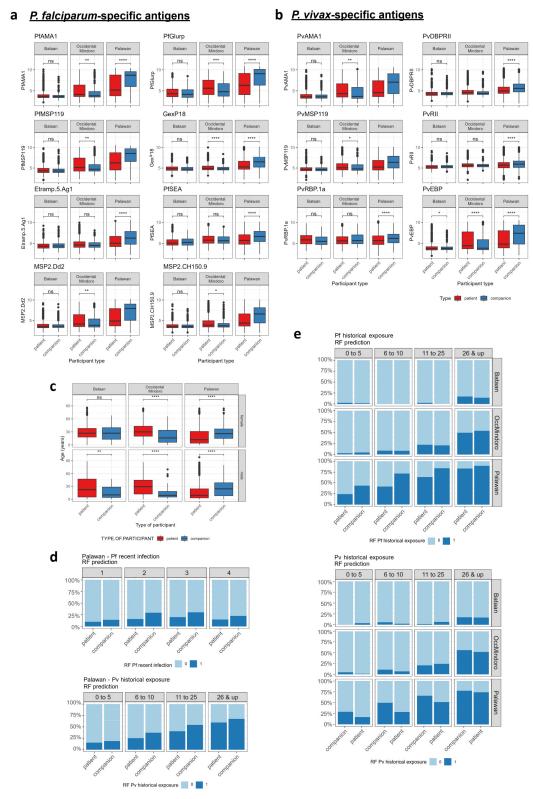
Figure S6. Comparing the antibody responses of health facility-based survey participants and its effect on recent and historical exposure predictions.

a-b. Antibody responses to *P. falciparum* (a) and *P. vivax* (b) serological markers presented as box plots of log10 net MFI values by participant type and study site. Statistical difference of overall antibody responses between males and females were determined using t-test.

c. Box plots showing the age distribution of health facility-based survey participants (patients and companions). Statistical differences of age distribution were determined using t-test.

d. Machine learning model predictions by participant type and age group for Palawan study site of recent exposure to *P. falciparum* based on the RF 4-covariate model (upper graph) and *P. vivax* based on SL 6-covariate model (lower graph).

e. Random Forest predictions of historical exposure by gender, age group, and study site, with PfAMA1, PfMSP1$_{19}$ as covariates for *P. falciparum* (upper graph) and PvAMA1, PvMSP1$_{19}$ as covariates for *P. vivax* (lower graph).
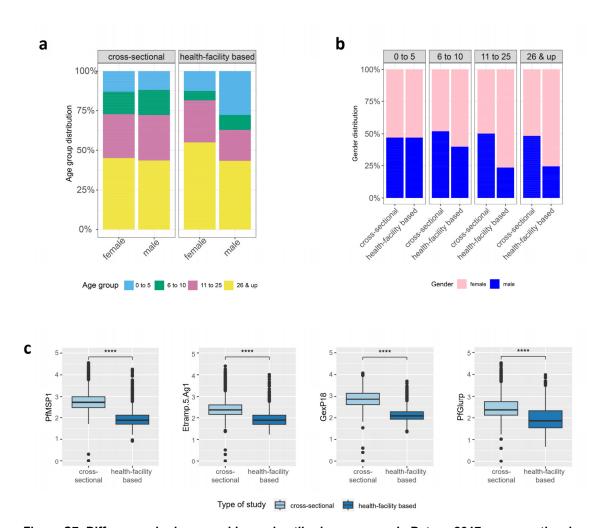
Figure S7. Differences in demographics and antibody responses in Bataan 2017 cross-sectional and health facility-based surveys.

a-b. Age (a) and gender (b) distributions of cross-sectional and health facility-based cross-sectional surveys conducted in Bataan.

c. Comparison of antibody levels (presented as box plots of log10 net MFI values) by gender and age group for *P. falciparum*-specific antigens PfMSP1$_{19}$, Etramp5.Ag1, GEXP18, and PfGLURP R2 of Bataan samples from 2017 cross-sectional study29 and the health facility-based survey analyzed in this study (collected 2017-2018).

Table S1. Seropositivity rates based on Pf-specific antigens and SuperLearner predictions for recent Pf exposure

Model		Seropositivity cutoff value for antigens	AUC – validation data	AUC – study data	Positive in Pf+ samples (n=625)		Positive in Palawan, n=6572 (%)		Positive in Occidental Mindoro, n=1683 (%)		Positive in Bataan, n=877 (%)	
PfAMA1	FMM	129·552007	0·9075 (0·8866-0·9284)	0·6921 (0·6771-0·7072)	509	(85·5%)	3842	(58·5%)	599	(35·6%)	91	(10·4%)
	Negpop	146·705091	0·9115 (0·892-0·9311)	0·6948 (0·6795-0·71)	507	(85·2%)	3792	(57·7%)	582	(34·6%)	82	(9·4%)
PfMSP1$_{19}$	FMM	315·978321	0·9321 (0·9182-0·9459)	0·6849 (0·6696-0·7002)	503	(84·5%)	3872	(58·9%)	639	(38·0%)	85	(9·7%)
	Negpop	294·522036	0·9301 (0·9152-0·9449)	0·6821 (0·667-0·6973)	508	(85·4%)	3940	(60·0%)	658	(39·1%)	89	(10·1%)
Etramp5.Ag1	FMM	343·006451	0·9075 (0·8918-0·9232)	0·7556 (0·7389-0·7722)	477	(80·2%)	2659	(40·5%)	267	(15·9%)	60	(6·8%)
	Negpop	254·493948	0·9111 (0·8941-0·9281)	0·7397 (0·7235-0·7559)	489	(82·2%)	2963	(45·1%)	362	(21·5%)	87	(9·9%)
GEXP18	FMM	402·149776	0·8848 (0·8663-0·9033)	0·7456 (0·7278-0·7633)	452	(76·0%)	2524	(38·4%)	203	(12·1%)	65	(7·4%)
	Negpop	267·001733	0·9037 (0·8849-0·9225)	0·7317 (0·7154-0·748)	487	(81·8%)	3067	(46·7%)	326	(19·4%)	126	(14·4%)
PfGLURP R2	FMM	346·692889	0·9206 (0·901-0·9401)	0·6849 (0·6701-0·6997)	516	(86·7%)	3817	(58·1%)	747	(44·4%)	147	(16·8%)
	Negpop	361·031502	0·9254 (0·9074-0·9435)	0·6855 (0·6706-0·7004)	512	(86·1%)	3810	(58·0%)	738	(43·9%)	143	(16·3%)
MSP2 CH150/9	FMM	137·354929	0·8558 (0·8325-0·879)	0·6849 (0·6665-0·7034)	437	(73·4%)	3093	(47·1%)	414	(24·6%)	76	(8·7%)
	Negpop	141·708302	0·8541 (0·8307-0·8774)	0·4971 (0·4921-0·5022)	437	(73·4%)	3074	(46·8%)	406	(24·1%)	75	(8·6%)
MSP2 Dd2	FMM	121·316261	0·9177 (0·9027-0·9326)	0·6762 (0·6598-0·6926)	484	(81·3%)	3673	(55·9%)	657	(39·0%)	126	(14·4%)
	Negpop	110·971531	0·9202 (0·9054-0·935)	0·6798 (0·6632-0·6965)	482	(81·0%)	3607	(54·9%)	615	(36·5%)	105	(12·0%)
PfSEA	FMM	733·501223	0·8207 (0·7975-0·844)	0·6714 (0·6515-0·6912)	384	(64·5%)	2387	(36·3%)	470	(27·9%)	131	(14·9%)
	Negpop	867·158828	0·8121 (0·7918-0·8324)	0·6614 (0·6411-0·6816)	357	(60·0%)	2203	(33·5%)	409	(24·3%)	112	(12·8%)
Random Forest: 4 covariates (RF.covar4)		(Etramp5.Ag1, GEXP18, PfGLURP R2, PfMSP1$_{19}$)	0·9983 (0·996-1)	0·9591 (0·9497-0·9684)	552	(92·8%)	1081	(16·4%)	5	(0·3%)	1	(0·1%)
Random Forest: 8 covariates (RF.covar8)		All 8 Pf-specific antigens	0·9898 (0·998-1)	0·9682 (0·9605-0·9759)	555	(93·3%)	1065	(16·2%)	3	(0·2%)	0	(0·0%)
SL: 3 covariates (SL:3-covar)		(Etramp5.Ag1, GEXP18, PfGLURP R2)	0·918 (0·9067-0·9293)	0·879 (0·8658-0·8921)	265	(44·5%)	809	(12·3%)	0	(0·0%)	0	(0·0%)
SL: 4 covariates (SL:4-covar)		(Etramp5.Ag1, GEXP18, PfGLURP R2, PfMSP1$_{19}$)	0·9338 (0·9214-0·9462)	0·8967 (0·8867-0·9067)	297	(49·9%)	687	(10·5%)	0	(0·0%)	0	(0·0%)
SL: 8 covariates (SL:8-covar)		All 8 Pf-specific antigens	0·955 (0·9486-0·9614)	0·914 (0·9055-0·9226)	317	(53·3%)	828	(12·6%)	0	(0·0%)	0	(0·0%)
SL: 9 covariates (SL:9-covar)		All 8 Pf-specific antigens and age	0·9898 (0·9874-0·9921)	0·9197 (0·9093-0·9302)	423	(71·1%)	1245	(18·9%)	4	(0·2%)	0	(0·0%)

*Abbreviations used: Pf: *P. falciparum;* FMM: Finite mixture model; Negpop: Negative population model; SL: Super Learner prediction model using different sets of covariates (including the 8 Pf antigens) for predicting recent and historical malaria exposure.

Table S2. Seropositivity rates based on 6 Pv-specific antigens and SuperLearner predictions for recent Pv exposure

Model		Seropositivity cutoff value for antigens	AUC – validation data	AUC – study data	Positive in Pv+ samples (n=172)		Positive in Palawan, n=6572 (%)		Positive in Occidental Mindoro, n=1683 (%)		Positive in Bataan, n=877 (%)	
PvAMA1	FMM	149·776739	0·9185 (0·8934 - 0·9436)	0·6744 (0·6424 - 0·7064)	130	(75·6%)	2991	(45·5%)	650	(38·6%)	84	(9·6%)
	Negpop	172·486298	0·9164 (0·8895 - 0·9434)	0·6720 (0·6391 - 0·7049)	127	(73·8%)	3035	(46·2%)	626	(37·2%)	77	(8·8%)
PvMSP1$_{19}$	FMM	395·678911	0·9429 (0·9229 - 0·9629)	0·7308 (0·6997 - 0·7618)	134	(77·9%)	2588	(39·4%)	430	(25·5%)	63	(7·2%)
	Negpop	666·248684	0·9234 (0·8935 - 0·9533)	0·7049 (0·6692 - 0·7406)	114	(66·3%)	2135	(32·5%)	295	(17·5%)	38	(4·3%)
PvDBP.RII	FMM	403·658933	0·8036 (0·7546 - 0·8527)	0·7065 (0·6697 - 0·7434)	105	(61·0%)	1699	(25·9%)	165	(9·8%)	50	(5·7%)
	Negpop	285·09444	0·8143 (0·7668 - 0·8618)	0·7037 (0·6681 - 0·7392)	115	(66·9%)	2087	(31·8%)	221	(13·1%)	72	(8·2%)
PvRBP·1a	FMM	777·354467	0·7367 (0·6865 - 0·7869)	0·6248 (0·5871 - 0·6625)	92	(53·5%)	1979	(30·1%)	416	(24·7%)	242	(27·6%)
	Negpop	2986·35245	0·5343 (0·5006 - 0·5679)	0·5127 (0·4901 - 0·5352)	17	(9·9%)	507	(7·7%)	104	(6·2%)	61	(7·0%)
PvRII	FMM	843·409764	0·7706 (0·7195 - 0·8218)	0·6302 (0·5935 - 0·6670)	67	(39·0%)	1079	(16·4%)	104	(6·2%)	38	(4·3%)
	Negpop	377·466964	0·8007 (0·7530 - 0·8484)	0·6322 (0·5955 - 0·6688)	107	(62·2%)	2717	(41·3%)	489	(29·1%)	102	(11·6%)
PvEBP	FMM	147·668259	0·7735 (0·7247 - 0·8224)	0·6471 (0·6144 - 0·6799)	128	(74·4%)	3328	(50·6%)	719	(42·7%)	106	(12·1%)
	Negpop	661·785062	0·7133 (0·6616 - 0·7649)	0·6184 (0·5809 - 0·6559)	101	(58·7%)	2608	(39·7%)	566	(33·6%)	63	(7·2%)
SL: 6 Pv antigens		All Pv-specific antigens as covariates	0·8857 (0·8429-0·9284)	0·6332 (0·5979-0·6686)	57	(33·1%)	603	(9·2%)	35	(2·1%)	2	(0·2%)
RF: 6 Pv antigens			1	0·6918 (0·6542-0·7293)	81	(47·1%)	802	(12·2%)	57	(3·4%)	5	(0·6%)
GBM: 6 Pv antigens			0·9043 (0·8647-0·9439)	0·6462 (0·6095-0·6828)	67	(39·0%)	851	(12·9%)	80	(4·8%)	7	(0·8%)

*Abbreviations used: Pv: *P. vivax;* FMM: Finite mixture model; Negpop: Negative population model; SL: Superlearner prediction model using different sets of covariates (including the 8 Pf antigens) for predicting recent and historical malaria exposure.

Table S3. Collated dataset for external validation of RF 4-covariate model for predicting Pf recent exposure.

Datasets	N	Pf+	Predicted Pf recent exposure	AUC (CI)
Bataan 2017 cross-sectional survey[30]	1926	0	2 / 1926 (0·10%)	
Malaysia 2015 study[29]				
Cross-sectional survey	8163	0	38 / 8163 (0·47%)	0·9300
Naïve and negative controls	506	0	4 / 506 (0·79%)	(0·8818 - 0·9782)
PCR-validated cases - Pf	47	47	41 / 47 (87·23%)	
PCR-validated cases - Pv, Pm, Pk	428	0	71 / 428 (16·59%)	

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Chapter 5 : Persistence of immune memory to malaria infection in the absence of transmission - relevance for elimination

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COVID-19 Impact Statement

It was about 5 months into my PhD that travel restrictions and lockdowns started being implemented due to the Covid19 pandemic. A cohort study to characterize the immunological memory responses to malaria in humans was initially planned to be pursued in the Philippines as part of the final PhD thesis. The project is in collaboration with Research Institute for Tropical Medicine, Department of Health, Philippines, where I am a staff (on study leave), and the study sites will be in 2 provinces where a previous malaria surveillance project was also implemented. We have applied and received a grant from the 2021 Department of Science and Technology (DOST) Philippines & Japan Society for the Promotion of Science (JSPS) Joint Research Program to support this research.

However, the Philippines and Japan closed their borders in early 2020, and traveling carried the risk of not being able to return to Japan to finish my laboratory-based PhD work. Nagasaki University also implemented very strict travel restrictions on students, which did not allow for travel to high-risk countries (including the Philippines) unless extremely necessary. Being optimistic that the pandemic would have been over after more than a year, the data collection was planned to commence in 2021 after I have finished substantial work in my mouse studies. It had not been anticipated that the pandemic would continue, and that quarantine measures being implemented would entail months' worth of quarantine days if fieldwork is to be pursued. This study was ultimately decided to be put on hold due to the circumstances brought about by the pandemic, and will be pursued as a 're-entry' project post-PhD. The approved protocol, prepared and developed during the tenure of the PhD project, is presented here, along with initial experiments to determine feasibility of the conduct of the study in the Philippines.

Abstract

An observational cohort study is planned to investigate the mechanisms underlying naturally acquired immunity to *Plasmodium falciparum (Pf)* malaria infection, in order to address basic questions relating to the induction of *Pf*-specific immunological memory in the current era of malaria elimination. The approved protocol is presented here. A prospective analysis of the *Pf*-specific immune responses of individuals in various transmission areas in the Philippines is proposed. The study will make use of existing data from the health facility surveys discussed and analyzed in Chapter 4. These include areas where the intensity of malaria transmission (i) is low but stable, (ii) has been very limited in recent years, or (iii) has been eliminated. Various components of the anti-malarial memory response will be dissected by delivering antigen-specific read-outs of antibodies, memory B cells (MBCs) conventional T cells and follicular T helper (Tfh) cells in defining the magnitude, breadth and persistence of responses; the stability of phenotypes of B and T cell populations will also be characterized. The project will benefit from newly discovered antigenic targets of recent and long-term exposure to malaria infection, refined multiplex-based protocols for measuring antibodies in serum, improved methods for quantifying MBCs, and high dimensional flow cytometry methods for both B and T cells. Initial experiments to determine feasibility of the conduct of the study in the Philippines are presented. Understanding the generation and persistence of immunological memory is essential for the rational development of malaria vaccines.

IRB-approved protocol:

IMMORTAL: PERSISTENCE OF IMMUNE MEMORY TO MALARIA INFECTION IN THE ABSENCE OF TRANSMISSION – RELEVANCE FOR ELIMINATION

Research Protocol

Version date: 13 June 2022

	Philippines – RITM, United Kingdom – LSHTM, Japan – NU
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Sponsors	Nagasaki University Doctoral Programme for World-leading Innovative & Smart Education (NU-WISE) Programme, Japan Society of Promotion of Science (JSPS)
Duration	14 months

1. Introduction

Malaria remains to be a major public health problem globally, with an estimated 228 million cases reported in 2018 (World Health Organization, 2019). A consensus observation in malaria immunological studies is that naturally acquired humoral and cellular immunity to blood-stage malaria require repeated exposure, and a decline in transmission and parasite genetic diversity may affect the persistence of immune memory in populations (Langhorne *et al*, 2008, Fowkes *et al*, 2016, Ryg-Cornejo *et al*, 2016, Ly and Hansen, 2019). Although there is a debate on the longevity of antibody responses following malaria infection, there is evidence that antibody and memory B cells capable of inducing immune responses can be maintained despite infrequent exposure, but results vary depending on study populations (Wipasa *et al*, 2010, Tran *et al*, 2013, Fowkes *et al*, 2016, Mugyenyi *et al*, 2017). Meanwhile, insights on the role of cell-mediated or T cell immunity in relation to humoral responses following malaria infection point to the immune system's balancing act between cytokine production for elimination of the pathogen and protection of the host from immunopathology, resulting in modulation of immune responses (*e.g.* B and T cell exhaustion) (Illingworth *et al*,

2013, Van Braeckel-Budimir *et al*, 2016).

Immunological studies also make use of mouse models to study mechanisms of immunity in a controlled environment. Ongoing mouse studies in the Immunology laboratory in Nagasaki University (NU) focus on the generation and maintenance of immunological memory to malaria, and the findings suggest a critical role of the regulatory cytokine IL-27 in the immune modulation following malaria infection (Sukhbaatar *et al*, 2020). It was observed that transgenic mice with defects in IL-27 receptor signaling had malaria antigen-specific T cells with enhanced memory responses. These immune mechanisms are currently being studied further in this malaria infection model to characterize the phenotype and function of malaria-specific T cells, to analyze the responses of antibody and B cells, as well as to determine how IL-27 receptor signaling affect induction and maintenance of T cell exhaustion.

While mouse models are able to provide insights into immune mechanisms of malaria, it remains essential to analyze the link of the important findings from the mouse model to human immune processes. Of interest for countries aiming for elimination is evaluating the impact of a decrease in transmission on the immunity or vulnerability of a population, especially in differing endemic settings (Mugyenyi *et al*, 2017, World Health Organization, 2017b). In relation to this, a health facility-based survey was conducted in the Philippines from 2016-18 (PH-UK ENSURE Project, MR/N019199/1) in provinces of varying transmission status (Palawan, Occidental Mindoro and Bataan). The study analyzed malaria-specific antibody levels to estimate malaria transmission (unpublished), and it confirmed the high transmission intensity in Rizal, Palawan, as indicated by the >20% PfAMA1 and $PfMSP1_{19}$ seropositivity rates, in children under 5 years of age and >80% in adults 25 years old and up. In contrast, Bataan and Occidental Mindoro had <8% seropositivity in younger age groups and 18.7% to 52% seropositivity rates in adults, respectively, suggesting varied levels of maintenance of naturally acquired immune memory responses in the 3 study sites. The decrease in seropositivity rates in the adult population in previously endemic Bataan could imply that humoral responses were affected by the lack of malaria exposure, and could thereby increase the risk of these populations for developing symptomatic malaria infections if reintroduction occurs.

By investigating the mechanisms of host immunity in areas with different elimination stages, it is possible to elucidate mechanisms of immune memory mechanisms for malaria, and identify potential biomarkers that can predict the immune status of endemic populations for

assessing risk (Langhorne *et al*, 2008, Hafalla *et al*, 2011, Mendonca and Barral-Netto, 2015, Fowkes *et al*, 2016, World Health Organization, 2017a). This study aims to evaluate the effect of varying degrees of malaria transmission on the acquisition and maintenance of anti-malarial immunological memory. It will be an observational study of malaria-positive or malaria-exposed individuals from 3 types of communities in the Philippines: municipalities with stable low malaria transmission (Puerto Princesa, and Aborlan, Palawan), recently had stable transmission (Narra, Palawan), and a malaria-free area (Morong, Bataan). This study, which is a component of a PhD project that looks into the generation and maintenance of immunological memory to malaria infection in mouse models and human populations, will also evaluate how the underlying mechanisms observed in mouse models can be applied in the settings of natural human infection in the Philippines.

2. Rationale/Significance

To date there is little information on the mechanisms of anti-malarial immunity and immunological memory of endemic populations in Asia-Pacific countries aiming for elimination. In the Philippines, where malaria is coming to the elimination stage (targeting malaria-free certification by 2030), there exist areas with varying degrees of malaria transmission. Palawan contributed >90% of the cases (~4800) in the country in 2018, most of which came from South Palawan, but within the province are municipalities like Roxas and Taytay in the north, as well as Narra and other municipalities near Puerto Princesa where malaria transmission is controlled. Of the 81 provinces and regions in the Philippines, 4 other provinces still reported local malaria cases, 26 provinces are in elimination phase, and 50 including Bataan were declared malaria-free as of 2018. This stratification provides an opportunity to compare the acquisition and maintenance of malaria-specific immunity in individuals living under differing levels of malaria transmission and exposure. As it has been observed that decrease in exposure to malaria affects the vulnerability of populations, investigating these humoral and cellular immune correlates of protection that affect immunological memory to malaria can help in surveillance and identification of populations that are most at risk of infection and clinical disease in the event of resurgence of transmission, and also in determining whether the interventions in place are effective. Immune biomarkers can also be applied to countries with declining malaria transmission such as the Philippines, and its utility can contribute to understanding the cause for the persistence of cases in particular regions (Richards *et al*, 2013, Helb *et al*, 2015, Fowkes *et al*, 2016). This study will then build on the broader perspectives with regards to malarial immunity, and will aim to

examine further the underlying mechanisms of anti-malarial immune responses that can aid in immunological surveillance for elimination, vaccine development and other strategic interventions that can be applied in a local context.

3. Objectives

General objective: The primary objective of this study is to investigate the induction and maintenance of immunological memory responses to *Plasmodium falciparum* (*Pf*) malaria infection in human cohort studies in varying endemic settings in the Philippines.

Specifically, the study will aim to:

a. Conduct field cohort studies to observe and investigate the longevity of cellular and humoral responses of adult populations in 3 areas of varying malaria endemicity in the Philippines – municipalities with stable transmission (Puerto Princesa and Aborlan, Palawan), municipalities with limited malaria exposure (Narra, Palawan), and a malaria-free area (Morong, Bataan) – in order to determine the effect of the differential exposure to natural malaria infection on the maintenance of malaria-specific immunity, and

b. Clarify the mechanisms of modulation of humoral and cellular immune responses through T cell, B cell and antibody assays to inform the development and deployment of intervention strategies for elimination.

4. Hypothesis

The development of protective immunity against malaria has been shown to be suppressed by malaria infection – the mechanisms of which have yet been widely studied. It has been observed in previous (mostly paediatric) cohort studies that complete protection from infection and clinical disease is not observed even after repeated malaria episodes (Tran et al, 2013), but continued exposure is needed for maintenance of antibody titers and memory B cells (Weiss et al, 2010, Ayieko et al, 2013). There is also differential production of pro-inflammatory and anti-inflammatory cytokines of CD4$^+$T cells, suggesting their immunomodulatory role in protection against malaria (Jagannathan et al, 2014, De Jong et al, 2017). The specific roles of these immune cells are also being explored using mouse models, and through this human cohort study, it can be determined

whether the trends and mechanisms observed in the controlled setting in the mouse model can be comparable to what is observed in natural human infections.

Adults will have a different immune profile from children, such that data to be obtained from this study would not be comparable to previous cohort studies; but being able to observe how similar or different these populations would be in relation to the duration of absence of malaria exposure in their area will be able to provide information on possible trends that can be useful in an elimination setting. It is expected that for this study on 3 areas with different levels of exposure to malaria, marked differences would be observed in the T cell, B cell and antibody profiles, as well as the produced cytokines in these adult populations. Potential biomarkers that distinguish these populations can then be identified, and evaluated further.

5. Materials and methods

5.1 Design

This is an observational cohort study for a year-long prospective analysis of the Pf-specific immune responses of adult populations (25 years old and above) living in varying levels of malaria transmission. Data on malaria history of individuals will be obtained through previously collected serological data as well as pre-survey analysis, and only those who meet the inclusion criteria (defined below) will be enrolled in the study. The differences in antimalarial memory responses will be investigated through analysis of T cell and B cell populations, as well as malaria-specific antibodies. Analysis will be done in different time points to determine kinetics of the immune cells.

5.2 Study Sites

The Philippines is a malaria-endemic country in Southeast Asia, with >90% of its malaria cases concentrated in 7 of 81 provinces. The study will have 4 municipalities in the Philippines as study sites: Puerto Princesa, Aborlan and Narra in the province of Palawan, and Morong in the province of Bataan. These sites were selected based on their malaria endemicity, availability of historical malaria data for potential subjects, as well as accessibility to existing laboratory facilities. Further details about the selected sites are presented in the table below.

Table 5.1. Profile of municipalities selected as study sites.

Malaria endemicity	Municipalities	No. of indigenous malaria cases (2018)	No. of indigenous malaria cases (2019)	Malaria incidence (2019)	Population (2015 Census)	No. of Barangays
Stable low transmission	Puerto Princesa, Palawan	157	59	0.23	255,116	66
	Aborlan, Palawan	50	8	0.23	35,091	19
Recently had stable transmission (limited malaria exposure)	Narra, Palawan	36	8	0.11	73,212	23
Malaria-free (eliminated)	Morong, Bataan	0	0	0	29,901	5

A. Stable low transmission areas: Puerto Princesa and Aborlan, Palawan

Palawan is the westernmost island of the Philippines, which reports more than 90% of malaria cases in the country since 2016. Puerto Princesa is its capital city, while Aborlan is an adjacent municipality, which is 70 kilometers away from the city. Although South Palawan would have been the ideal study area to represent an endemic area with stable transmission, it was decided that for logistical reasons, these 3 municipalities will be more suitable sites since the laboratory facility where the whole blood samples can be processed is located in the city at the Provincial Health Office.

B. Areas with limited malaria exposure: Narra, Palawan

The municipality of Narra is located 100 kilometers away from Puerto Princesa City, and despite its proximity to the city reported lower trends of annual parasite indices in its population compared to Puerto Princesa and Aborlan (Table 1). It was then chosen to represent populations with limited exposure to malaria.

C. Malaria-free area: Morong, Bataan

The province of Bataan is located about 200 kilometers northwest of the country's capital Manila. It received malaria-free certification in 2017, with its last indigenous cases reported in the municipality of Dinalupihan in 2011. Previous malaria researches by RITM were conducted in the municipality of Morong, including recent health facility and community surveys (conducted 2017-18) that utilized serological assays to measure age-specific malaria-specific antibody levels as

proxy for malaria transmission. Morong had its last indigenous malaria case in early 2000s, with occasional imported cases from travelers from Africa and other malaria-endemic regions.

5.3 Study Population

The study will focus on the adult age group of 25 years old up for a comparative analysis of the immune responses in the selected populations. This was based on the results of the 2017-18 ENSURE study, where it was observed that the municipality of Morong, Bataan (last malaria case reported in the 2000s) had a PfAMA1 antibody seropositivity rate of 18.7% for the participants in the adult age range of 25 years old and above, while the other study sites Abra de Ilog, Occidental Mindoro (last reported cases in 2016) and Rizal, South Palawan (most endemic municipality in the country) had >50% and >80% seropositivity for the same age group, respectively.

A total of 45 participants per site (mixed sex) will be recruited and enrolled upon their informed consent. Potential subjects will be screened through available information on their malaria history, based on accessible health-related data. For Morong, Bataan, sample selection will base on results of serological surveys conducted previously, while conduct of pre-surveys prior to collection will be necessary for the Palawan municipalities. After confirming the malaria infection and exposure status of adults from the pre-survey, eligible individuals will be randomized for a final list of selected cohort study participants. Eligibility criteria will vary for each study site, as detailed in the next section. As a negative control population, blood samples will also be collected from Metro Manila residents (n=10) who has no history of travel to malaria-endemic areas (naïve/control population). RITM staff who fit the eligibility criteria will be recruited as participants. For recruitment within RITM, the institute's email blasts will be utilized to call for participants, and sample collection will be conducted during their lunch break or before or after office hours.

5.4 Selection criteria

Inclusion criteria:
   a. Adults of either sex, above 25 years of age
   b. Site-specific criteria:

- ❖ For Puerto Princesa, and Aborlan: Currently has malaria or diagnosed to have malaria in the past 90 days PTE
- ❖ For Narra: Had known history of malaria, but with no malaria episode for the past 5 years; malaria antibody-positive in serological survey
- ❖ For Morong: Resided in Morong from 1992 onwards, or at least resided for at least 6 to 9 months each year in Morong since 1992; malaria antibody-positive with relatively higher antibody titers in 2017 serological survey,
- ❖ For Manila: Born and currently residing in Manila; have not ever traveled to malaria-endemic provinces in the Philippines or malaria-endemic countries, &

c. Signed written informed consent

Exclusion criteria:

a. Patients with fever other than caused by malaria, pallor, with chronic and/or serious illnesses that require urgent care

b. Had intake of antimalarial treatment in the past 30 days

c. Pregnant women

6. Study procedures

6.1 Pre-survey data collection

In order to screen for potential cohort study participants, we will coordinate with local health offices to acquire, if available, some historical malaria distribution data, *i.e.,* known foci of malaria transmission where pre-surveys can be conducted, or if possible, line lists of malaria cases in the selected Palawan municipalities in recent years. For stable transmission areas, patients with current infection will be prioritized for recruitment, but with the very low number of cases reported in 2019, the study will also recruit those who had malaria 90 days before the scheduled collection. We will then coordinate with health facilities for line lists of recent cases. Line lists of historical malaria cases (>5 years ago from planned commencement of collection) from Narra, Palawan will also be obtained. The team will seek assistance from the local health units to locate and contact the eligible individuals for recruitment. In Morong, Bataan, eligible participants will be selected based on previous RITM projects' serological survey data from 2017, where antimalarial antibody (PfAMA and PfMSP) titers will be ranked, and

those with the highest levels will be recruited. A final list of cohort participants will be informed of the results of the selection process.

6.2 Data and sample collection

Informed consent will be obtained for venous blood extraction from the chosen participants for the cohort study. Licensed and trained medical technologists will perform the blood extraction procedures. About 25 mL of peripheral blood will be collected in EDTA vacutainer (purple-top) tubes and PAXgene tubes, to be stored frozen while on transport (on dry ice or liquid nitrogen), and immediately transported within 3 hours to the laboratory for processing and storage. We will make use of the laboratory set up by RITM and NU in the Palawan Provincial Health Office (PHO) in Puerto Princesa, while samples from Morong will be stored and processed in RITM. An aliquot of the collected blood will be blotted onto filter paper to acquire DBS samples, on a slide for a malaria smear and/or malaria RDT for microscopic diagnosis. For malaria-positive individuals, blood will be collected (Month 0) before they are referred to health personnel for administration of antimalarial treatment. Plasma and blood cells will be separated by density gradient centrifugation. Peripheral blood mononuclear cells (PBMCs) will be isolated from the individual blood samples, stored at -80°C freezer, and will be used for the phenotypical and functional assays. ELISA and ELIspot assays will be performed in Palawan PHO for Palawan samples, and in RITM for Bataan and Manila samples. Aliquots of the PBMC samples from Palawan will be transported (while stored in dry ice or liquid nitrogen) to RITM for the cytokine assays requiring the use of a flow cytometer. Patients will be followed up in the indicated time points below, during which venous blood will once again be extracted.

In the stable low transmission areas, where malaria-infected patients will be recruited, blood samples will be collected 1, 4 and >6 months after initial collection (Month 0) to analyze the change in immune responses over time after malaria infection, and determine the longevity of observed responses. This will then be compared to the immune responses of participants from the limited malaria exposure areas and malaria-free area with no malaria infection, who will also be followed up after >6 months to analyze immune response longevity. The naïve population samples will not have any follow-up collections:

Table 5.2. Schedule of follow-up in study sites

Study site type	Month 0	Month 1	Month 4	Month 6+
Stable low transmission areas	✓	✓	✓	✓
Limited malaria exposure areas	✓			✓
Malaria-free area	✓			✓
Naïve population	✓			

During Month 0, participants will be interviewed using a pilot-tested c-based e-questionnaire on Android tablet units. ODK is capable of recording geographical data, which will be used to record the location of residence of participants for mapping and follow-up purposes. Age, gender, axillary temperature, malaria history, travel history, and the date and location of collection will be recorded. Data entries will be checked for inconsistencies and verified with the concerned participants.

To ensure the safety of all individuals involved in the study during the Covid19 pandemic, the study team will ensure that field personnel will be trained on strict biosafety protocols before commencement of any field activity. The team will ensure proper coordination and consultation with the local government units and the Inter-Agency Task Force (IATF) on Emerging Infectious Diseases of the Department of Health (DoH), Philippines regarding the house visits, informed consent process, and data/sample collection procedures to be performed, which will be adjusted and updated accordingly on a case-by-case basis (e.g., arrangement of place of meeting, adjustment of informed consent procedure to minimize face-to-face interactions, etc).

6.3 T cell assays

PBMCs will be cultured in the presence of malaria antigens or anti-T cell receptor antibody (anti-CD3, anti-CD28), along with negative controls. Red blood cell lysates that are infected with *P. falciparum* parasite (iRBCs) and uninfected RBCs (uRBCs) as control will be prepared, to be used for *in vitro* stimulation of PBMCs and proliferation of T cells (Portugal et al, 2014, Kijogi et al, 2018). The supernatant will be assayed to determine levels of cytokine production (IFN-γ, IL-2, IL-10, IL-27). This will be done by sandwich enzyme-linked immunosorbent assay (ELISA) or cytokine enzyme-linked immune

Potentially eligible study participants:
Adults of either sex, above 25 years of age

Stable low transmission areas: Puerto Princesa, and Aborlan, Palawan	Limited malaria exposure areas: Narra, Palawan	Malaria-free area: Morong, Bataan	Naïve population: Metro Manila

Eligibility screening

Stable low transmission areas:
AT MONTH 0:
A1. Malaria-positive, untreated
A2. Malaria RDT confirmation
OR
B. Diagnosed with malaria within the past 90 days

Limited malaria exposure areas:
Pre-survey – 1 month before actual survey:
1. Historical malaria data from health office
2. Informed consent, questionnaire
3. Collection of dried blood spot sample
4. Malaria RDT, multiplex serological assay for malaria exposure

Malaria-free area:
Screening using historical data:
a. Historical malaria data from health office
b. FAME / ENSURE 2017 serological survey data for past exposure

Naïve population:
Screening at RITM/nearby health facilities:
a. Born and currently residing in Manila
b. Have not ever traveled to malaria-endemic provinces in the Philippines or malaria-endemic countries

Month 0

A. Malaria-positive patient, untreated: Recruit on-site at attending health facility

B. Diagnosed with malaria within 90 days: contact/ visit household for recruitment

Inform eligible participants:
Had known history of malaria, but with no malaria episode for the past 5 years; malaria antibody-positive in serological survey

Contact and recruit eligible individuals:
Resided in Morong from 1992 onwards,; malaria antibody-positive with high antibody titers in 2017 serological survey

Recruit eligible individuals on-site at health facility

Consenting individuals

Sample and Data collection at Month 0
1. Informed consent form completion
2. Collection of 25mL venous blood: ~3 EDTA tubes, 1 PAXgene tube
1. DBS sample collection
2. Questionnaire
3. Antimalarial treatment by health personnel for malaria-positive
4. Transport of sample to Palawan PHO
5. Storage at -20degC for DBS, PAXgene tubes
6. PBMC isolation for whole blood in EDTA tubes

Sample and Data collection at Month 0
1. Additional questionnaire form completion
2. Collection of 25mL venous blood: ~3 EDTA tubes, 1 PAXgene tube
3. DBS sample collection
4. Questionnaire
5. Transport of sample to Palawan PHO
6. Storage at -20degC for DBS, PAXgene tubes

Sample and Data collection at Month 0
1. Informed consent form completion
2. Collection of 25mL venous blood: ~3 EDTA tubes, 1 PAXgene tube
3. DBS sample collection
4. Questionnaire
5. Transport of sample to RITM lab
6. Storage at -20degC for DBS, PAXgene tubes

Sample and Data collection at Month 0 (one-time collection)
1. Informed consent form completion
2. Collection of 25mL venous blood: ~3 EDTA tubes, 1 PAXgene tube
3. DBS sample collection
4. Questionnaire
5. Transport of sample to RITM lab
6. Storage at -20degC for DBS, PAXgene tubes

Follow-up visits

Folllow-up blood sample collection (25mL venous blood)
a. After 1 month
b. After 4 months
c. After 9 months

Folllow-up blood sample collection (25mL venous blood)
a. After 9 months

Folllow-up blood sample collection (25mL venous blood)
a. After 9 months

Laboratory processing

Laboratory processing at Palawan PHO:
- DBS samples and whole blood in PAXgene tubes stored at -20degC
- PBMC isolation from whole blood
- Culturing of PBMCs, with or without stimulation
- Cytokine ELISA
- Aliquoting of PBMC samples
- B cell ELIspot assay
- Cytokine ELIspot assay

Laboratory processing at RITM:
- Malaria PCR of DBS samples
- PBMC isolation from whole blood
- Culturing of PBMCs, with or without stimulation
- Cytokine ELISA
- Cytokine ELIspot assay
- Storage at -80degC
- Aliquoting of PBMC samples
- B cell ELIspot assay
- Luminex assay

Transport to RITM / **Transport 2 frozen aliquots to RITM**

Transport aliquot to SLH

Laboratory processing at RITM:
- DBS samples and whole blood in PAXgene tubes stored at -20degC
- Storage at -80degC
- Thawing of PBMC aliquot
- Malaria PCR of DBS samples
- Luminex assay

Laboratory processing at SLH:
- Antibody staining of thawed PBMCs
- Run in flow cytometer

Figure 5.1. Flowchart of methodology for field data collection and sample processing in cohort study.

absorbent spot (ELISpot). PBMC samples from the 3 sites will also be transported to and stored in RITM for conduct of other immunoassays. Cellular compositions of PBMCs and their phenotypes will be evaluated by staining the mononuclear cells with specific antibodies and analyzing them using flow cytometry. Cultured cells will be harvested and surface-stained with fluorochrome-conjugated monoclonal antibodies (mAbs) for flow cytometry analysis to determine proportions of cell subsets. This fluorescence-activated cell sorting (FACS) analysis will be performed in San Lazaro Hospital, which houses a flow cytometer.

6.4 B cell and antibody assays

Memory B cells (MBCs) will be analyzed using B cell enzyme linked immunospot (ELISPOT) assay (Jahnmatz et al, 2013) for analyzing antibody secreting B cells (ASCs). PBMCs will be prepared for stimulation with TLR7/8 ligand R848 in the presence of IL-2 in culture, for proliferation of MBCs and differentiation into plasma cells, through which the malaria-specific antigen activity can be quantified. ELISPOT plates will be sent to collaborators in Sweden for analysis using a CTL Immunospot reader. To analyze cellular and antigen-specific antibody responses, a multiplex bead-based assay (Luminex system) (Fujii et al, 2014, Assefa et al, 2019) will be performed using serum or eluted DBS samples. Recombinant malaria-specific antigens will be coupled to magnetic beads/microspheres. All antigen-coupled beads will be mixed in one well per sample for simultaneous analysis on a Luminex 200 system using the xPONENT software.

6.5 Future Use of Samples: Exploring immunologic and genetic biomarkers

Depending on the results of the immunoassays, we can further explore specific mechanisms for memory maintenance to identify biomarkers that can be utilized as therapeutic and strategic interventions to aid elimination efforts. With the observation that immunity declines along with a decrease in malaria exposure, biomarkers related to malaria memory maintenance can potentially distinguish which populations are at which stage of immunity, to inform malaria programs of risk areas or populations based on their immune status, as well as to confirm the absence of transmission in elimination areas. The collected dried blood spot and PAXgene blood sample tubes will be used for this purpose. Host factors identified through immunological assays that vary depending on malaria endemicity will be analyzed further through genetic and serological analysis to identify markers that may be related to disease phenotype during malaria infections

(Yamazaki et al, 2011, Sepulveda et al, 2017). Genotyping for analysis of immune system-related gene polymorphisms can be explored, as well as gene expression and serological profiling.

7. Data analysis and statistical considerations

PBMCs are examined for cellular and humoral immune responses and its maintenance over time in 3 areas with varying endemicity to elucidate the mechanism of immunological memory following malaria infection in Philippine samples. Palawan has municipalities representing areas with ongoing and limited transmission, while Bataan represents areas with no known transmission, and the conduct of cohort studies in these sites allows for comparative analyses of the longevity of their response to malaria. The study will analyze malaria-specific immune cells, which have very low frequencies in the blood – with memory B cells in particular as limiting factors (Nduati et al, 2010, Kleiveland, 2015). Using the reported frequencies of MBCs for sample size calculation will result in a logistically difficult high sample size requirement. An alternative is to utilize relative spot volume (RSV) measurements, which reflect the antibody production and antigen affinity of antibody-secreting cells (ASCs), and provides another metric that may be able to differentiate study populations. RSV values are known to range from 8000-800000 in preliminary studies, and hypothesizing that the RSV means for currently- and previously-exposed individuals are 90000 and 16000, respectively, with standard deviations of 150000 and 50000, respectively, the sample size required will be 35 individuals per group to detect a significant difference in power at a 95% confidence level ($\alpha=0.05$). The sample size was then adjusted to a maximum of 45 individuals per study site to account for attrition rate.

Before application in the cohort studies, all data collection forms will be pilot tested. All questionnaires and case report forms (CRFs) will be double-encoded before analysis, and backups in cloud drives and external hard drives of the encoded databases will be created before cleaning and validation. Clinical information and other field-collected data from study participants will be merged with the immunoassay results for analysis of risk factors affecting malaria immunity. All statistical analyses will be conducted through the open-source R statistical software in RStudio, and Graphpad PRISM software. FACS data will be analyzed using Flowjo software. For determining statistical significance of results, the Mann-Whitney non-parametric test or the *t*-test will used for pairwise comparisons of differences in antibody and cytokine levels, as well as phenotype. For comparing more

than 2 groups, analysis of variance (ANOVA) with Tukey HSD post hoc tests will be performed. Correlations will be analyzed using Spearman's rank correlation coefficient. Comparisons with p values <0.05 will be considered statistically significant.

8. Timeline

Table 5.3. Timeline for the project activities.

Tentative schedule of events	Month														
	-4	-2	0-1	2	3	4	5	6	7	8	9	10	11	12	13+
1. Research ethics approval and permits	▓	▓													
2. Preparation for cohort study 2.1 Coordination with local stakeholders	▓														
2.2 Project orientation of stakeholders in Palawan			▓												
2.3. Data collection: pre-surveys in Palawan			▓												
3. Sample collection (community surveys)				▓	▓	▓			▓				▓	▓	
3.1 Puerto Princesa, Aborlan									M.0	M.1			M.4		*M.6/9*
3.2 Narra									M.0	M.1					
3.3 Morong				M.0						*M.6*			M.9		
3.4 Manila (Naïve)				M.0											
4. Laboratory work for cohort studies a. Immunoassays of field samples				▓	▓	▓			▓				▓	▓	
b. Differential gene expressions									▓	▓	▓	▓	▓	▓	▓
c. Multiplex serological analysis (Luminex)				▓	▓	▓			▓	▓	▓	▓	▓	▓	▓
5. Feedback meetings with stakeholders						▓									▓
Data / Statistical Analysis	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓

9. Ethical considerations

9.1 No local data collection will commence without a favorable written approval from the RITM, NU and LSHTM ethics review boards. Applicable guidelines of the Declaration of Helsinki (2008), International Ethical Guidelines for Biomedical Research Involving Human Subjects, and laws and regulations of the Philippines will be complied with. Any planned use of collected samples for human genetic research will adhere to ethical guidelines as described in the National Ethical Guidelines for Health and Health-Related Research. Written approval for conducting the research will be

obtained from different Palawan province agencies (i.e., NCIP, PCSD, LGUs, etc.) where required, and will be sought prior to implementation of any research activity. Moreover, all study documents (research protocol, English and translated versions of information sheets and written informed consent forms, and data collection forms) will be submitted to RITM IRB for ethical approval.

9.2 Risk-benefit ratio

Local health and government unit staff from the selected municipalities will be oriented about the study objectives and procedures, and will be consulted regarding community reception, if necessary. Also, the study will be conducted in areas with indigenous peoples (IPs), and we will enter their ancestral domain while performing the survey; nevertheless, they will be able to benefit from the study as we are addressing a health problem that affects their communities.

The research activities will involve a maximum of 45 adult males and females from each municipality (total of 135 participants for the whole study), and the 25 mL of blood will be collected by venipuncture. This large amount of blood is necessary for the various immunoassays that will be performed, since PBMCs constitute a small percentage in peripheral blood, and live cells need to be recovered for the culture assays. Transfer of blood samples will always accompany a Materials Transfer Agreement (MTA) between concerned institutions.

9.3 Process of obtaining written informed consent

Informed consent will be obtained from all individuals prior to any procedure specific to this study. An independent witness shall be identified to participate in the informed consent procedure for illiterate study participants. A copy of the information sheet and informed consent form shall be given to the participants, and any changes to the procedure of data collection and sampling that will affect their involvement will be made known to the participant. A re-consent will be made, if necessary. If a participant decides to withdraw from the study, their data and information will be purged from the database and their samples will also be destroyed.

9.4 Incentives and payments for participation

The selected cohort study participants will be given incentives in kind (*e.g.*, lunch for RITM staff participants) for their participation in the study. Any project-related expenses of the participants (*e.g.,* travel expenses) will be reimbursed, as necessary.

9.5 Confidentiality/anonymity

The identity of individuals from whom information is obtained in the course of this project shall be kept strictly confidential in accordance with the Republic Act 10173 (Data Privacy Act of 2012). Access to this information will be restricted to the investigators and study staff. The results from the immunoassays of study participants will be disseminated through a report to be submitted to the concerned municipal and provincial health offices and other stakeholders (e.g., DoH National Malaria Control and Elimination Program), and through a feedback meeting scheduled after the completion of the field and laboratory work (Table 3). No information revealing the identity of any individual or in the aggregated analysis, the country or facility, shall be included in the final report or in any other communication prepared in the course of the study. Each enrolled individual will be given a study identification number that will be used on all specimen labels. This proposed confidentiality measure shall not be applied to patients with notifiable diseases which should be officially reported to the clinician in order to aid in diagnosis, case management and treatment.

Appendix: Preliminary work on PBMC assays

A. Identification of study participants in an area where malaria has been eliminated: selection of individuals with previous exposure

Purpose and initial results: To select potential participants in Morong, Bataan, an area where malaria has been eliminated, the analysis of serological markers described in Chapter 4 was utilized. The low antibody responses observed in Bataan, specifically the 25-year-old above age group, implicated waning antibody responses due to loss of exposure. To explore this, the profiles of Pf and Pv historical positives based on the SL 2-covariate model (with AMA1 and MSP1 for Pf and Pv as covariates) were determined, specifically the duration of residence in the sites (Figure 5.2). It was confirmed that for the older age groups (26 years old and above), >80% of the predicted historically exposed individuals have been residing in Bataan for >10 years (questionnaire had limited options for duration of stay, with the longest option being >10 years). This was also observed in the Palawan and Occidental Mindoro study sites.
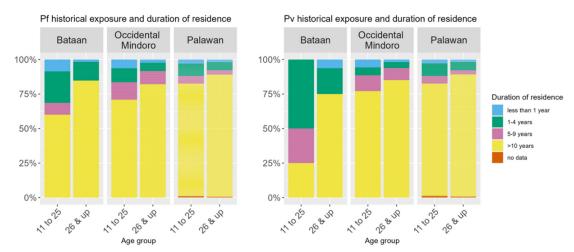
Figure 5.2. Residence profiles of predicted historically exposed populations in the health facility-based survey study sites. Duration of residence in the province among the historically exposed individuals aged 11 and up from the 3 study sites.

Outcome: These results suggested that the predicted historical exposure based on AMA1 and MSP1 seropositivity rates was likely due to past exposure for those who have resided in Bataan while there was ongoing transmission in the area. On the other hand, the higher proportions of predicted historical exposure in the 11- to 25-year-old age group may be attributed to background seropositivity. The list of individuals invited for the cohort study was then narrowed down to those with predicted Pf historical exposure, and have resided in Bataan for >10 years.

B. Flow cytometry analysis of PBMC samples: impact of storage conditions and the use of heparin in blood collection

Purpose and initial results: Since logistical difficulties are being anticipated for the field collection of samples, preliminary experiments with PBMCs were performed to determine the effect of storage conditions on the surface marker expression in lymphocytes.

Palawan is a province with municipalities of varying malaria transmission, ranging from having limited/locally imported transmission, to low but stable transmission (the most endemic municipality, Rizal, had an API of 5.7 in 2018). It is located in a separate island in the Philippines, where there is no liquid nitrogen storage facility and is a flight away from Manila. Since there is only 1 dry shipper available for the transport of samples, it might then be possible that some samples will be stored for more than a month while sample collection is being completed. Moreover, K_2-EDTA tubes were previously used by other studies collecting serum samples, while it has been documented that heparin tubes are recommended for analyzing immune cell function (Mallone *et al*, 2011).

For the flow cytometry analysis, results from a freshly collected sample (own sample) was used as baseline, and sample aliquots were stored at -80 °C and liquid nitrogen for 10 weeks before thawing. The total CD3+ population seem to have been maintained, while there was a slight decrease in the CD3+CD8+ subset. Proportions of CD45RA-CD45RO+ memory CD4+ T cells were comparable among the 3 samples, as well as the expression of CD27 and CD62L memory markers. This suggests that the phenotype of the immune cells was maintained even after a 10-week storage in sub-optimal conditions at -80 °C. The use of heparin and EDTA collection tubes was also assessed. Between heparin and EDTA tube-collected samples, CD62L was decreased in the heparinized sample, and so other markers will be considered for characterizing memory T cells. Otherwise, the proportions of immune cells analyzed were comparable to published studies. This latter experiment (Figure 5.3B) was performed in the Nagasaki University-San Lazaro Hospital Collaborative Research laboratory in Manila, Philippines, confirming that this experiment can be done on site.

Outcomes: A 10-week storage in sub-optimal conditions at -80 °C did not alter the phenotypes of immune cells studied. The use of heparin when collecting blood resulted in the reduction of CD62L staining, but has no effect on other surface markers.
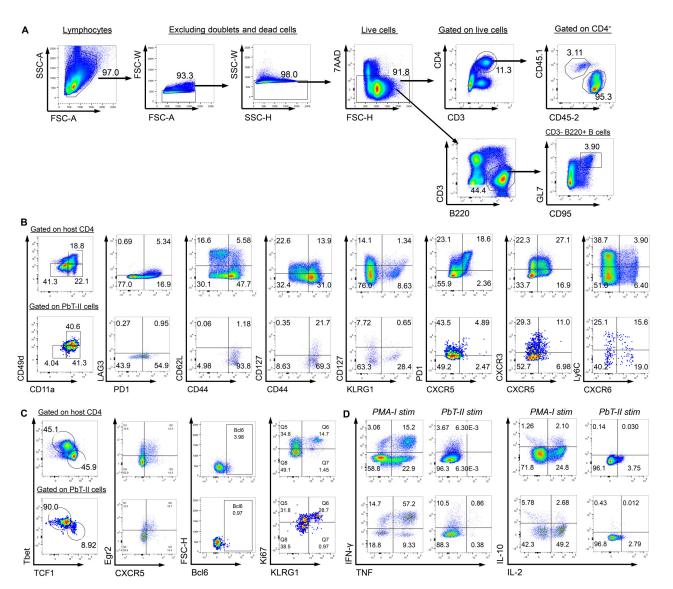
Figure 5.3. Effect of storage and collection conditions on the cell surface marker expression of PBMCs.

A. Lymphocytes in a PBMC sample were analyzed on collection day (fresh PBMC), and 10 weeks after storage in -80°C freezer and in a liquid nitrogen tank. Flow cytometry analysis was performed using BD LSR II Fortessa analyzer in Nagasaki University, Japan. Lymphocytes were gated on single cells, live cells, and $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. $CD4^+$ T cells were characterized using their expression of CD45RA, CD45RO, CD27 and CD62L markers.

B. Blood from the same donor was collected in heparinized and K_2 EDTA tubes to compare surface marker expression of lymphocytes based on collection method. Flow cytometry analysis was performed using FACS Lyric analyzer in San Lazaro Hospital, Philippines. As in A, lymphocytes were gated on single cells and live cells, but TCRαβ was used instead of CD3 to gate for $CD4^+$ and $CD8^+$ T lymphocytes. CD45RA, CD45RO, CD27 and CD62L markers were also analyzed for $CD4^+$ T cells.
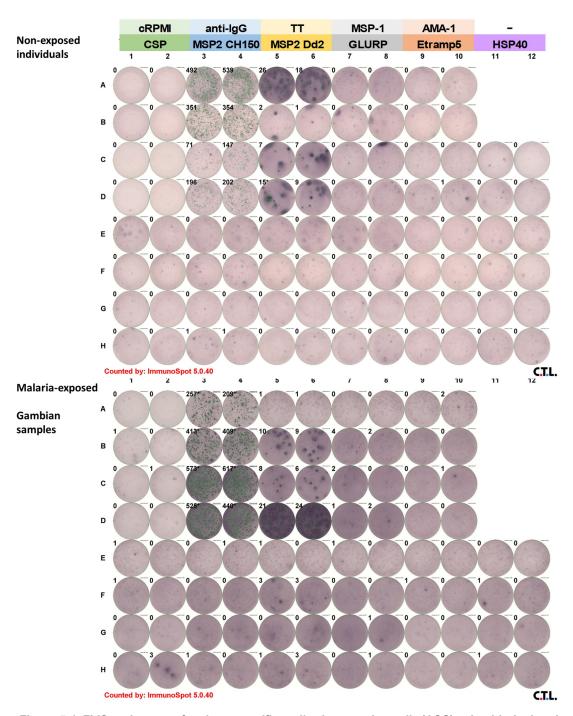
Figure 5.4. ELISpot images of antigen-specific antibody-secreting cells (ASC) using biotinylated antigens.

Plates were coated with malaria-specific antigens, as well as negative and positive controls. Each plate analyzed 2 samples each for non-exposed (above) and malaria-exposed (below) individuals, with 2 technical replicates for each antigen tested (alternating rows).

C. Measurement of memory B cell maintenance

Purpose and initial results: It is important to establish the assay protocol that would be consistent and can later be compared to available studies that look into malaria-specific memory B cell responses (Ayieko *et al*, 2013, Jahnmatz *et al*, 2013, Scholzen *et al*, 2014). Hence, an optimized protocol for the PBMC stimulation and memory B cell ELISpot assay using Millipore polyvinylidene fluoride (PVDF) plates was performed twice on 2 malaria non-exposed samples and 2 malaria-exposed PBMC samples from The Gambia to first test known samples with antigens used in other studies (MSP1, AMA1, CSP), together with evaluating new antigens that were used in the multiplex serological assay.

As described briefly in Chapter 5, PBMCs were first cultured with IL-2 and R848 for pre-stimulation for 5 days (incubation at 37°C in 5% CO_2) to allow for differentiation of memory B cells into plasma cells. PBMCs are plated in 6-well plates at 5×10^6 cells per well. Ethanol-treated (activated) PVDF plates were coated overnight (as duplicates for each sample) with selected malaria-specific antigens MSP1, AMA1, CSP, MSP2.Ch150, MSP2.Dd2, Glurp, Etramp5 and HSP40, along with cRPMI negative control and anti-IgG (mAbs MT91/145). Pre-stimulated PBMC cultures (containing the antibody-secreting plasma cells) were harvested after 5 days. The ELISpot plates were blocked with cRPMI for at least 1 hour and washed, wherein cells were plated and cultured at 37C for stimulation. After a 16- to 20-hour incubation, plates were then processed to detect the spots corresponding to the memory B (IgG-producing) cells responding specifically to the coated malaria-specific antigen/s. To observe the spots, detection biotinylated mAbs MT78/145 (2-hour incubation) followed by streptavidin-ALP (1-hour incubation in the dark) were used for detection, with BCIP/NBT subtrate used for development of the spots (Figure 5.5). Malaria-exposed samples were shown to have some spots for stimulations with malaria-specific antigens, while it was mostly 0 for the unexposed samples. Quantification of total antibody-secreting cells was done using the anti-IgG detection mAb, with the number of ASCs for each antigen stimulation computed as the proportion within the total IgG-producing ASCs normalized to 1×10^6 cells (Table 5.4).

Outcomes:
Results of the memory B cell ELISpot assay will need to later be optimized for conduct in the Philippines. For the assay, about 200,000 cells per well were plated for the malaria-specific antigens, and 10,000 cells per well for the total IgG and negative control. It can be observed that there are numerous spots on the total IgG wells, and a lower count is being considered for future experiments. Also, depending on the amount of PBMC recovered

during the cohort study, the measurement of malaria-specific antigen activity might be limited to priority antigens, which will also be assessed later on. In August 2023, the B cell ELISpot protocol was also performed in RITM Immunology laboratory, and we confirmed that the assay can be conducted without a problem in the available facilities.

Table 5.4. Computation of antigen-specific MBCs based on total IgG-producing ASCs from MBC ELISpot assay.

Sample	anti-IgG average total IgG+ per 1x10e6 cells	complete RPMI average # spots in 1x10e6	antigen-specific MBCs	TT average # spots in 1x10e6	antigen-specific MBCs	MSP1 average # spots in 1x10e6	antigen-specific MBCs	AMA1 average # spots in 1x10e6	antigen-specific MBCs	CSP average # spots in 1x10e6	antigen-specific MBCs
Gambia1	23,300	0	-	5	0.0215	0	-	5	0.0215	2.5	0.0107
Gambia2	41,100	2.5	0.0061	47.5	0.1156	15	0.0365	0	-	2.5	0.0061
Gambia3	59,500	2.5	0.0042	35	0.0588	5	0.0084	2.5	0.0042	0	-
Gambia4	48,250	0	-	112.5	0.2332	7.5	0.0155	0	-	7.5	0.0155
Naive1	51,550	0	-	110	0.2134	0	-	0	-	0	-
Naive2	35,250	0	-	7.5	0.0213	0	-	0	-	0	-
Naive3	10,900	0	-	35	0.3211	0	-	0	-	0	-
Naive4	19,900	0	-	60	0.3015	0	-	2.5	0.0126	0	-

Sample	MSP2 CH150 average # spots in 1x10e6	antigen-specific MBCs	MSP2 Dd2 average # spots in 1x10e6	antigen-specific MBCs	GLURP average # spots in 1x10e6	antigen-specific MBCs	Etramp5 average # spots in 1x10e6	antigen-specific MBCs	Hsp40 average # spots in 1x10e6	antigen-specific MBCs
Gambia1	2.5	0.0107	2.5	0.0107	2.5	0.0107	0	-	0	-
Gambia2	0	-	15	0.0365	0	-	2.5	0.0061	2.5	0.0061
Gambia3	0	-	0	-	2.5	0.0042	0	-	0	-
Gambia4	2.5	0.0052	10	0.0207	2.5	0.0052	0	-	2.5	0.0052
Naive1	0	-	0	-	0	-	0	-	0	-
Naive2	0	-	0	-	0	-	0	-	0	-
Naive3	0	-	0	-	0	-	0	-	0	-
Naive4	5	0.0251	0	-	0	-	0	-	0	-
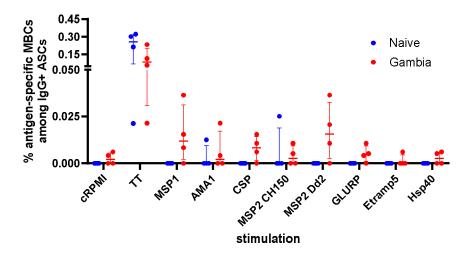
Figure 5.5. Summary of quantification of memory B cells specific to indicated antigen stimulations for samples from malaria-exposed individuals from a Gambia study (blue) and naïve/unexposed samples (red). Error bars indicate median and interquartile range.

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Chapter 6 : Discussion

Summary and implication of findings

The main aim of this PhD project was to address gaps in our understanding of the mechanisms of immunological memory, particularly of CD4$^+$ T cells and B cells, against blood-stage malaria infection. The suboptimal generation and maintenance of immunological memory to malaria is observed in studied mammalian hosts, including humans and mice, and notable studies that have given important insights into this are discussed in **Chapter 1**. The utility of mouse models in studying malaria immunology was highlighted in this chapter, and previous studies that led to the focus on studying immune cells in a chronic malaria infection model, as well as the potential role of the cytokine IL-27 in memory generation and maintenance was presented.

The availability of the MHC class II-restricted TCR-transgenic mouse line PbT-II provided the opportunity to study *Plasmodium*-specific CD4$^+$ T cell responses alongside other immune cells, as is described in **Chapter 3**. With the past findings of other members of the laboratory regarding IL-27 activity during malaria infection (Sukhbaatar *et al*, 2020) and characterization of CD4$^+$ T cell responses using PbT-II cells (Jian *et al*, 2021, Ntita *et al*, 2022), my thesis looked into the further investigation of the effect of IL-27 on malaria-specific immunity in the mouse model. Results of antibody neutralization experiments highlighted an important role of this regulatory cytokine in inhibiting Th1 differentiation during the initial response, which affected the protective capacity of cell-mediated and antibody-mediated memory response. Moreover, the observed kinetics of malaria-specific responses during the chronic infection support the existing notion that the inhibition of Th1 memory is associated with decreased protective immune responses during repeated infections. In the flow cytometry as well as scRNA-seq analysis, unique Th1-like malaria-specific CD4$^+$ T cell subsets were observed to be maintained during the chronic/memory phase in mice transiently treated with IL-27 mAb early on. These results implicate on the possible mechanisms that cause the interruption of memory maintenance during malaria infection, such as the suppression of this protective Th1 memory response, that are discussed further in the succeeding sections.

In **Chapter 4**, the availability of multiplex serological data of antimalarial antibody responses from 3 areas of varying endemicity in the Philippines allowed for an opportunity to look into the effect of declining exposure on the presence and possible maintenance of antibodies in malaria-endemic areas. The chapter focused on exploring different methods of analysis to determine recent and historical malaria exposure, with the intent to use serological surveys for evidence-based elimination strategies. Although the study was limited to the analysis of

antibody responses, the comparison of endemic areas within the same country was a unique opportunity to be able to observe the responses upon declining transmission in areas within the same geographical setting. Also, the identification of malaria-exposed individuals in these endemic areas in the Philippines was intended to be used for the purpose of recruitment for study participants to a planned cohort study in Bataan and Palawan, and the protocol is described in **Chapter 5**. The ethics-approved study intends to explore the cellular basis of immune memory to human malaria, which will be related to the observed antibody responses.

The work presented in this thesis then provided new insights into how chronic malaria infection affects immunological memory in both the mouse model and in humans. This chapter delves further into the implications of my findings for current malaria intervention strategies, as well more questions that need to be answered through future research that was beyond the scope of this thesis.

Characterizing malaria-specific CD4$^+$ T cell responses using the PbT-II system

What makes studying CD4$^+$ T cells challenging is their heterogeneity and functional plasticity upon response to infections, which were dependent on the nature of pathogen involved (Lees and Farber, 2010, Gasper *et al*, 2014). Cytokines, transcription factors and costimulatory signaling present during priming dictate the Th phenotype and lineage commitment upon T cell differentiation, which would also alter the expression of cell surface markers (Mahnke *et al*, 2013, Kurup *et al*, 2019, Duckworth and Groom, 2021). Flow cytometry is the classical approach in characterizing these phenotypes, and the use of TCR transgenic lines such as PbT-II in mouse models allows for determining pathogen-specific profiles through adoptive transfer experiments. Previously reported markers of effector and memory CD4$^+$ and CD8$^+$ T cell subsets (Joshi *et al*, 2007, Stephens and Langhorne, 2010, Opata and Stephens, 2013, Lönnberg *et al*, 2017, Martin and Badovinac, 2018, Hope *et al*, 2019) were explored for their utilization in characterizing PbT-II cells and the effect of the cytokine IL-27 (Appendix Table 3.1).

In the preliminary experiments shown in Appendix B, results show that PbT-II cells from the spleen and peripheral blood exhibited similar trends for some prevailing populations, and as expected, PB had higher proportions of effector subsets and minority of Tfh cells. Also, the host CD4$^+$ T cell profiles were analyzed along with the PbT-II profiles, and observed phenotypes were found to be consistent when gating on activated host cells. There were differences in the subpopulations of persisting malaria-specific CD4$^+$ T cell subsets for acute

and chronic infections, or when antimalarial treatment has been administered, as described for *Plasmodium*-specific B5 Tg CD4^{+} T cells (Stephens and Langhorne, 2010, Opata *et al*, 2015) and also in PbT-II cells (Soon *et al*, 2020) using other cell surface markers. Antimalarial treatment during the acute phase of infection resulted in increased Il7r (CD127) expression, while the maintenance of a chronic infection resulted in a dominantly CD44^{hi}CD62L^{lo} effector memory (Tem) phenotype (CD62L data not shown). The differentiation of CD4^{+} T cell effectors into Th1 and Tfh cells was also observed through the expression of surface markers, transcription factors, and cytokine production after stimulation. This Th1/Tfh differentiation was consistent with results from previous studies on developmental trajectories of PbT-II cells (Lönnberg *et al*, 2017, Soon *et al*, 2020). CXCR6 and CXCR5 are the main surface markers used to identify Th1 and Tfh cells, respectively, along with the expression of CD49d, Tbet, and IFNy by Th1, and PD1, Bcl6 and TCF1 by Tfh. As the PbT-II cell characterization for our experiments' wild-type controls was mostly consistent with previous reports, they were able to better highlight the effect of IL-27 neutralization on the maintained malaria-specific CD4^{+} T cell subsets during chronic infection, as unique Th1-like effector and memory subsets were induced.

Th1 memory modulation by IL-27 during malaria infection

It has long been established that immunity against malaria does not develop efficiently, especially when compared to other diseases to which the immune system is able to mount completely protective memory responses (Cowman *et al*, 2016, Ryg-Cornejo *et al*, 2016, Wykes *et al*, 2017). Although maintenance of immunological memory is the main underlying concept in developing vaccines, the mechanisms of the development and maintenance of protective immunity against malaria infections, particularly the cell-mediated responses, are still incompletely studied. My research using the mouse model then focused on a specific aspect of the immune response based on findings of previous studies using the Pcc mouse model – which was elucidating on the mechanisms of IL-27 during malaria infection (Freitas do Rosario *et al*, 2012, Sukhbaatar *et al*, 2020).

The working hypothesis was that IL-27 inhibits the generation and maintenance of CD4^{+} T cell memory during *Plasmodium* infection, and I aimed to investigate mechanisms underlying the inhibition of CD4^{+} T cell memory by IL-27, and to describe how memory CD4^{+} T cells are maintained better in the absence of IL-27. In our chronic infection model, administering anti-IL-27 mAb during the acute phase of infection resulted in slightly higher proportions of CD127^{+}KLRG1- PbT-II cells starting at Day 7 pi, which steadily increased in proportion along

with CD127⁻KLRG1⁺ cells, and by Day 28 pi became the dominant populations, as opposed to control mice having predominantly CD127⁻KLRG1⁻ PbT-II. The increase of these populations was driven by the second wave of expansion observed only in these chronically infected anti-IL27 mAb-treated mice, and since anti-IL27 mAb treatment after the acute phase did not have the same occurrence, it is clear that IL-27 present during the initial phase of infection limited this differentiation. When comparing antimalarial-treated mice, the CD127⁺KLRG1⁻ and CD127⁻KLRG1⁺ proportions were higher in anti-IL27 mAb-treated mice at Day 28 pi than control IgG-treated mice, albeit at lower total numbers compared to antimalarial-untreated mice, suggesting that these subsets can persist without chronic infection. In CD8⁺ T cells, the differential expression of CD127 and KLRG1 has been used to determine memory potential, by distinguishing CD127ʰⁱKLRG1ˡᵒ memory precursor effector cells (MPECs), and CD127ˡᵒKLRG1ʰⁱ short-lived effector cells (SLECs) (Joshi *et al*, 2007); however, these well-defined subsets are not typically observed in CD4⁺ T cells, although CD127ʰⁱ CD44⁺ cells are considered memory cells (Gasper *et al*, 2014). Interestingly, the IL-27 neutralization in mice enriched these SLEC-like and MPEC-like phenotypes for PbT-II cells during the chronic phase of infection, and through transcriptome analysis, both populations were found to also exhibit Th1-like phenotype. The effect of chronic stimulation is explored further in the next section.

It has been established that IL-27 is a pleiotropic cytokine that can induce either pro-inflammatory or anti-inflammatory effects in a context-dependent manner (Yoshida and Hunter, 2015, Jafarzadeh *et al*, 2020, Yui and Inoue, 2020). Although early studies showed its role in promoting Th1-type immunity, subsequently IL-27 was found to inhibit Th1 differentiation and the accumulation of pathogenic T cells in the case of malaria. WSX1⁻ᐟ⁻ (Il27rα⁻ᐟ⁻) mice retained T cells with high Tbet and IFNy expression, suggesting Th1 preference, as well as KLRG1, which is linked to effector phenotype and terminal differentiation (Findlay *et al*, 2010, Villegas-Mendez *et al*, 2013, Sukhbaatar *et al*, 2020). These were also observed in other IL-27 deficient mouse strains, namely p28⁻ᐟ⁻ (Il27⁻ᐟ⁻) and EBI3⁻ᐟ⁻ mice, used for my preliminary studies (Appendix Figures S3 to S5). PbT-II cells in these Pcc-infected IL-27 deficient mice reached and maintained at higher total numbers by Day 28 pi than WT mice, with higher proportions of CD11aʰⁱCD49dʰⁱ, CD127⁺, KLRH+, CXCR6+, and T-bet+ phenotypes. These were also observed in the antibody neutralization experiments, wherein despite both IgG- and anti-IL-27 mAb-treated mice having similar Th1/Tfh differentiation profiles during the peak response (Day 7 pi), anti-IL-27 mAb-treated mice increased their proportions and total PbT-II numbers of Tbet+ Th1-like populations, while

majority of the persisting PbT-II in control mice expressed TCF1, likely composed of memory-like and Tfh-like populations. Although these results suggest a drastic change affected by IL-27 neutralization. However, when total numbers in the spleen were taken into account, there was in fact no significant difference in Tfh and TCF1+ PbT-II cell numbers of IgG- and anti-IL-27 mAb-treated mice, indicating that these subsets were maintained, and the proportions were changed because of the proliferation of Th1-like subsets. The higher proportions of Th1 phenotype were also confirmed through the higher proportions of IFNy-producing PbT-II, contributed mostly by CD127⁻KLRG1⁺ effector-like cells. There is a concern that TCR-transgenic cells may exhibit subset differentiation biases (i.e., toward Th1 or Tfh), such as the inclination of OT-II cells to be Tfh or SMARTA cells to be Th1 (Kunzli and Masopust, 2023). Based on previous studies and this study, PbT-II cells in the Pcc infection model are able to differentiate into Th1 and Tfh subsets, showing a Th1/Tfh fate bifurcation during the acute phase (Lönnberg *et al*, 2017, Soon *et al*, 2020). Although the same trend of PbT-II activation and differentiation can be observed during acute infection in anti-IL-27 mAb-treated mice, the IL-27 inhibition caused a gradual shift to the formation of Th1 memory subsets after the contraction phase that is not observed in control mice.

The maintenance of SLEC-like and MPEC-like subsets were previously described for both CD4⁺ and CD8⁺ T memory cell pools in acute and chronic infection in mouse models. In CD8⁺ T cell studies, KLRG1ʰⁱ populations were found to have increased and persisted to the memory phase after repeated boosting in LCMV, *Listeria monocytogenes* and vaccinia virus infection models, and also had better protective capacity despite its reduced proliferative capacity (Olson *et al*, 2013). In the acute LCMV infection model, KLRG1ʰⁱ effector-like CD8⁺ cells were identified as a unique population that is maintained during the memory phase along with the CD127ʰⁱ memory precursor cells (Renkema *et al*, 2020). Transcriptome analysis of KLRG1ʰⁱ CD8⁺ T cell subsets during acute LCMV infection showed high expression of CX3CR1, transcription factors Tbet, Id2 and Zeb2, and also acquired Bcl2 expression, which is associated with enhanced survival (Dominguez *et al*, 2015, Renkema *et al*, 2020). Zander et al (2019) and Hudson et al (2019) also described a protective cytotoxic CX3CR1+ or CD101-Tim3+ CD8⁺ T cell subset, respectively, that expressed the aforementioned genes along with Klf2, whose differentiation relied on IL-21 signaling from CD4⁺ T cells. A CD4⁺ T cell population with KLRG1ʰⁱ phenotype was likewise observed in a colitis model, where IFNy-producing CD4⁺ T cells that induce chronic inflammation were found to also express Klrg1, Tbx21, and Zeb2 like terminally differentiated CD8⁺ T cells (Shin *et al*, 2018). It is interesting to note that the KLRG1ʰⁱ cells described in these previous studies share a similar gene

expression pattern as this study's CD127$^-$KLRG1$^+$ PbT-II subset (cluster 1** from Figure 3.5A) from the Day 28 anti-IL-27 mAb-treated sample. Based on the transcriptome (scRNA-seq) data, the Th1-like cluster 1** also differentially expresses Klrg1, Zeb2, Cx3cr1 and Klf2, suggesting shared characteristics of this KLRG1-expressing subset with all these previously documented populations. It should be noted however that the studies with transcriptome analysis of KLRG1^{hi} subsets during the memory phase (Olson *et al*, 2013, Renkema *et al*, 2020) used the LCMV acute infection model, and as shown by our analysis of antimalarial-treated groups, this phenotype comprises a low proportion of PbT-II (CD4$^+$ T) cells compared to CD8$^+$ T cells as these studies reported. This highly suggests that the IL-27-modulated Th1-like KLRG1^{hi} PbT-II subset we observed is of a unique phenotype.

The CD127$^+$KLRG1$^-$ MPEC-like subset (Day 7 cluster 1 from Figure 3.4A) was significantly higher in anti-IL-27 mAb-treated mice at Day 7 pi, and a major CD127$^-$KLRG1$^+$ subset was observed in PbT-II cells from anti-IL-27 mAb-treated mice (cluster 1* from Figure 3.5A) on day 28 of infection. This population, however, did not express other key memory-associated genes such as Tcf7, Ccr7 and Slamf6 (Zander *et al*, 2022) except for *Cd2*, and are unlikely to be precursors of memory cells. On the other hand, the CD127$^+$ cluster in PbT-II cells from anti-IL-27 mAb-treated mice on day 28 of infection (based on scRNA-seq and CITE-seq data) highly expressed the Th1-related genes *Cxcr6, Id2, Ifng,* and *Cxcr3,* consistent with their CD127$^+$CXCR6+IFNγ+ flow cytometry profile and memory-related genes (Il7r). To date, this memory-like subset has not been reported in other infection or inflammation models, suggesting the emergence of a unique Th1 memory phenotype that is also likely limited by IL-27 regulation during other infections. This is probably part of the anti-inflammatory role of IL-27 that limits the Th1 differentiation, to the detriment of the pathogen-specific memory formation.

Interestingly, when the Day 7 cluster 1 of PbT-II cells from IgG- and anti-IL-27 mAb-treated mice were compared for average gene expressions, there was an increased expression of the Th1-related genes (Cxcr6, Id2, Cxcr3) in PbT-II cells from anti-IL-27 mAb-treated mice, suggesting inhibition of an early establishment of Th1 fate commitment by IL-27. Analysis of the common conserved and upregulated genes in each cluster suggested that the Day 7 cluster 1 (Th1) further developed into the 2 distinct clusters 1* and 1** starting Day 14 pi, with both showing high Th1 signature scores, and with cluster 1** (CD127$^-$KLRG1$^+$ subset) also exhibiting a T central memory precursor (Tcmp) signature (Ciucci *et al*, 2019). Moreover, cluster 1* expressed co-inhibitory genes (Tigit, Lag3 and Pdcd1), such that gene ontology

analysis showed an enrichment of negative regulation pathways, while cluster 1** enriched leukocyte migration and activation pathways, further highlighting the functional differences of these persisting Th1 subsets. The cluster analysis of Day 7 PbT-II identified a third major cluster that exhibited a memory T (Tmem) cell signature, which was reduced to a minor population in PbT-II cells from anti-IL-27 mAb-treated mice on Day 28 of infection. When an integrated clustering was applied to the Day 28 datasets from IgG and anti-IL-27 mAb-treated mice, this memory cluster 3 was maintained as a major population (63.4% vs. 0.6%) in the control IgG PbT-II profile. Moreover, mapping the Day 28 PbT-II datasets to a CD4+ T cell reference atlas (Andreatta *et al*, 2022) highlighted the effect of IL-27 neutralization on the persisting subsets, as the IgG control had Tcm and Tfh memory as the major populations, while anti-IL-27 mAb PbT-II had Th1 memory and Th1 effector (Fig EV3E).

Requirement of chronic stimulation for better protective responses

Previous studies have shown that chronic or repeated exposure to malaria is required for development of protective immunological memory (Beeson *et al*, 2008, Doolan *et al*, 2009, Stephens and Langhorne, 2010). Active infection was interrupted by antimalarial treatment to characterize the memory responses based on differential exposure in the Pcc infection model. Antimalarial-treated mice had a decreased maintenance of PbT-II cells compared to chronically infected mice when antimalarial treatment started 6 days after infection. In my experiments comparing IgG- and anti-IL-27 mAb-treated mice that were treated (or not) with antimalarials, treatment before the peak response (Day 6 pi) prevented the second wave of expansion of the Th1-like populations observed at Day 21 pi in untreated mice. Still, compared to antimalarial-treated control mice, treated anti-IL-27 mAb-treated mice had significantly higher PbT-II cell numbers maintained at Day 28 pi and had a similar phenotype as PbT-II from untreated anti-IL-27 mAb-treated mice: the PbT-II population was mostly Tbet^{hi}TCF^{lo} and CXCR6+, but with a lower proportion of CD127$^-$KLRG1$^+$ compared to its untreated counterpart, suggesting that the early lineage commitment by IL-27 neutralization is established prior to the antimalarial treatment. Moreover, the persistence of PbT-II cells after late treatment (Day 21 pi) of anti-IL-27 mAb-treated mice despite a decrease in splenic CD4 total numbers suggested that antigen stimulation is not required for the persistence of these Th1 memory PbT-II cells. The Tfh proportions were again found to maintain its total numbers. The B cell profile was also characterized in relation to Tfh cells (Chapter 3, Fig 3.1.2J-K), and it was observed that antimalarial-treated groups had higher proportions of B cells, while GC B cells were higher for antimalarial-untreated groups, which suggests enhanced B cell

response due to the chronic stimulation, as also observed in previous Pcc studies (Stephens *et al*, 2009, Chen *et al*, 2022).

The PbT-II adoptive transfer system was also used to determine the proliferation and differentiation potential of memory PbT-II cells, although the experiments were preliminary (Chapter 3, Fig 3.1.3). Results of memory PbT-II cell transfer to naïve lymphopenic mice suggest that memory PbT-II cells can be maintained in a non-inflammatory environment such as in naïve Tcrb$^{-/-}$ mice, although its proliferation was not observed, consistent with previous studies (Stephens and Langhorne, 2010, Pepper and Jenkins, 2011, Opata *et al*, 2018). A similar experimental scheme was performed in IL-27 neutralization experiments, and the Tcrb$^{-/-}$ transfer experiments showed how the inflammatory environment in infection-matched recipients allowed for better maintenance of transferred PbT-II cells (compared to naïve recipients), which is consistent with the observation in the antimalarial treatment experiments. It can be speculated that PbT-II cells transferred to or maintained in infection-matched Tcrb$^{-/-}$ recipients and antimalarial-treated mice, respectively, were able to be sustained through key factors and survival signals that are present in a malaria antigen-experienced environment. However, we cannot discount the possibility of the presence of residual antigens in this system, which would once again suggest the seeming requirement of continuous antigen stimulation for malaria-specific immunological memory. Identifying the key factors that distinguish the antigen-experienced from antigen-free environment would elucidate on the appropriate conditions for immunological memory maintenance.

Lastly, an examination of polyclonal CD4$^+$ T cells of IgG- and anti-IL-27 mAb-treated mice was also performed during the memory phase at 9 weeks pi. Results did not show significant differences in the proportions of CD127$^-$KLRG1$^+$ and CD127$^+$KLRG1$^-$ populations, although trends of higher proportions were observed for CD11a+ (activated) CD4$^+$ T cells from anti-IL-27 mAb-treated mice, even in those treated with antimalarials at Day 14 pi. The protective responses against reinfection were evaluated through heterologous challenge of Pcc-primed mice with PbA. For WT versus IL-27$^{-/-}$ mice, IL-27$^{-/-}$ mice had significantly lower parasitaemia, higher weight loss, but comparable ECM clinical scores and higher survival rates, suggesting that there was increase in immunopathology but better control of parasitaemia. However, none of the mice survived by the 3rd week, although Il27$^{-/-}$ mice had slightly prolonged survival (not significant). The experiment using WT mice administered with anti-IL-27 mAb or IgG control exhibited similar results for IgG control mice but better disease outcomes for anti-IL-27 mAb-treated mice (100% survival rate), suggesting that IL-27 immunoregulatory

mechanisms could have played a role in preventing excessive immunopathology after PbA challenge in mice treated with anti-IL-27 mAb during the initial infection with Pcc. Notably, chronically infected anti-IL-27 mAb-treated mice had the highest IFNγ production by $CD4^+$ T cells, suggesting the maintenance of malaria-specific Th1-type memory $CD4^+$ T cells. Pathogenesis of these Th1 cells during secondary infection with heterologous malaria parasites may have prevented by IL-27 produced during the infection. Moreover, anti-Pcc and anti-PbA total IgG levels, as well as anti-Pcc IgM levels were highest for the chronically infected anti-IL-27 mAb-treated mice, suggesting that IL-27 neutralization promoted the production of antibody by LLPCs, which may have contributed the antibody-mediated protective responses against a heterologous challenge. Previous studies have reported that IL-27 improves humoral immunity by enhancing IL-21 production and consequently Tfh responses (Batten *et al*, 2010), and that IL-27 signaling is required for optimal immune responses to subunit vaccination (Pennock *et al*, 2014). Iin the context of malaria, malaria-specific CXCR3+Ly6C+T-bet+ memory Th1-like (Zander *et al*, 2017) or IFN-γ+IL-21+CXCR5+ Th1/Tfh memory cells (Carpio *et al*, 2015) were reported to be able to improve protective antibody responses against malaria. Whether the proliferation of the unique Th1 memory subsets had a role in enhancing antibody responses, or the IL-27 inhibition had a direct effect on antibody-producing B cells, the mechanisms by which IL-27 modulates both cell-mediated and antibody-mediated malaria immunity warrants further investigation.

Longevity of memory responses against malaria in humans

For the analysis of antibody data from the Philippines, I assessed the predictive ability of individual responses or combined analyses of IgG antibody levels to malaria-specific antigens for determining recent and historical malaria exposure. The study was able to demonstrate the utility of serology in showing the heterogeneity of malaria transmission in malaria-endemic populations in the Philippine setting. The classical approach in analyzing serological data, typically obtained through ELISA, is to determine cutoffs of seropositivity based on populations of known negatives for each individual analyte or antigen assayed. Seroconversion rates can then be generated based on age-specific seroprevalence of cumulative exposure markers such as AMA1 and $MSP1_{19}$. using mathematical models. The antibody levels in the multiplex bead-based assay simplifies the processing of samples from eluted DBS samples, and the higher dynamic range of the Luminex platform allowed for a better resolution in the quantitative antibody responses even in an elimination setting, where most responses are expected to be low. The antibody levels to the serological markers for *P.*

falciparum and *P. vivax* exposure clearly showed significantly different levels for Palawan, Occidental Mindoro and Bataan. Moreover, the species specificities of all the malaria antigens in the panel were assessed by comparing the net MFI levels of Palawan samples based on the malaria diagnosis (mono-infections of *P. falciparum* and *P. vivax*, *P. falciparum* plus *P. vivax* mixed infections, negative for both). Encouragingly, the *P. falciparum*-positive group as well as those with mixed infections had significantly higher net MFI values than the *P. vivax*-positive and malaria-negative populations for *P. falciparum* antigens, with the same trends of specificity observed for the *P. vivax* antigens (i.e., higher antibody levels for *P. vivax* positive groups compared to non-vivax groups). Still, antibody responses were also observed to be similar between *P. falciparum*-positive and *P. vivax*-positive samples in particular age groups for some antigens in the panel (*i.e.*, PfAMA1, PvAMA1, PfGlurp, etc.).

When assessing the malaria-specific antigens individually, *P. falciparum*-positive and *P. vivax*-positive samples were observed to have higher antibody levels to the *P. falciparum*- and *P. vivax*-specific antigens, respectively, suggesting species-specific responses (Figure 4.3). However, strong correlations were also observed among some of the *P. falciparum*- and *P. vivax*-specific antigens– for example, for the homologues of AMA1 and MSP1$_{19}$. Although some may argue that this may somehow be attributed to cross-reactivity, similar levels of correlation were also observed between different antigens that should not be cross-reactive. It may then be better interpreted as attributable to the combined risk of *P. falciparum* and *P. vivax* exposure in Rizal, Palawan. Since the area is endemic for both malaria species, a considerable portion of the population has likely been historically and recently exposed, especially the older populations. This endemicity could also explain the significant differences in the antibody levels between male and female participants (Figure S4 & S5), and whether they were the consulting patient or a companion (Figure S6), as observed for Palawan (and not in Occidental Mindoro and Bataan). Being male, and being a companion of the health facility attendee were identified as 2 of the risk factors for malaria positivity in our previous analysis of the survey data (Reyes *et al*, 2021), and this epidemiological aspect is planned to be analyzed for the serology data. Nonetheless, the simultaneous cumulative increase in species-specific antibodies has been previously explored for its possible role in the development of cross-protective immunity (Gnidehou *et al*, 2019, Mitran and Yanow, 2020, Muh *et al*, 2020, Raghavan *et al*, 2023), and can be an area for further investigation, as this could potentially explain the observed asymptomatic PCR-only cases (including mixed infections) in Rizal, Palawan. Through such studies an important question that could also be addressed would be the effect of the genetic diversity of the prevalent parasites on the

antibody repertoire of exposed populations (Raghavan *et al*, 2023).

Although analysis of antibody data to each antigen is a more straightforward approach, the availability of multiplex data allowed for an exploration of new analytical approaches that involved the simultaneous analysis of these identified historical and recent exposure markers. The results show alternative approaches using a multiplex analysis of serological markers that was able to improve the computation of binary outcomes for accurately predicting recent malaria infection. Although the *P. falciparum* recent exposure markers have already been applied in identification of recently exposed individuals (Dewasurendra *et al*, 2017, van den Hoogen *et al*, 2020c, Wu *et al*, 2020a), machine learning models were only used before for identifying variable importance of these markers (Helb *et al*, 2015), and not for binary classification. On another hand, machine learning models were recently employed for screening and classification of recent exposure using *P. vivax* serological markers (Longley *et al*, 2020), as well as SARS-CoV-2 recent infections (Rosado *et al*, 2021), which both highlighted the robust classification outcomes that can be obtained using statistical analysis of multiplex antibody data.

Machine learning models were evaluated for their predictive capacity in classifying recent and historical malaria exposure, and from our panel of antigens, we found that the analysis of the mixture of 4 Pf antigens Etramp5.Ag1, PfGLURP R2, GEXP18 and PfMSP1$_{19}$ using the Random Forest model had the highest accuracy in predicting recent falciparum malaria infection, including subpatent PCR-only cases. On the other hand, the prediction for recent vivax exposure was not improved using machine learning classifications, which is likely a limitation in our antigen panel. Historical Pf and Pv exposure were also not improved, with AMA1 and MSP1$_{19}$ antibody levels expectedly driving the predictions. Still, the machine learning models were able to predict the absence of recent exposure in Bataan and Occidental Mindoro, demonstrating their potential to provide evidence of the absence of transmission in areas targeting elimination, and identifying areas or populations at risk. The next step forward would be to develop an analytical platform that can be easily used by programs, to be able to propose its use-case scenarios, especially in countries aiming for elimination, where multiplex serology can be a novel tool for assessing the elimination status of endemic areas. This can also potentially simultaneously provide assessment for other diseases of public health concern, and can be integrated into local health programs (Fujii *et al*, 2014, Chan *et al*, 2022).

The longevity of IgG antibody responses to malaria-specific antigens can provide information on the development of naturally acquired immunity in populations (King *et al*, 2015) – although if and how assay responses translate to immune protection is not well understood (Crompton *et al*, 2010, Stanisic *et al*, 2015, Proietti *et al*, 2020). From the 3 sites in the study, we were able to detect *Plasmodium* infections only in Rizal, Palawan. Asymptomatic infections comprised 44.5% of the malaria cases detected, and were observed in all age groups, which suggests the development of clinical immunity in this endemic population with relatively stable transmission. Our results showed that >80% of the PfAMA1 seropositives in Bataan had lived there for >10 years, and had net MFI levels that were comparable with adults in Occidental Mindoro and Palawan, suggesting that what we observed could be long-lived antibody responses to *P. falciparum* and *P. vivax* malaria in the population. In assessing the individual performances of recent exposure markers, it can be observed that younger age groups in Bataan reported <10% seropositivity, although this province has not reported indigenous cases since 2011, and this may be attributed to background seropositivity. Moreover, the lower seropositivity rates observed in younger age groups in Palawan may be attributed to children who have not yet developed adequate IgG responses for detection in a serological assay. Still, the lower seropositivity rates in Bataan and Occidental Mindoro, areas which had high transmission 20 and <10 years ago, respectively, may highlight the impact of a reduced transmission in malaria immune responses. Still, our data supports the notion that long-lived antibodies can be maintained in the absence of ongoing transmission. The planned cohort study was intended to relate these antibody results to the maintenance of immune memory cells in malaria-exposed individuals.

Research on T and B cell memory responses during malaria infection in humans are limited. The protocol developed for the human cohort study describes how to obtain a characterization of the immune responses to malaria infection of individuals living in areas with declining transmission (at differing levels) in Bataan and Palawan, Philippines, all of which will be related to the time since exposure that will allow for an observation of the durability of responses over time.

Our findings in the mouse studies show how the antimalarial treatment negatively affected the maintenance of T cell memory as well as the GC B cell numbers and malaria-specific IgG levels, although memory T cell responses were maintained after parasite clearance from antimalarial treatment. A phenotypic analysis of the immune cell profiles of PBMC samples will be performed, alongside functional assays for malaria-specific responses of memory T

and B cells through stimulation with malaria antigens, to gain insights on the differences in these immune cells' characterization based on malaria exposure. Serum malaria-specific IgG antibody levels and cytokine levels will also be measured, and will include analyzing IL-27 levels. Very few studies have assessed IL-27 expression in relation to malaria infection. A notable recent study looked into plasma IL-27 levels in adult patients with malaria and/or HIV (Otterdal *et al*, 2020), where they found a strong correlation for increased IL-27 levels with parasitaemia, as well as the indicator for endothelial cell activation. Since our findings point to the early activity of IL-27 during T cell activation and differentiation upon malaria infection, our study design might not allow us to observe potentially elevated levels of IL-27 even from recently infected individuals, but it is still planned to be analyzed using available assay kits. Nonetheless, the planned cohort study, which is still being pursued outside the scope of this PhD project, will be the first to characterize the cellular basis of immunological memory to malaria in the Philippines in relation to the decline in transmission.

In order to investigate the observed humoral responses analyzed in this study (Chapter 4) in relation to the malaria-specific T and B cell responses, the human cohort study (Chapter 5) will analyze the presence of antigen-specific functional memory T and B cells. Bone marrow-residing LLPCs that produce the circulating antibodies observed in serum and MBCs that are also maintained in circulation, are developed to have different functions. The combined use of the assays planned for the cohort study, which will analyze the serum antibody levels and their avidity, and characterize antigen-specific T and B cell phenotype and function, is expected to provide insights on how T and B cell memory responses are influenced by cumulative malaria exposure or interruption of exposure.

Implications for intervention strategies

Understanding the generation and maintenance of effective immune responses during natural infection, particularly in the era of changing malaria epidemiology, is crucial for the rational development and evaluation of future interventions and vaccines. The mouse studies provided the means to examine the effect of cytokine regulation on immunological memory, and our results show promise for transient IL-27 neutralization as a means to improve malaria vaccines. It would prove useful if a proper vaccine regimen can be developed to accommodate early anti-IL-27 mAb treatment with an adequate number of boosts to elicit the desired development of *Plasmodium*-specific Th1 memory while balancing immunopathology to provide improved protective immunity.

Still, the mechanisms of how malaria infections affect the development and maintenance of protective immunity have not been widely studied in humans, more so in regions that are nearing elimination like in the Asia-Pacific. With the decrease in transmission in endemic areas and as the focus shifts towards elimination, it is then important to consider how the reduction in local transmission could affect the immunity of endemic populations, as a possible increase in their vulnerability can pose a threat to elimination efforts. Investigating the mechanisms that affect immunological memory to malaria can help in surveillance and identification of populations that are most at risk of infection and clinical disease in the event of resurgence of transmission, and also in determining whether the interventions in place are effective. It is hoped that the findings in this PhD work contributed to providing a better understanding of the mechanisms of cellular and humoral immune memory responses against malaria.

Limitations and Future Research Directions

This thesis had some limitations that must be noted. Firstly, for the experimental studies in the mouse model, although the use of a PbT-II adoptive transfer system enabled the elucidation of malaria-specific CD4$^+$ T cell generation and maintenance, it also limited the analysis to this MHC class II-restricted transgenic CD4$^+$ T cell line specific for the *P. berghei* heat shock protein 90 (PbHsp90$_{484-496}$) epitope (Enders *et al*, 2021). From recent literature it has become more apparent that the diversity of the CD4$^+$ T cell effector and memory differentiation depends on a variety of factors, including the nature of the antigen, such that it is important to consider that the PbT-II responses characterized in this thesis presented a limited glimpse into malaria-specific CD4$^+$ T cell responses. Nonetheless, the phenotypic analysis together with the transcriptomic analysis had consistent results with the other studies that made use of PbT-II cells. Also, the identification of this cognate epitope of PbT-II cells could allow for studies on endogenous CD4$^+$ T cell responses with the same specificity to confirm our observations. The use of other available TCR-transgenic *Plasmodium*-specific CD4+ T cells like the MSP1-specific B5 Tg cells can also be explored (Stephens *et al*, 2005). Another limitation of the mouse studies is that the observation of memory responses was limited to 4 weeks (1 month) post-infection (pi) for majority of experiments, and the farthest examined time point was only up to 9 weeks (>2 months) pi. Although the use of PbT-II cells allowed for a prolonged observation of malaria-specific memory maintenance, PbT-II cells still tend to be observed at very low proportions in wild-type mice at day 50 onwards, such that these experiments did not have enough analyzable number of cells (hence also not included

in the thesis), and the 9-week experiments were limited to observing polyclonal endogenous responses gated on CD11a^{hi} antigen-experienced cells. Also, although there was no parasitemia observed at week 9 in the mice in these conducted experiments, Pcc infections have been documented to persist for up to 3 months (Achtman *et al*, 2007). It would then be more accurate to state that our data reflects the chronic memory responses during malaria infection. Future investigations can potentially explore the maintenance of endogenous epitope-specific CD4^{+} T cell responses to determine the feasibility of analyzing long-term memory maintenance.

As we looked into different under-explored aspects of malaria-specific immunological memory, more questions arose with regards to how persistence of exposure to malaria affects the maintenance of memory. In particular, the use of the PbT-II transfer model also allowed us to highlight the substantial effect of the early inhibition of IL-27 activity on the persisting malaria-specific CD4^{+} T cell memory populations, and the chronic exposure. IL-27 activity is context-dependent, and its inhibition has been found to be either beneficial or detrimental to immune memory development. In the Pcc infection model, the early IL-27 inhibition in B6 mice increased the maintenance of 2 phenotypically distinct Th1-like subsets with higher protective capacity, as well as higher serum anti-Pcc antibody levels than similarly chronically infected control mice. IL-27 is known to be produced mainly by dendritic cells and macrophages, while IL-27Rα (WSX1) is expressed by a variety of immune cells, including T and B cells, as well as non-immune cells such as epithelial and endothelial cells (Povroznik and Robinson, 2020), such that further studies need to identify the direct or indirect effect of the transient disruption of IL-27 signaling. It would also be interesting to explore the underlying epigenetic programming that the changes in transcriptional programming entailed, but this is outside the scope of this thesis. It is hypothesized that there are significant changes in the chromatin accessibility of Th1-related transcription factors in CD4^{+} T cells when IL-27 activity is inhibited, in addition to the effect of chronic infection. The concept of trained immunity for innate immune cells could then also be investigated, as it was observed that the innate immune cell profiles had some differences, albeit not significant, for anti-IL-27 mAb-treated mice during the memory phase. Of interest would be looking into how IL-27 affects dendritic cells, macrophages, and NK cell subsets, as previous studies reported the cytokine's modulation of their activity (Matsui *et al*, 2009, Villegas-Mendez *et al*, 2013, DeLong *et al*, 2019).

Another aspect not investigated further was the effect of IL-27 neutralization on malaria-

induced pathology. A consistent observation when examining mouse spleens from Pcc-infected mice was that control mice always had more pigmented homogenized spleens than anti-IL-27-treated mice, suggesting a higher accumulation of hemozoin in the former (data not shown). This difference was noted only through visual evaluation after removal of RBCs from the homogenized spleen samples, and was observed for Day 7 up to Day 28 experiments. Since hemozoin crystals can bind to molecules that can incite inflammatory activity and also affect innate immune responses (Lamikanra *et al*, 2009, Mota and Rodriguez, 2017b, Lakkavaram *et al*, 2020), it is possible that the improved protective responses observed when IL-27 is inhibited could be related to a better control of hemozoin accumulation through increased activity of innate immune cells such as macrophages. Also, since anti-IL-27 mAb-treated mice exhibited higher pro-inflammatory responses during the acute phase, it begs the question of what the effect in the human setting would be if IL-27 transient inhibition is applied as a therapeutic target for improving vaccine efficacy. As with any other vaccine strategies, the balance of the pro-inflammatory responses and the protective capacity must be determined.

We also endeavored to examine the fate trajectories of the novel PbT-II subsets maintained during the memory phase; however, the adoptive transfer of memory PbT-II cell subsets resulted in poor survival and maintenance in naïve and infection-matched TCRb$^{-/-}$ recipients for both control and IL-27 mAb-treated donor mice. More advanced techniques such as *in vivo* lineage tracing approaches that merge clonal lineage and transcriptomic datasets could be employed to better determine T cell fate commitment (Raju *et al*, 2021, Kasmani *et al*, 2023). Individuals in malaria communities would typically be advised to seek treatment when they have malaria symptoms, such that the effect of antimalarial treatment on the memory formation was investigated. In the mouse study, as already mentioned, fewer lymphocyte numbers were observed in antimalarial-treated mice versus untreated, while IL-27 mAb-treated (treated with antimalarials at Day 6 pi – just around the peak of blood-stage infection) still retained the Th1 memory subsets. It can be speculated that the CD127^{hi}KLRG1^{lo} and CD127^{lo}KLRG1^{hi} subsets observed during the memory phase (Day 28 pi) in anti-IL-27 mAb-treated mice were differentiated PbT-II cells that did not undergo apoptosis even when they did not encounter parasites in the treated mice. A possible transcriptomic and epigenetic reprogramming could have allowed for the better survival of these cells, such as the higher expression of Il7r (CD127) in one subset, and high Klf2 expression in the CD127^{lo}KLRG1^{hi} subset. Since CD4$^+$ and CD8$^+$ T cell fate commitment and differentiation has been described for other chronic infection models (Crawford *et al*, 2014, Zander *et al*, 2022, Kasmani *et al*,

2023), these existing datasets can be utilized to further investigate T cell development in the context of malaria infection.

For the human studies, the PhD work was heavily disrupted by Covid-19 travel restrictions, such that the planned analysis of immune cells in a cohort study in the Philippines cannot push through within the thesis timeline. The focus was shifted to analyzing available antibody data from the Philippines, which also looked into the effect of different levels of exposure on the maintenance of antimalarial antibody responses. The cross-sectional survey with the multiplex serology data from *P. falciparum* and *P. vivax*-specific antigens did not have data on their actual malaria history, and analysis solely relied on anecdotal self-reporting. Nonetheless, the significant differences in the IgG antibody levels for the 3 sites of varying endemicity clearly illustrate that declining exposure results in reduced maintenance of malaria-specific antibodies. This can still be inferred from the results despite also lacking more specific data on the duration of residence of survey participants to relate to their seropositivity outcomes. The improved classification outcomes provided by machine learning techniques was a highlight of the antibody data analysis, despite the training data also being limited by the available positives and which samples to consider negatives for the classification model. In datasets tested from the Philippines and neighboring Malaysia, the Random Forest model was able to correctly predict the absence of recent infections in elimination areas using the multiplex data of recent and historical markers. Still, its usability in classifying recent and historical malaria exposure in other areas of similar endemicity or geographical setting remains to be seen. For the Philippine cohort study, the same area in Bataan was selected as a study site, and a line list of the seropositives with self-reported malaria history was generated for potential recruitment. It is hypothesized that malaria-specific memory B cells and T cells would be observed in these individuals who have maintained their antibody responses to historical exposure markers.

One of the main reasons that the area of cellular immunology in humans has not yet been extensively studied is the difficulty in observing lymphocytes and other immune cells in the peripheral blood collected from humans. T and B lymphocytes are typically found in lymphoid organs, and its presence in the blood mostly depends on the day at which the blood sample was collected. For the cohort study protocol developed in this PhD project, the age group was limited to adults 25 years old and above, to minimize the effect of age on the immune responses to be observed. However, this also limited the number of cases expected in study sites representing areas with ongoing and limited transmission. To ensure that we acquire

the appropriate sample size, the study site with ongoing transmission need not recruit currently malaria-infected individuals, but those who have been infected within 3 months before the scheduled date of sample collection. The gaps in the malaria exposure requirement in the 3 chosen study sites is expected to still show differences in immune cell profiles of recently infected, historically exposed from 5 years back, and from >20 years back. Such immune biomarkers can be applied to countries with declining malaria transmission, and characterizing the protective function of these memory cells can contribute to understanding the cause for persistence of cases in particular regions.

Conclusions

This PhD thesis contributes to a better understanding of the development of immunological memory against chronic malaria infection. It sheds light on different aspects of antimalarial immunity through the mouse studies and analysis of human antibody data, while a protocol was also developed to investigate further the cellular basis of immunological memory in the context of malaria elimination in the Philippines. The use of TCR-transgenic PbT-II cells allowed for determining the action of the regulatory cytokine IL-27 during malaria infection, wherein its transient inhibition during the early phase of infection led to the generation and maintenance of 2 unique and functional Th1 memory-like CD4$^+$ T cell subsets, as well as higher antibody levels, suggesting a likewise enhanced B cell function. IL-27 is possibly limiting the development of these protective immune responses, and can therefore be explored as a therapeutic target to enhance vaccines. Additionally, although it was observed that antimalarial treatment can drastically reduce the numbers of maintained memory T and B cells and also have lower antibody responses, memory cells are still able to persist despite the absence of antigens. Similarly in the human context, it was observed that antibody levels of historically exposed individuals can also be maintained, and it is hypothesized that the cohort studies will provide evidence that malaria-specific memory T and B cells can also be maintained. Another important contribution of this thesis is the exploration of different methods of analysis of multiplex antibody response data, wherein the clear differences in antibody levels were shown, depending on the status of malaria transmission. The use of machine learning in analyzing a panel of malaria-specific serological markers significantly improved the classification of recent and historical malaria exposure in populations of varying endemicity. These developed prediction models can potentially be used by control programmes in estimating recent and historical infections and proving the absence of transmission in elimination areas. This highlights the potential use of serology in integrated surveillance efforts to estimate the disease burden of populations not just for malaria but also for other diseases. Altogether, these research findings addressed important gaps in our knowledge of immunological memory to malaria, that provide insights to more gaps that remain.

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