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Exploration of Extracellular Vesicles as a Novel Approach for Antigen Discovery and Vaccine Development against *Plasmodium vivax* Malaria

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‘What we observe is not nature itself,
but nature exposed to our method of questioning’

— **W. K. Heisenberg**

Abstract

Plasmodium vivax is the most geographically widespread human malaria parasite. Research on this parasite needs to be expanded in order to develop adequate tools for its control such as a highly effective vaccine. One particular feature of *P. vivax* is its preference to invade immature red blood cells, also known as reticulocytes. Interestingly, ultrastructural studies performed on reticulocytes enabled the discovery of exosomes, extracellular vesicles (EVs) of endocytic origin. These vesicles were initially seen as a selective cargo-disposal pathway, but later works showed the involvement of exosomes, and other extracellular vesicles, in a variety of biological processes. Importantly, exosomes derived from reticulocytes infected with *P. yoelii*, a murine reticulocyte-prone parasite that resembles *P. vivax*, contained parasite proteins. When used in CpG-adjuvanted immunizations, exosomes were able to elicit long-lasting protective responses. This thesis hypothesizes that exosomes derived from *P. vivax*-infected reticulocytes contain parasite antigens and stimulate immune responses. We evaluated the potential of circulating extracellular vesicles from *P. vivax* infections as a source of antigens and as activators of T-cell responses, and explored human reticulocyte-derived exosomes as a vaccine platform against *P. vivax* malaria.

We isolated EVs from plasma of *P. vivax*-infected patients and determined their protein composition by mass spectrometry-based proteomics in order to unveil their potential use in antigen discovery. We found parasite proteins associated to these vesicles that could serve as antigens. Indeed, two of the identified vivax proteins present promising cytotoxic T-cell epitopes as evidenced by *in silico* analysis. Moreover, we detected HLA class I molecules and observed an altered protein cargo in vesicles from vivax patients compared to

healthy donors, thus suggesting that circulating EVs might affect the course of *P. vivax* infection. Next, we evaluated the *in vitro* interaction of these vesicles with leukocyte populations from the human spleen, given the importance of this organ in the induction of adaptative immune responses. Remarkably, we observed a significant interaction of monocytes, B-cells and T-cells with vesicles from patients compared to healthy individuals. We studied the capacity of these vesicles to activate T-cells, and preliminary results indicate that circulating vesicles from infections might stimulate CD8+ T-cell responses. Recent studies highlighted the role of cytotoxic CD8+ T-cell responses against *P. vivax* blood-stage parasites. In parallel, we studied the proteomic composition of exosomes derived from human reticulocytes and analyzed their ability to interact with antigen-presenting cells. We identified over 300 proteins in these vesicles, including HLA class I molecules, and found that these exosomes could be taken up by antigen-presenting cells, thus suggesting their contribution to the presentation of antigens.

Collectively, our results indicate that EVs from vivax infections can be used in antigen discovery and might contribute to cell-mediated immune responses that could be critical for vivax control. In particular, reticulocyte-derived exosomes represent a potential vaccine platform to be furtherly explored. We believe this work has provided novel insights for vaccine development against *P. vivax* malaria.

Resumen

Plasmodium vivax es el parásito que causa malaria humana más extendido geográficamente. Se ha de ampliar la investigación sobre este parásito para desarrollar herramientas adecuadas para su control, entre ellas, una vacuna altamente efectiva. Una característica particular de *P. vivax* es su preferencia por invadir glóbulos rojos inmaduros, también conocidos como reticulocitos. Curiosamente, estudios ultraestructurales realizados en reticulocitos permitieron el descubrimiento de exosomas, vesículas extracelulares (VEs) de origen endocítico. Los exosomas y otras vesículas extracelulares, fueron vistos inicialmente como una vía selectiva de eliminación de proteínas obsoletas, pero en la actualidad, se sabe que participan en una gran variedad de procesos biológicos. Los exosomas derivados de reticulocitos infectados con *P. yoelii*, un parásito propenso a invadir reticulocitos murinos que se asemeja a *P. vivax*, contienen proteínas parasitarias. Cuando estos exosomas se usan en inmunizaciones con adyuvante de CpG son capaces de provocar respuestas protectoras duraderas. Esta tesis plantea la hipótesis de que los exosomas derivados de reticulocitos infectados con *P. vivax* contienen antígenos del parásito y pueden estimular respuestas inmunes. Evaluamos el potencial de las VEs circulantes en infecciones de *P. vivax* como fuente de antígenos y como activadoras de respuestas de células T. Además, exploramos los exosomas derivados de reticulocitos humanos como una plataforma de vacunación contra la malaria vivax.

Aislamos VEs del plasma de pacientes infectados con *P. vivax* y determinamos su composición proteica mediante proteómica basada en espectrometría de masas para investigar su potencial uso en el descubrimiento de antígenos. Encontramos proteínas del parásito asociadas a estas vesículas, las cuales podrían actuar como antígenos. De hecho, el análisis *in silico* de dos de estas proteínas

reveló prometedores epítopos citotóxicos de células T. Además, detectamos moléculas HLA clase I y observamos un alterado contenido de proteínas en las vesículas de pacientes con vivax en comparación con donantes sanos, lo que sugiere que los VEs circulantes podrían afectar el curso de la infección por *P. vivax*. A continuación, evaluamos la interacción *in vitro* de estas vesículas con poblaciones leucocitarias del bazo humano, dada la importancia de este órgano en la inducción de respuestas inmunes adaptativas. Observamos una interacción significativamente elevada de monocitos, células B y células T con vesículas de pacientes en comparación con VEs de individuos sanos. Estudiamos la capacidad de estas vesículas para activar las células T, y los resultados preliminares indican que las vesículas circulantes de infecciones de vivax podrían estimular las respuestas de las células T CD8+. Recientes estudios han destacado el posible papel de las respuestas citotóxicas de las células T contra los parásitos de la etapa sanguínea de *P. vivax*. Paralelamente, analizamos la composición proteómica de los exosomas derivados de reticulocitos humanos y determinamos su capacidad para interactuar con células presentadoras de antígenos. Identificamos más de 300 proteínas en estas vesículas, incluidas las moléculas HLA de clase I, y descubrimos que estos exosomas podían ser internalizados por células presentadoras de antígenos, lo que sugiere su contribución a la presentación antigénica.

En conjunto, nuestros resultados indican que las VEs de las infecciones por vivax pueden usarse en el descubrimiento de antígenos y pueden contribuir a respuestas inmunes mediadas por células que podrían ser críticas para el control de vivax. En particular, los exosomas derivados de reticulocitos representan una potencial plataforma de vacuna. Creemos que este trabajo ha proporcionado nuevas ideas para el desarrollo de vacunas contra la malaria por *P. vivax*.

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Table of Contents

List of Figures

Figure **Page**

List of Tables

List of Abbreviations

aa aminoacid

ACT artemisinin-based combination therapy

Alix ALG-2 interacting protein X

AMA1 apical membrane antigen 1

APC antigen-presenting cell

BCA bicinchoninic acid

BCB bead-coupling buffer

BLAST basic local alignment search tool

BSA bovine serum albumin

CHMI Controlled Human Malaria Infection

CI confidence interval

CpG cytosine and guanine separated by one phosphate

CSP circumsporozoite protein

CVC caveola-vesicle complex

DARC Duffy Antigen/Chemokine Receptor

DC dendritic cell

DLS dynamic light scattering

DMEM Dulbecco's Modified Eagle Medium

DNA deoxyribonucleic acid

DTT dithiothreitol

dUC differential centrifugation

EBV Epstein-Barr virus

EM electron microscopy

ER endoplasmic reticulum

ESCRT endosomal sorting complex required for transport

EV extracellular vesicle

FBS fetal bovine serum

G6PD glucose-6-phosphate dehydrogenase

GC B-cell germinal center B-cell

GLA-SE glucopyranosyl lipid adjuvant-stable emulsion

GPI glycosylphosphatidylinositol

HCT hematocrit

HD healthy donor

***h*EVs** circulating EVs from healthy donors

HIV/AIDS human immunodeficiency virus infection and acquired immune deficiency syndrome

HLA human leukocyte antigen

HuRex human reticulocyte-derived exosomes

ICAM-1 intercellular adhesion molecule-1

IgG immunoglobulin G

IFN-γ interferon-γ

IL-12 interleukin-12

IL-1β interleukin-1β

ILV intraluminal vesicle

iRBC infected RBC

LC-MS/MS liquid chromatography tandem mass spectrometry

LPS lipopolysaccharide

mDC mature dendritic cell

MFI median fluorescence intensity

MHC major histocompatibility complex

mRNA messenger RNA

MS mass spectrometry

MSP merozoite surface protein

MV microvesicle

MVE multivesicular endosome

NSAF Normalized Spectral Abundance Factor

NTA nanoparticle tracking analysis

O/N overnight

PAMP pathogen-associated molecular pattern

PALS periarteriolar lymphoid sheaths

PBMC peripheral blood mononuclear celL

PBS phosphate buffered saline

Pf *P. falciparum*

PSM peptide-spectrum match

Pv *P. vivax*

PV *P. vivax* infected patient

PvDBP *P. vivax* ligand Duffy Binding Protein

***Pv*EVs** circulating EVs from *P. vivax* infected patients

RBC red blood cell

RBP reticulocyte-binding protein

RESA ring-infected erythrocyte surface antigen

Rex reticulocyte-derived exosomes

RNA ribonucleic acid

RPMI Roswell Park Memorial Institute

RT room temperature

Sal-I Salvador Strain 1

SEC size exclusion chromatography

SD standard deviation

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SVP synthetic vaccine particle

TBV transmission-blocking vaccine

TCR T-cell receptor

TEM transmission electron microscopy

T_H1 cell T helper 1 cell

T_{FH} cell T follicular helper cell

TfR transferrin receptor

TLR toll-like receptor

TNF tumor necrosis factor

TRAg tryptophan rich antigen

TRAP thrombospondin-related adhesion protein

UC ultracentrifugation

VLP viral-like particle

WHO World Health Organization

Chapter

1 Introduction

Since the beginning of humankind, malaria has been one of most devastating infectious diseases. While traditional and contemporary control strategies have made a huge progress to reduce malaria burden, there is still a high number of malaria cases worldwide (WHO, 2018). Undoubtedly, a vaccine would be a useful tool to reduce the morbidity and mortality associated to this disease. In fact, vaccines have been proven to be the most cost-effective interventions to provide protection against infections and reduce their transmission (Ada, 2005). However, the design of an effective vaccine against malaria is hampered by the complicated biology of malaria parasites. There are several species of malaria parasites that can affect humans and all of them have an intricate life cycle. Furthermore, malaria parasites have co-evolved with our immune system during thousands of years and have *learned* to evade our immune responses, so well, that we still do not fully understand how our defenses try to stop the infection (Cowman, Healer, Marapana, & Marsh, 2016).

Plasmodium vivax is the most widely distributed of the species that cause malaria to humans, putting around one-third of world's population at risk of infection (WHO, 2018). Yet, this parasite has received less attention than *Plasmodium falciparum*, the most virulent malaria species. Much of the neglect of *P. vivax* malaria is due to the historical misconception that *P. vivax* cannot cause severe malaria (Baird, 2013). In addition, the lack of an *in vitro* culture for *P. vivax* represents a great obstacle to study this parasite. Research on *P. vivax* needs to be substantially expanded in order to develop adequate tools for its control (Mueller et al., 2009).

One of the particular characteristics of *P. vivax* is that it preferentially invades reticulocytes, immature red blood cells, during the blood stage of infec-

tion (Kitchen, 1937). Curiously, the release of exosomes, membrane nanovesicles of endocytic origin, was originally described in these young red blood cells (Harding, Heuser, & Stahl, 1983; Pan & Johnstone, 1983). In those seminal studies, these small vesicles were considered as a cargo-disposal mechanism of the reticulocyte to get rid of obsolete proteins. However, the perspective on the function of exosomes and other extracellular vesicles has changed over the years. First, not only reticulocytes, but virtually any kind of cell, might be able to secrete extracellular vesicles (Yáñez-Mó et al., 2015). Multiple works on exosomes released by immune cells have highlighted their role on the promotion of immune responses (Raposo et al., 1996; Théry et al., 2002). In addition, there is an increasing evidence on the contribution of extracellular vesicles to intercellular communication (Valadi et al., 2007), even between malaria parasites (Regev-Rudzki et al., 2013).

The role of exosomes in antigen presentation in malaria was explored for the first time in a reticulocyte-prone murine malaria model resembling *P. vivax* (Martin-Jaular, Nakayasu, Ferrer, Almeida, & Del Portillo, 2011). This work demonstrated that exosomes derived from malaria-infected reticulocytes contained parasite components and could elicit anti-malarial immune responses.

This thesis explores the potential of (i) extracellular vesicles from human *P. vivax* infections in antigen discovery as well as in the promotion of immune responses, and (ii) human reticulocyte-derived exosomes as a vaccine platform to control *P. vivax*.

1.1 Malaria

Malaria is an ancient parasitic disease. Records from early Greek philosophers already describe unhealthy people with an enlarged spleen tormented by fever. There are even earlier references to a debilitating disease in Chinese writings, to what was almost certainly malaria (Cox, 2010). In 1880, scientific understanding of this disease began thanks to the discovery of its causative agent by Alphonse Laveran. He was perceptive enough to observe pigmented granules in the blood of malaria patients and managed to discover malaria parasites under an unrefined microscope (Laveran, 1881)(see Figure 1.1).

Malaria parasites can also affect other species (Garnham, 1966). We can study those potentially comparable parasite-host interactions and extrapolate that knowledge to tackle human malaria. This was the case of Ronald Ross' studies. In 1897, his work on avian malaria uncovered the mode of malaria transmission: the bite of infected mosquitoes (Ross, 1898). Two years later, Grassi, Bigmani and Bastianelli proved the mechanism on humans showing that mosquitoes that had fed on malaria patients could infect healthy individuals in subsequent blood meals (Grassi, Bignami, & Bastianelli, 1899). However,

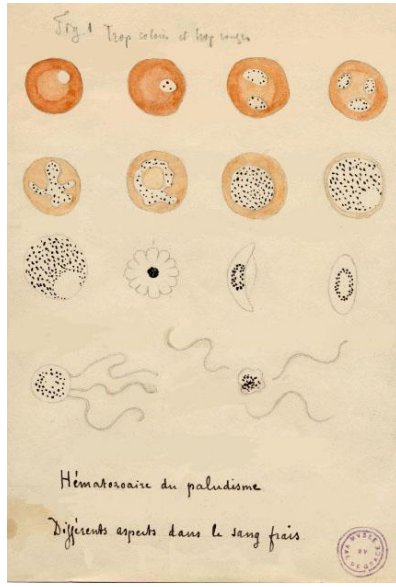


Figure 1.1 **Illustration of malaria parasites drawn by Laveran.** Drawings of malaria parasites observed on fresh blood by Laveran. Pigmented granules were observed in most of the forms. Source of figure: Laveran, 1881.

malariologists wondered during the following 50 years why malaria parasites where not present in blood until 10 days after infection. Finally, in 1947, Shortt and Garnham discovered parasites dividing in the liver prior to the development of parasites in blood.

Knowledge about malaria has rapidly and extensively spread since the mentioned early findings but still, malaria represents one of the major infectious diseases for humankind.

1.1.1 *Malaria Burden*

Almost half of the world population lives at risk of malaria infection. Investment in malaria control programmes enabled a global decrease of 37% of malaria incidence rates between the years 2000 and 2015 (Cibulskis et al., 2016). Even though this represented a huge progress towards malaria elimination, the improvement has slowed down in the recent years. For the second year in a row the number of cases has plateaued (WHO, 2018) having still 219 million of estimated cases of malaria and 435,000 deaths in the year 2017 (WHO, 2018).

The distribution of malaria cases is geographically heterogenous. Approximately half of the global malaria cases occurred only in five countries: Nigeria (25%), Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%) (WHO, 2018).

From the five species that can cause human malaria, *Plasmodium falciparum* and *Plasmodium vivax* are the major contributors to malaria morbidity (WHO, 2018). The differential spatial distribution of these parasites influences the cause of malaria cases around the WHO regions. Model-based maps of the endemicity of *P. falciparum* (D. J. Weiss et al., 2019) and *P. vivax* (Battle et al., 2019) have been recently published (see Figure 1.2). Of note, *P. vivax* is the most geographically widespread human malaria parasite, putting nearly 2.5 billion people at risk of infection (Howes et al., 2016).

In 2017, *P. falciparum* accounted for the majority of malaria cases in most of WHO regions (see Figure 1.3). Still, *P. vivax* was responsible for at least 7.5 million cases according to the last WHO estimates. *P. vivax* is the most prevalent parasite in the WHO Region of the Americas, representing 74.1% of malaria cases, and accounts for an important percentage of cases in the WHO regions of South-East Asia (37.2%), Eastern Mediterranean (31%) and Western Pacific (28.1%) (WHO, 2018) (see Figure 1.3). Remarkably, just five countries (India, Pakistan, Ethiopia, Afghanistan and Indonesia) concentrated around 80% of the estimated vivax malaria cases in 2017 (WHO, 2018).

Even if these estimates are the best ones we can get with the current methodologies, we should not forget that *P. vivax* infections are likely to be underestimated. Many *P. vivax* infections go unnoticed. Clinical symptoms occur even though there is no detectable parasitemia in circulation (Moreira, Abo-Shehada, Price, & Drakeley, 2015). In addition, to a much lower yet significant extent, co-infections with *P. falciparum* tend to be classified as *P. falciparum* mono-infections since the higher density of *P. falciparum* parasite masks *P. vivax* (Mayxay, Pukrittayakamee, Newton, & White, 2004). Without an accurate estimation of *P. vivax* infections, it is unlikely to develop and implement efficient control strategies.

1.1.2 *Malaria Disease and Control*

Individuals who contract malaria often experience fever, headache and chills when they are non-immune. These first symptoms are similar to the ones of other diseases, which makes it difficult to diagnose malaria. If malaria is not diagnosed and treated, it might lead to severe complications and death. Children, pregnant women, HIV/AIDS patients and non-immune migrants are the groups at highest risk (WHO, 2018).

Severe malaria episodes might cause seizures, coma and failure in multiple organs. Main complications include cerebral malaria, severe anemia and respiratory acidosis. It is been always believed that only *P. falciparum* can cause severe malaria, but reports from the last decade indicate that *P. vivax*, which was wrongly qualified as "benign", can lead to severe malaria as well (Baird, 2013).

P. falciparum cases are treated with artemisinin-based combination therapy

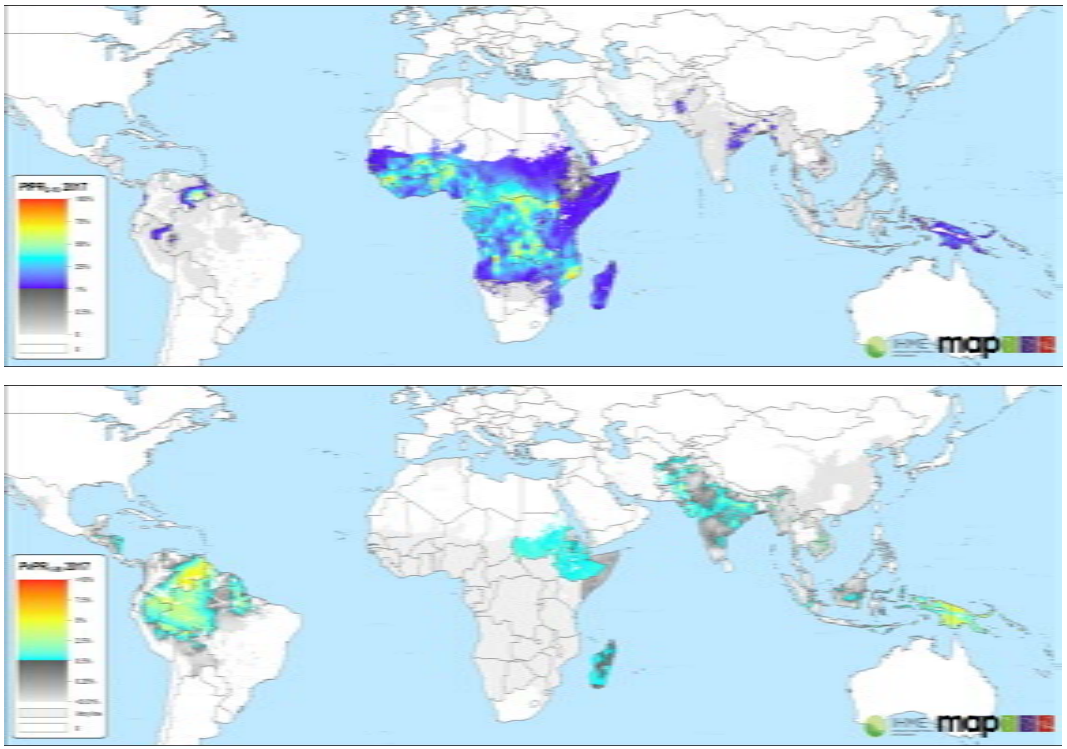


Figure 1.2 **Global endemicity of *P. falciparum* and *P. vivax* in 2017.** Upper map shows the predicted age-standardized parasite prevalence rate for *P. falciparum* malaria in two to ten-year olds (*Pf*PR2-10). Bottom map shows all-age parasite prevalence rate for *P. vivax* malaria (*Pv*PR1-99). Source of figure: The Malaria Atlas Project.

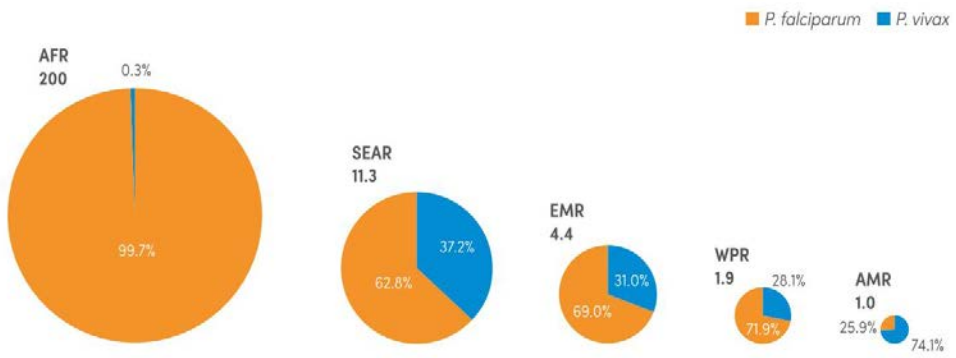


Figure 1.3 **Estimated malaria cases caused by *P. falciparum* and *P. vivax* by WHO region in 2017.** Area of the circles represents estimated number of cases and shows percentage of the contribution of *P. falciparum* and *P. vivax*. AFR: WHO African Region; AMR: WHO Region of the Americas; EMR: WHO Eastern Mediterranean Region; SEAR: WHO South-East Asia Region; Source of figure: WHO, 2018.

(ACT) while *P. vivax* cases are normally managed with a combination of chloroquine and primaquine. However, in areas where there has been emergence of cloroquine-resistant *P. vivax* parasites, treatment with ACT should be provided (Douglas, Anstey, Angus, Nosten, & Price, 2010).

P. vivax poses additional challenges for malaria control and prevention compared to other *Plasmodium* spp. For instance, *P. vivax* parasites can develop dormant stages in the liver that, if not properly treated with primaquine, could eventually reawake and lead to a relapse. However, primaquine administration will likely cause hemolysis to patients with a hereditary deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD) (Baird, Valecha, Duparc, White, & Price, 2016). Therefore, the dose and frequency of the administration of primaquine treatment should be guided by the patient's G6PD enzyme activity.

For the specific control of *P. vivax*, the development of affordable and reliable diagnostic tools that detect liver-stages and G6PD deficiency is crucial. Novel tools should also be applied to detect *P. vivax* low blood parasitemia (The malERA Consultative Group on Diagnoses, 2011) and target its particular vectors (Adapa et al., 2019).

To aid reducing *P. vivax* disease and transmission, scientific community should make a greater effort on the development of highly effective vaccines (Mueller, Shakri, & Chitnis, 2015; Reyes-Sandoval & Bachmann, 2013), that also applies to *P. falciparum* (Draper et al., 2018). Still, less than ten *P. vivax* vaccine candidates have undergone or are under clinical trial compared to the approximately 200 that have been carried out for *P. falciparum*[1].

[1]WHO Malaria Vaccine Rainbow Table and clinicaltrials.gov

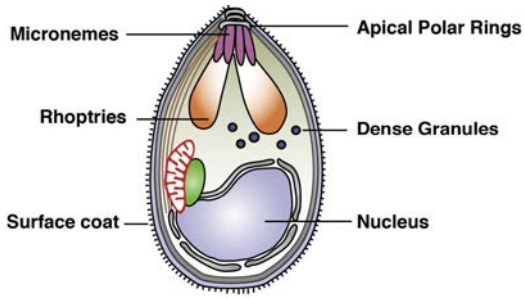


Figure 1.4 **Schematic representation of a *Plasmodium* parasite at merozoite stage.** Several of the major structures and organelles are represented. Source of figure: Tham, Beeson, and Rayner, 2017 https://creativecommons.org/licenses/by-nc-nd/4.0/.

Overall, despite the great efforts applied to control malaria, there is still a huge burden associated to this disease (WHO, 2018). Weak health systems of many of malaria endemic countries, emergence of resistance to antimalarial drugs and to insecticides, gaps in the understanding of the biology of the malaria parasites and the immunity against them are some of the factors accountable for this situation. Current and future malaria control programs already face multiples challenges to maintain the obtained gains (J. M. Cohen et al., 2012) and will require renewed efforts of funding, research and policy-making to advance towards malaria elimination (WHO, 2018).

1.1.3 *Biology of Malaria: focus on P. vivax Parasite*

Parasites that cause malaria have an extraordinarily complex biology. A lot of understanding is still required to develop efficient strategies against them. Malarial parasites change appearance throughout their intricate life cycle and are resilient against the attacks of the hosts.

Malaria is caused by obligate intracellular parasites from the genus *Plasmodium*, which belongs to the phylum Apicomplexa (N. Levine, 1970). Apicomplexan parasites were named after their shared organelles (micronemes, rhoptries and dense granules) at the apical end of some life stages (N. D. Levine & Ivens, 1981), see an example in Figure 1.4. Besides *Plasmodium* spp., approximately other 6000 species have been classified within the phylum Apicomplexa (Adl et al., 2007). Among those, *Toxoplasma* spp. (Dubey & Beattie, 1988) and *Cryptosporidium* spp. (Clode, Koh, & Thompson, 2015) also largely affect humans, domestic animals and/or livestock.

From the nearly 250 species of *Plasmodium*, five are known to infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* (Garnham, 1966; B. Singh et al., 2004). Other *Plasmodium* spp. are able to infect rodents and simians among other animals (Garnham, 1966). Phylogenetic analysis of human and non-human

malaria parasites provided insights into the evolution of *Plasmodium* genus (Escalante & Ayala, 1994). These studies also guide the choice of non-human parasites of rodents or non-human primates to model processes of malaria disease that are impossible to assess in human subjects (Langhorne et al., 2011). Many non-human malaria models have been of great value to understand malaria pathogenesis and host immune responses to malaria parasites (Joyner, Barnwell, & Galinski, 2015; Wykes & Good, 2009). Although these animal models are extraordinarily informative, we must bear in mind that they represent an approximation of certain aspects of human malaria infections.

Malaria community has traditionally prioritized the study of the parasite *Plasmodium falciparum* since it is the most virulent human malaria parasite. In the last decade, *Plasmodium vivax* has withdrawn attention due the recently acknowledged tendency of this parasite to become prevalent in the areas where it is endemic (WHO, 2018). However, research on *P. vivax* has been very limited due to the lack of a continuous *in vitro* culture for this parasite (Noulin, Borlon, Van Den Abbeele, D'Alessandro, & Erhart, 2013). In the following section we describe some aspects of its fundamental biology, which hopefully would be expanded in the future with renewed research efforts.

1.1.3.1 Life Cycle of *Plasmodium* spp.: Emphasis on *P. vivax* Unique Features

From the mosquito to the human liver

The first plasmodial form that naturally enters a human host is called sporozoite (see Figure 1.5). Sporozoites are injected into the skin during the blood meal of infected female *Anopheles* mosquitoes. These parasites need to reach the bloodstream to make their journey from the skin to the liver using mechanisms that are not fully understood yet (Mota & Rodriguez, 2004). Thanks to their great gliding motility and capacity to traverse cells, sporozoites are able to pass through endothelial and Kupffer cells before they find suitable hepatocytes (Tavares et al., 2013). Still, a large fraction of sporozoites does not enter the blood circulation. Instead, those parasites will presumably end up in the lymphatic system, where some of the first immune responses against the parasite occur (Sinnis & Zavala, 2012).

Once in the liver, sporozoites change their migrating behavior into an invasive one. The circumsporozoite protein (CSP), the main protein of the surface of the sporozoite, is key for this switch. This protein has to be cleaved by a parasite protease in order to unveil an adhesive domain located in its carboxy-terminus (Coppi et al., 2011). When sporozoites enter hepatocytes, they induce the formation of a parasitophorous vacuole where they will undergo development and parasite schizogony. Rapid divisions will arise thousands of

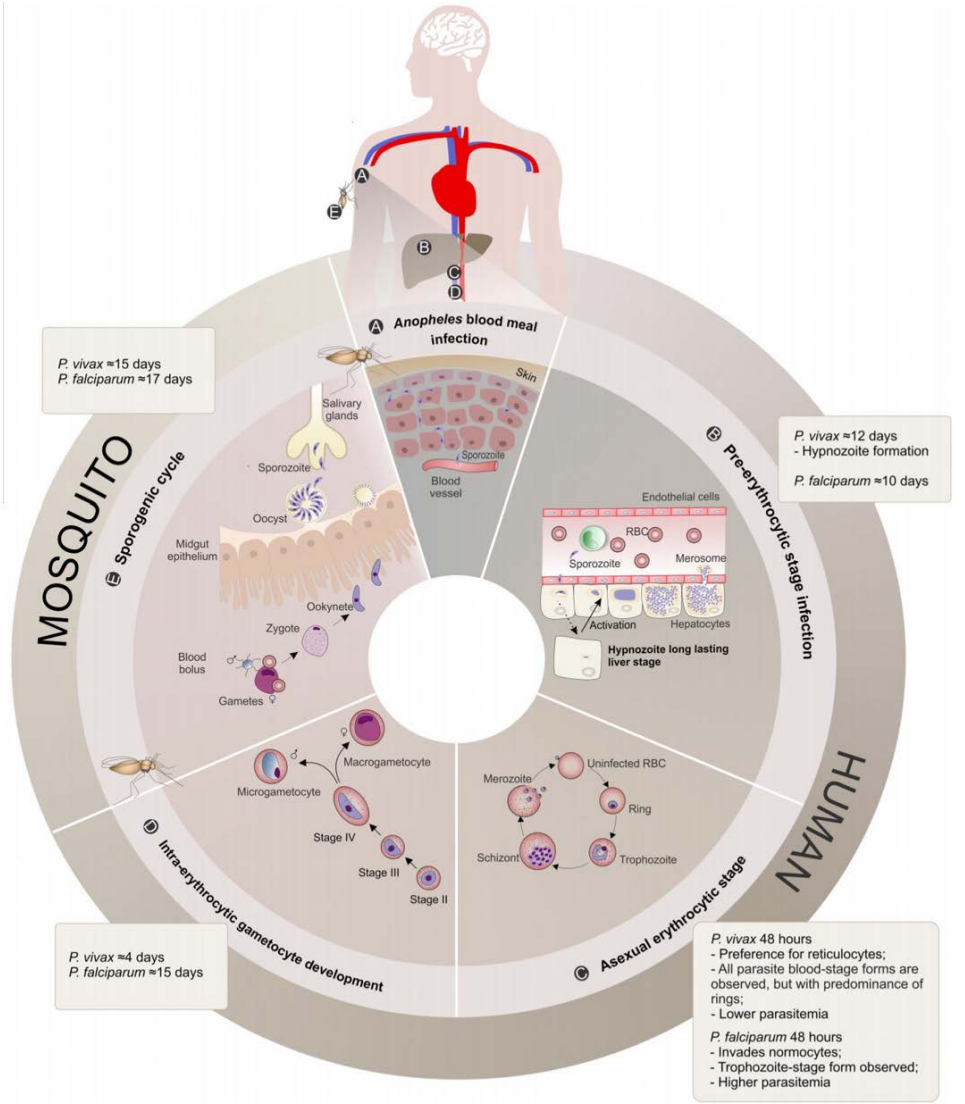


Figure 1.5 **Comparative life cycle of *Plasmodium vivax* and *Plasmodium falciparum*.** Source of figure: Bourgard, Albrecht, Kayano, Sunnerhagen, and Costa, 2018. https://creativecommons.org/licenses/by/4.0/.

daughters merozoites that will be released in "packet" of merosomes into the bloodstream within approximately 10 days (Sturm et al., 2006).

A critical difference between *P. vivax* and *P. falciparum* is the capacity of *P. vivax* parasites to develop dormant stage forms in the liver called hypnozoites. Months or even years after the initial infection, these forms can be activated leading to a clinical relapse (Krotoski, 1985). Hypnozoites represent an important parasite reservoir and greatly contribute to *P. vivax* burden

(Betuela et al., 2012). The determinants behind the development into an active schizont or a dormant hypnozoite remain unknown. Also, the factors that contribute to the awakening of the hypnozoites are yet to be discovered.

Erythrocytic infection

Merosomes are released into the liver capillaries avoiding the phagocytosis by Kupffer cells. After some hours, merozoites are released into the bloodstream, where they invade red blood cells (RBCs). The entrance of any *Plasmodium* parasite into a red blood cell is a highly complex process, that involves several merozoite proteins, host receptors and invasion pathways, many of them still unknown (Cowman et al., 2016).

Predilection to invade immature red blood cells, reticulocytes, is a peculiarity of *P. vivax* parasites (Kitchen, 1937). *P. falciparum* is not restricted by the age of red blood cells. Reticulocytes are relatively transient in erythropoiesis and represent between 1-2% of the RBCs in peripheral blood. This tropism for reticulocytes is one of the main factors contributing to the lack of a continuous *in vitro* culture for *P. vivax*. Why *P. vivax* parasites prefer reticulocytes is still unclear (Galinski & Barnwell, 2008).

Studies have shown that invasion of *P. vivax* relies on the interaction between the red blood cell Duffy receptor (also known as Duffy Antigen/Chemokine Receptor, DARC) and the *P. vivax* ligand Duffy Binding Protein (PvDBP) (Chitnis & Miller, 1994; Miller, Mason, Clyde, & Mcginniss, 1976). Indeed, the interaction of this parasite with ancient African human populations might have contributed to the current dominance of Duffy-negative individuals in Africa (Zimmerman, Ferreira, Howes, & Mercereau-Puijalon, 2013). However, recent reports of *P. vivax* infection in Duffy-negative individuals have turned the essentiality of the Duffy receptor for *P. vivax* invasion into a matter of debate (Mendes et al., 2011; Woldearegai, Kremsner, Kun, & Mordmü ller, 2013).

Many other determinants of the parasite invasion should be studied. Figure 1.6 shows some of the known invasion ligands and their host receptor specificities (Bermúdez, Moreno-Pérez, Arévalo-Pinzón, Curtidor, & Patarroyo, 2018). Of note, more than 50 parasite proteins have been described to be involved in *P. falciparum* invasion (Cowman et al., 2016) whereas around 20 have been identified in *P. vivax* (Gunalan, Niangaly, Thera, Doumbo, & Miller, 2018). Remarkably, the transferrin receptor, a long-suspected reticulocyte-specific receptor for *P. vivax*, has eventually been proven as one of the molecular keys for its tropism (Gruszczyk et al., 2018).

Once parasites have invaded RBCs, parasites will undergo an asexual cell cycle where they transform and proliferate during 48 hours. Few hours after invasion the first stage that can be observed is known as ring-stage. During

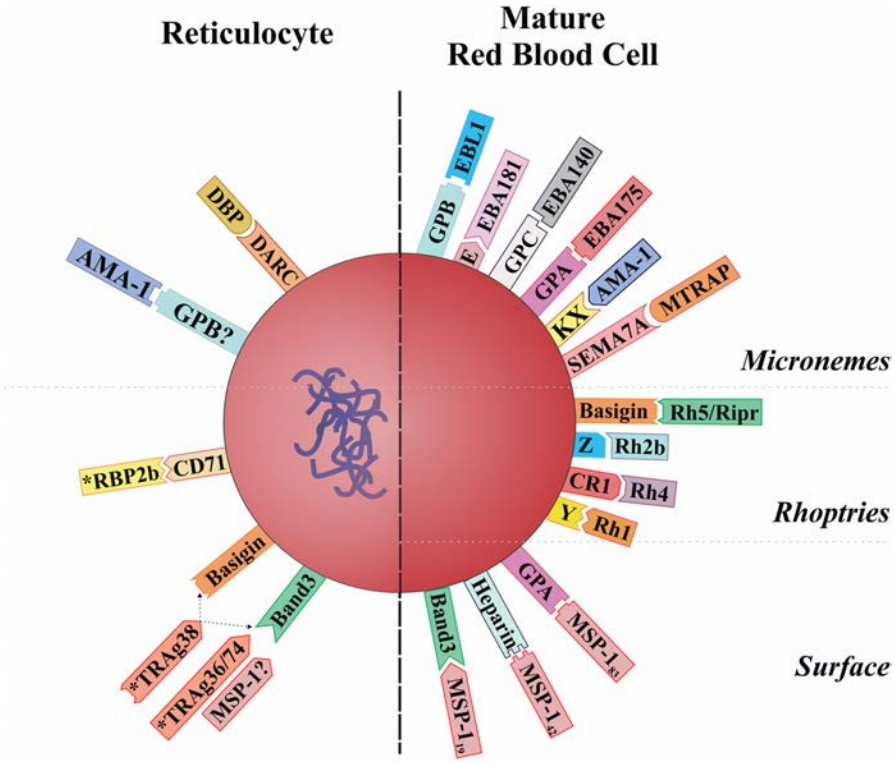


Figure 1.6 **RBC receptors of *P. vivax* (left) and *P. falciparum* (right) merozoite proteins.** Half reticulocyte depicted on the left side and half mature RBC on the right side. Merozoite binding proteins for each receptor are shown with their corresponding location on the merozoite. Proteins marked with an asterisk have unknown location. Source of figure: Bermúdez, Moreno-Pérez, Arévalo-Pinzón, Curtidor, and Patarroyo, 2018. https://creativecommons.org/licenses/by/4.0/.

this early phase, many parasite proteins are being transported to the red blood cell surface. Later, the parasite enters the trophozoite-stage and subsequent schizogony. When schizonts rupture from the host cell, daughter merozoites will be able to invade new RBCs to sustain the infection (Cowman et al., 2016).

Parasites greatly modify their host cells. Many indentations, *caveolae*, are formed in the membrane of *P. vivax*-infected reticulocytes and are surrounded by vesicles forming caveola-vesicle complexes (CVCs) (Barnwell, Ingravallo, Galinski, Matsumoto, & Aikawa, 1990). These complexes accumulate in the surface of RBC membrane, giving it a dotted appearance commonly known as Schüffner's dots (Aikawa, Miller, & Rabbege, 1975). The function of the CVCs has not been elucidated yet.

Another relevant difference between *P. falciparum*-infected RBCs and the ones infected by *P. vivax* is their deformability. *P. vivax*-infected RBCs have an increased deformability that might favor their safe passage through the spleen (Suwanarusk et al., 2004).

The way back to mosquitos

A small proportion of the parasites can differentiate into gametocytes. These forms must be transmitted to an *Anopheles* mosquito to begin the sexual cycle that will produce sporozoites.

Gametogenesis occurs in the human host. For *P. falciparum* this process takes around 15 days, but for *P. vivax* only 4 days. This gives vivax an advantage in transmission since sexual parasites might be uptaken during *Anopheles* mosquitoes blood meal before infected people might notice any clinical symptoms. When gametocytes are uptaken by mosquitoes they further go fertilization, formation of ookinetes that will traverse the midgut epithelium of the mosquito and finally develop into a form that will multiply and yield sporozoites.

Of note, *P. vivax* mosquito vectors have more erratic behavior than the ones of *P. falciparum*. Vivax vectors tend to bite outdoors and are not so anthropophilic. Therefore, the regular campaigns that tackle *P. falciparum* vectors are likely not to be equally effective for the control of vivax vectors (Bousema & Drakeley, 2011).

1.1.4 *Immune Responses to Vivax Malaria*

In 1900, Robert Koch travelled to Indonesia and Papua New Guinea and observed that adults that were exposed to malaria were less susceptible to clinical illness than children living in the same area. Koch deduced that resistant individuals acquired that protection after high and continuous exposure to the infection (Koch, 1900).

During the next 100 years of malaria research, huge efforts have been carried out to uncover the necessary components to achieve an immunizing process against malaria. Many promising targets have been identified. Yet, the targets and mechanisms of natural acquired immunity to malaria infections are not well understood (Doolan, Dobano, & Baird, 2009; Long & Zavala, 2017). Undoubtedly, unveiling those targets and mechanisms is key to design rational vaccination strategies (Crompton, Pierce, & Miller, 2010).

Epidemiological studies have shown that natural immunity to *P. vivax* infection is acquired faster than immunity to *P. falciparum* infection. In places where both parasite are highly endemic, such as New Guinea island, most of the children are immune to vivax by the age of five, meanwhile they continue at risk of falciparum clinical disease (Mueller et al., 2013). There might be several explanations for the rapid acquisition of immunity to *P. vivax* infections. It has been suggested that a higher force of blood-stage infection, measured as number of genetically distinct blood-stage infections, could be one of the possible reasons (Koepfli et al., 2013). These observations together with the unique biological

features of each *Plasmodium* spp. remark that the mechanisms accounting for protection against *P. vivax* and *P. falciparum* might substantially differ.

Our immune system would try to defend us from *Plasmodium* parasites through multiple innate and adaptive immune responses. Still, it is unclear which of those are crucial for protection, and how they might be affected by age and level of malaria transmission. Despite this, evidence from both naturally infected patients and experimental infections suggests that for the generation of long-lasting protection both maintenance of cytophilic antibodies and induction of B-cell and T-cell memory are likely to be essential (Long & Zavala, 2017). In the following sections, we will describe first the innate and adaptive immune responses, focusing on responses targeted to *P. vivax* parasites. After that, we will discuss the relevant role of the spleen in immunity against malaria. We will finish the section by mentioning some of the inefficient immune responses to malaria parasites.

1.1.4.1 Innate Immune Response

The innate immune response is the first line of defense to any infection. Innate mechanisms involve dendritic cells (DCs), natural killer cells, granulocytes and phagocytic cells (monocytes/macrophages), among others (Gazzinelli, Kalantari, Fitzgerald, & Golenbock, 2014). Activation of the innate immune system is usually triggered by the recognition of microbial pathogen-associated molecular patterns (PAMPs) by host Toll-like receptors (TLRs) (Akira, Takeda, & Kaisho, 2001). In the case of malaria infections, some of described PAMPs are hemozoin (Coban et al., 2005), glycosylphosphatidylinositol (GPI) (Schofield & Grau, 2005) or immunostimulatory motifs present in the parasite nucleic acids (Pichyangkul et al., 2004). Of note, *P. vivax* contains around 2,500 CpG immunostimulatory motifs in its genome, a higher number than in falciparum genome (Auburn et al., 2016). This is likely to contribute to more pyrogenic responses against *P. vivax* infection (Anstey, Douglas, Poespoprodjo, & Price, 2012).

When innate immune cells get activated, they produce pro-inflammatory mediators, such as cytokines [(tumor necrosis factor (TNF), interleukin-1β (IL-1β), interleukin-12 (IL-12), interferon-γ (IFN-γ), type I interferons)] and chemokines (Gazzinelli et al., 2014). The release of these cytokines plays central role in the promotion of phagocytosis (Su, Fortin, Gros, & Stevenson, 2002) and opsonization (Kumaratilake, Ferrante, Jaeger, & Rzepczyk, 1992) of parasites or parasite-derived products.

Plasmodium infection modulates the function of monocytes and macrophages (Chua, Brown, Hamilton, Rogerson, & Boeuf, 2013) as well as dendritic cells (Amorim, Chagas, Sulczewski, & Boscardin, 2016). The multiple roles of these cells during *P. vivax* infection are not fully understood. Antonelli *et al* found

an increased frequency of circulating monocytes and, importantly, they showed that CD14+CD16+ subsets had increased effector function and might be relevant for *P. vivax* control (Antonelli et al., 2014). However, a previous study by Fernandez-Arias *et al* suggested an impaired clearance capacity of monocytes/macrophages (CD16+CD10-) related to a diminished expression of complement receptor 1 (Fernandez-Arias et al., 2013). Perhaps a consensus on the phenotyping of the cells and the assessment of their functions as well as a better knowledge on the infection status will improve our understanding on their role.

DCs are a fundamental part of the innate immune system but also the bridge to the adaptive immune response. Therefore, they have an extremely relevant role against infections. Studies have suggested that during *P. vivax* infections, maturation of DCs is inhibited (Gonçalves et al., 2010) and that the ratio between myeloid and plasmacytoid DCs is altered (Jangpatarapongsa et al., 2008). More recent works have studied immune responses to human *P. falciparum* infections applying a Controlled Human Malaria Infection (CHMI). *P. vivax* CHMI studies have been recently available and among other findings, they have shown a reduction of DC numbers and impairment of CD1c+ myeloid DC maturation (Vallejo et al., 2018; Woodberry et al., 2017).

1.1.4.2 Adaptive Immune Response

Evidence from studies on natural and vaccine-induced immunity as well as experimental animal models remarks the importance of both humoral and cellular immunity to control malaria infection. However, which one is the critical component of the adaptive immunity would largely depend on the malaria parasite species (Burel, Apte, McCarthy, & Doolan, 2016) and the parasite life-stage (López, Yepes-Pérez, Hincapié-Escobar, Díaz-Arévalo, & Patarroyo, 2017).

Humoral immunity

Seminal studies from Cohen *et al* highlighted the importance of antibodies in naturally acquired immunity to malaria (S. Cohen, McGregor, & Carrington, 1961). This work demonstrated for the first time that the transfer of sera from immune adults to children protect those children from clinical disease. This seminal observation was reinforced years later when purified immunoglobulins G (IgGs) from African individuals exposed to *P. falciparum* were transferred to Thai patients and their infections were cleared as fast or faster than with chemotherapy (Sabchareon et al., 1991). Parasite-specific antibodies have different effector functions against malaria infection: targeting sporozoites, merozoites and parasitized RBCs for antibody-dependent cellular cytotoxicity, blocking merozoite invasion from invading RBCs, opsonization of parasitized

cells to increase their phagocytosis as well as activating the classical complement pathway (Teo, Feng, Brown, Beeson, & Rogerson, 2016).

As mentioned in the life cycle of the parasite, PvDBP is an important protein for merozoite invasion and, therefore, it has always been considered a relevant target for vaccination (Chitnis & Sharma, 2008). More than twenty years ago it was already demonstrated that sera from individuals naturally exposed to infection could recognize the region II of the DBP (Fraser et al., 1997). Over the years, there has been accumulating evidence that antibodies directed against this protein block DBP-DARC interaction and inhibit RBC invasion by *P. vivax* (Grimberg et al., 2007; Michon, Fraser, & Adams, 2000). Despite the polymorphic nature of DBP, recent structural analysis over human-derived monoclonal antibodies from individuals with naturally acquired immunity revealed that they target broadly conserved epitopes on the DARC-binding domain (Urusova et al., 2019). Hopefully, structural vaccinology will help in the design of immunogens that mainly consist of protective epitopes (S. K. Singh, Hora, Belrhali, Chitnis, & Sharma, 2006).

Many works have studied the generation of antibody responses against other merozoite surface proteins, MSP1 being the one that has drawn most of the attention. While many studies have measured antibody responses against MSP1 and have found a high proportion of seropositive vivax infected patients, only few studies have been able to associate MSP1-specific antibodies with protection (Nogueira et al., 2006; Versiani, Almeida, Mariuba, Orlandi, & Nogueira, 2013). There are more studies on the naturally induced production of antigen-specific antibodies against blood-stage proteins including MSP3, MSP9, AMA1, various TRAgs, VIRs, RBPs, CSP and the gametocyte antigen GAM1 (reviewed in (Longley, Sattabongkot, & Mueller, 2016)). In a longitudinal study in children from Papua New Guinea, naturally-acquired humoral immune responses against PvMSP3 and PvMSP9 had associations with protection from clinical *P. vivax* malaria (Stanisic et al., 2013). In addition, it has been demonstrated that VIR proteins are targets of both humoral and cellular naturally-acquired immune responses in pregnant women (Requena et al., 2016), but their contribution to protection remains to be elucidated.

There are over 5,000 proteins encoded in *P. vivax* genome (Auburn et al., 2016) and it is not easy to determine which of them would be the best target for a highly efficacious vaccine. A recent study by França *et al* on naturally acquired humoral immunity has found IgG responses to several *P. vivax* proteins, such as EBP and RBPs, to be strongly associated with protection (França et al., 2017). Furthermore, this study investigated the potential protective efficacy of antibodies to multiple antigen-combinations in order to guide the development of multi-component *P. vivax* vaccines.

Cellular immunity

Cellular responses appear to be critical to limit both the asymptomatic liver-stage infection as well as the symptomatic blood-stage (Kurup, Butler, & Harty, 2019).

Most of the evidence of T-cell mediated immunity comes from rodent models that study protection against pre-erythrocytic stages. In the 1960s, Nussenzweig *et al* found that rodents vaccinated with radiation-attenuated sporozoites could be protected from a challenge with parasites from the same stage (Nussenzweig, Vanderberg, Most, & Orton, 1967). Years later, it has been demonstrated that CD8+ T-cells are critical for the observed protection (W. R. Weiss, Sedegah, Beaudoin, Miller, & Good, 1988). Despite that it is still unclear how and where those responses are primed, a recent publication has examined the nature of antigen-presenting cells (APCs) in the liver after sporozoite infection (Kurup, Anthony, et al., 2019). This study has revealed that monocyte-derived DCs uptake parasite antigens from infected hepatocytes and migrate to the liver-draining lymph nodes to prime naive CD8+ T-cells. After their activation, malaria-specific CD8+ T-cells would home to the liver and would eliminate infected hepatocytes. Still, the possibility that other DC subsets might contribute as well to the priming of *Plasmodium*-specific CD8+ T-cells should not be discarded (Villarino & Schmidt, 2014). Of note, *in vitro* studies have also shown specific CD8+ T-cell responses against *P. vivax* liver stages (Vichchathorn et al., 2006). However, to what extent CD8+ T-cell responses would limit natural primary liver infections is ambiguous. Few sporozoites are delivered in each mosquito bite compared to the dose administered in experimental infections or vaccinations.

CD8+ T-cell responses to blood-stage infection have been always overlooked due to the fact that mature erythrocytes do not express human leukocyte antigen (HLA) class I molecules that could present parasite antigens. However, unlike *P. falciparum*, *P. vivax* infects preferentially reticulocytes, cells that do retain the expression of HLA class I molecules (Silvestre, Kourilsky, Niccolai, & Levy, 1970). Burel *et al* explored for the first time the potential differential cellular responses of naive volunteers in CHMI with blood-stage parasites of *P. vivax* or *P. falciparum*. They discovered that only the volunteers infected with *P. vivax* had an expanded subset of CD8+ T-cells. This subset was associated to an activated phenotype and cytotoxicity (Burel et al., 2016). Furthermore, a recent study has demonstrated that *P. vivax*-infected reticulocytes can be recognized and killed by CD8+ T-cells through immunological synapses dependent on the reticulocyte HLA (Junqueira et al., 2018). We might be looking at a differential defense mechanism between *P. vivax* and *P. falciparum* infection. Further studies to determine the role of CD8+ T-cell responses against blood-stage *P. vivax* infection and its potential in vaccination should be pursued.

Studies on rodent malaria models have also remarked the importance of CD4+ T-cell responses to control malaria infection (Pérez-Mazliah et al., 2017). DCs present antigens to CD4+ T-cell via HLA class II molecules and produce cytokines that shift the differentiation of CD4+ T-cells towards T helper 1 (T_H1) cell and T follicular helper (T_{FH}) subsets. T_H1 cells produce IFN-γ that would enhance the phagocytic capacity of macrophages and induce the production of nitric oxide, toxic to the parasite. T_{FH} cell populations expand during both human and rodent blood-stage *Plasmodium* infections and are critical to promote protective antibody responses. T_{FH} cells engage parasite-specific B-cells and assist them during the germinal center reaction. T_{FH} cells promote the selection of the clone with the highest affinity for the antigen. Only these high-affinity B-cell clones undergo class switching and differentiate into long-lived antibody-secreting plasma cells and memory B-cells (Kurup, Butler, & Harty, 2019). Figueiredo *et al* have recently stood out the relevance of T_{FH} cells in the immune response to *P. vivax* infection. They found out that during the infection there is an expansion of the circulating T_{FH} cells that boost the immunoglobulin production by B-cells. They also demonstrated that vivax infections induce changes in B-cell compartments, and that re-exposure to the infection led to an increased frequency of classical memory B-cells that was accompanied with high levels of parasite-specific antibodies (Figueiredo et al., 2017).

Multiple experimental models have shown that B-cells as well as CD4+ and CD8+ T-cells are subject to regulation by other T-cells such as regulatory T-cells (Couper et al., 2008; Findlay et al., 2010; Zander et al., 2016). Augmented levels of natural regulatory T-cells have been observed in acute *P. vivax* infections in Thailand (Jangpatarapongsa et al., 2008) and Brazil (Gonçalves et al., 2010), but their role during *P. vivax* infection has not been elucidated yet.

$\gamma\delta$ T-cells are also activated in response to liver and blood-stage infection due to unknown signals. They are thought to recognize phosphoantigens on the *Plasmodium* apicoplast (Guenot et al., 2015). $\gamma\delta$ T-cells expand in patients and mice infected with *Plasmodium* spp. and produce relevant amounts of IFN-γ (Deroost & Langhorne, 2018). However, there is no clear answer whether these cells would contribute to a protective response or not.

1.1.4.3 Spleen-mediated Immune Responses to Malaria

Leon Weiss said "So powerful is the splenic response, so nicely does it interweave host and parasite, that one is moved to speculate that the very structure of the spleen, as that of hemoglobins, may have been evolutionarily driven by malaria." (L. Weiss, 1990). The spleen is a secondary lymph organ with multiple functions in physiology and immunity. It serves important roles

during malaria infections. It is the site where parasitized RBCs are cleared and where adaptive immune responses are generated (Engwerda, Beattie, & Amante, 2005). Its crucial role is confirmed by the inability of splenectomized rodents and humans to control malaria infections (Ho, Looareesuwan, Suntharasamai, & Webster, 1993; L. Weiss, 1989).

The architecture of the spleen reflects its complexity and specialization in multiple functions (Bowdler, 2002). The spleen is composed by two main functional compartments: the red pulp and the white pulp. The interphase between them is known as perifollicular zone in humans and as marginal zone in rodents. The red pulp contains neutrophils, monocytes, DCs, $\gamma\delta$ T-cells and macrophages and its primary function is to filter the blood of aged, damaged or pathogenic RBCs. The white pulp contains follicles enriched in B-cells and periarteriolar lymphoid sheaths (PALS), that are largely populated by naive and central memory T-cells. Parasite-specific B- and T-cell responses are developed in this compartment (Lewis, Williams, & Eisenbarth, 2019).

At the red pulp and the perifollicular zone, specialized leukocytes, including APCs, are perfectly placed to capture blood-borne antigens such blood-stage malaria antigens. Importantly, the blood flow is slowed down when it enters to the spleen, which facilitates the antigen capture by APCs. However, most of the lymphocytes are placed in the white pulp. There are several possible alternatives to facilitate low-probability interactions between APCs and lymphocytes, including migration of APCs and/or transfer of antigen-MHC complexes between cells or via extracellular vesicles (see Section 1.2). After adaptive immune responses against *Plasmodium* spp (or other agent) have been generated, there is limited knowledge on how antigen-specific T and B-cells are positioned in locations where they can function effectively (Engwerda et al., 2005).

Most of the reports on the development of splenic responses to malaria parasites come from rodent models and it is not clear to what extent we can translate those results to human infections (Del Portillo et al., 2012). Given the differential anatomy and distribution of the different cell populations of this organ among the different mammal species, functionality of the splenocytes might greatly differ (Steiniger, 2015). Studies on the spleen of malaria infected patients normally cannot be performed due to ethical and technical limitations. Still, it would be relevant to have a deeper understanding of the spleen of malaria-infected or non-infected individuals. Further studies on the interaction of the human spleen with human malaria parasites should be pursued to better understand the role of this crucial organ in malaria.

1.1.4.4 Inefficient Immune Responses to Malaria

During *Plasmodium* infections there are a lot of potential mechanisms

accountable for inefficient immune responses. We have already commented some of them: impairment of DC maturation and release of immunoglobulins against targets that are not protective, but there are many others.

Both antigenic variation of parasites and their high polymorphism represent a great barrier to control infections (McCollum et al., 2014). Moreover, even when cellular responses are primed, some reports have described atypical B-cell activation (Scholzen & Sauerwein, 2013) and exhaustion of T-cells (Gigley, Bhadra, Moretto, & Khan, 2012).

Additionally, unbalanced immune responses against the pathogen might be responsible for the deadliest consequences. Many symptoms of severe malaria have been associated with excessive inflammation (Gazzinelli et al., 2014).

In spite of the "dysfunctional" or exacerbated inflammatory responses that might be triggered by the infection, evidence suggest that memory T-cell responses (Jangpatarapongsa et al., 2006) as well as memory B-cell responses (Wipasa et al., 2010) can be generated against *P. vivax* parasites. Further investigation would be required to understand if those mechanisms are related to protection and to know how can they be induced and maintained.

1.1.5 *Vaccination against P. vivax: Candidates and Approaches*

Given the complexity of *Plasmodium* spp., the development of highly effective vaccine against them is a great challenge. The most advanced malaria vaccine candidate is the RTS,S/AS01 and it is aimed for the prevention against *P. falciparum* malaria. This vaccine is based on a virus-like particle that comprises 18 copies of the central domain and the C-terminal domain of the pre-erythrocytic antigen *P. falciparum* CSP fused to hepatitis B virus surface antigen. It is formulated with AS01, a potent liposomal adjuvant system from GlaxoSmithKline (GSK). RTS,S/AS01 is the only malaria vaccine candidate that has showed protective efficacy in a phase III trial. This trial enrolled approximately 15,000 children corresponding to two different age groups: children aged 5-17 months who received three doses of RTS,S administered at 1-month intervals, followed by a fourth dose 18 months later, and infants that received the vaccine at 6, 10 and 14 weeks of age. Results from this trial showed that the protection is partial. In children aged 5-17 months, the incidence of clinical malaria was reduced by 51% over the first year of follow-up post-dose three [95% CI 48%–55%]. Over 48 months of follow-up, efficacy was 26% [95% CI 21%–31%], and among children receiving a fourth dose at month 20 (18 months post-dose three), efficacy was 39% [95% CI 34%–43%]. In infants, protection was lower (RTS, 2015). Even with limited efficacy, the vaccine received a positive evaluation from European Medicines Agency and large-scale pilot studies will be carried out to provide additional data for its safety and efficacy as well as to check operational feasibility.

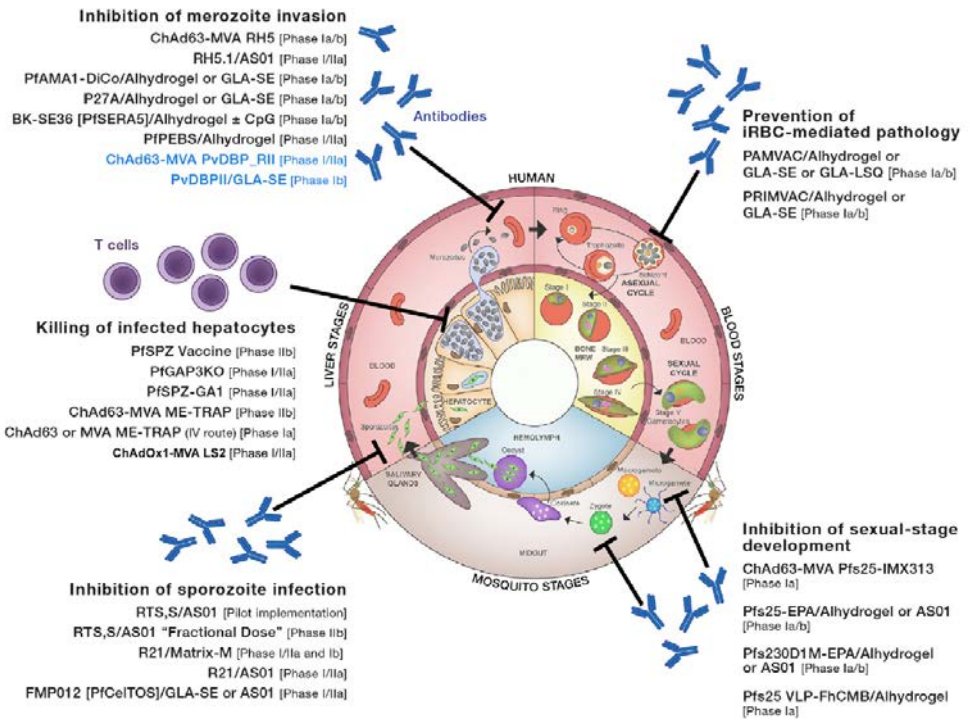


Figure 1.7 **Malaria vaccine candidates undergoing clinical trials in 2018.** In black, vaccines candidates designed to tackle *P. falciparum* malaria. In blue, vaccine candidates against *P. vivax*. Source of figure: Draper et al., 2018 https://creativecommons.org/licenses/by/4.0/.

In addition to RTS,S/AS01, several other human vaccine trials are underway, the majority against *P. falciparum* (Draper et al., 2018) (see Figure 1.7). The design for species-transcending vaccines would be incredibly practical, but they even more unlikely to be developed. For both malarial species, vaccine candidates have targeted all parasites stages in the human host: pre-erythrocytic as well as asexual and sexual erythrocytic stages (see Figure 1.8).

For *P. vivax*, a highly effective pre-erythocytic vaccine would be able to reduce primary infections and impair the establishment of hypnozoites and therefore prevent relapses as well. Proteins PvCSP and PvTRAP have been extensively targeted in many vaccination approaches due to their importance in the interaction between sporozoites and hepatocytes.

Two subunit vaccines targeting PvCSP have reached clinical trials. In one of them, immunogenic fragments of CSP have been evaluated as a recombinant vaccine formulated with GSK Adjuvant System AS01B (VMP001/AS01B). This vaccine candidate was shown to be immunogenic in U.S. volunteers, inducing antibody and cell-mediated immune responses and delaying the prepatency period. However, it was insufficient to confer sterile protection against *P. vivax*

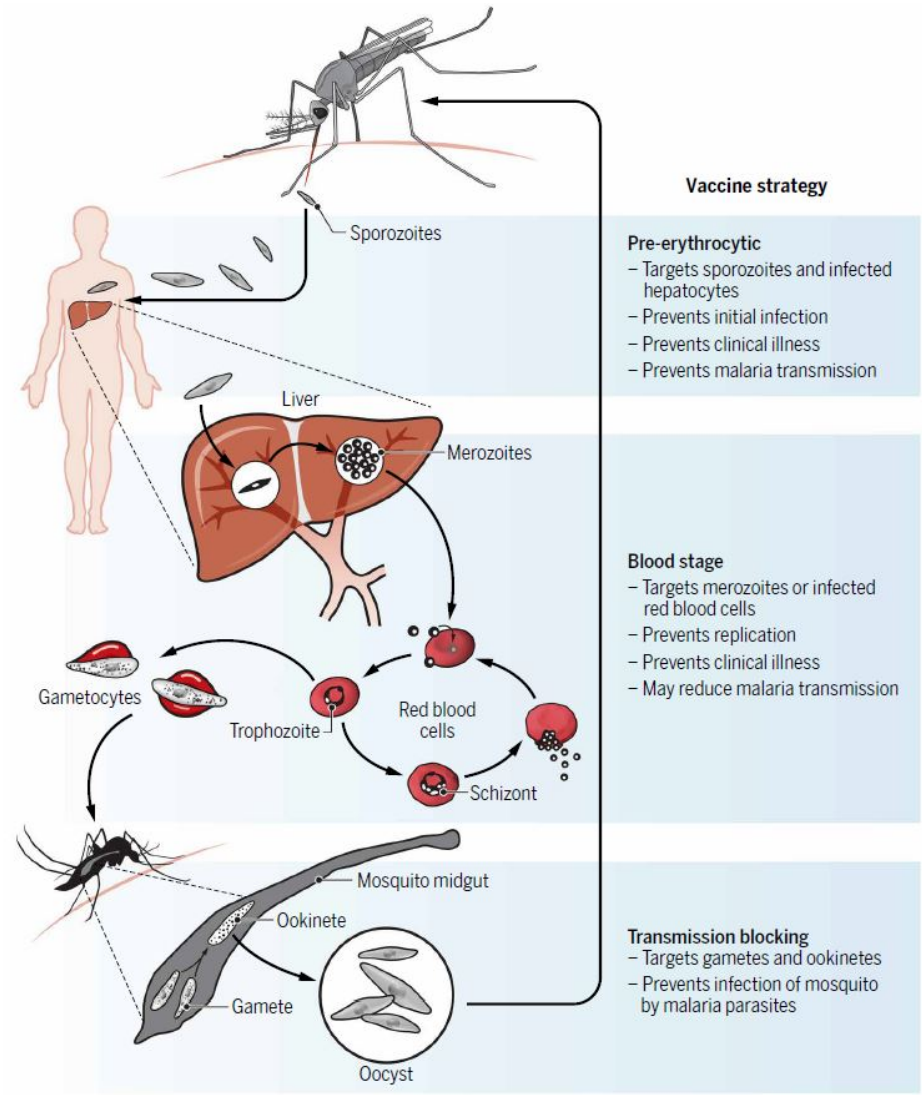


Figure 1.8 ***Plasmodium* life cycle and vaccine targets.** Source of figure: A. Kitterman /Science Translational Medicine, (Beeson et al., 2019). Reprinted with permission from AAAS.

mosquito-bite CHMI (Bennett et al., 2016). The other approach tested was a fusion protein between VMP001 and hepatitis B surface antigen adjuvanted with AS01 (CSV-S,S/AS01), a particulated form, versus a soluble form in rhesus monkeys. The particulated form showed an enhanced humoral response, but not progress to further clinical trials (Vanloubbeeck et al., 2013).

Immunizations with PvCSP-based vaccine using viral-like particles (VLPs) known as Rv21, conferred sterile protection against a stringent sporozoite

challenge in rodent models to malaria and highlights the potential of VLPs to increase the efficacy of vaccine candidates (Salman et al., 2017). More recently, a combined approach of PvCSP Rv21 and PvTRAP viral vectors enhanced immune response over the levels provided as single vaccine (Atcheson et al., 2018). Hopefully, these promising results would move forward in clinical development.

Vaccine strategies against the replicating asexual blood infection, the symptomatic phase of infection, would greatly reduce morbility and mortality in vivax infections. PvDBP is a relevant blood-stage target that has been subject of clinical trials. Specifically, the cysteine-rich region II, PvDBP_RII, which contains the receptor-binding domain has been under study. In one of the clinical trials, viral vectors ChAd63 and MVA encoding PvDBP_RII from the Salvador I (SalI) reference strain of *P. vivax* were shown to be safe and immunogenic in human vaccinees. Furthermore, elicited antibodies could inhibit *in vitro* the binding of vaccine homologous and heterologous variants of recombinant PvDBP_RII to its receptor (Payne et al. 2017). Another clinical trial in malaria-naive adults tested the safety and immunogenicity of PvDBP_RII formulated with glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) demonstrated the induction of strain-transcending antibodies as well (K. Singh et al. 2018). These results encourage to investigate if those responses would be protective against a *P. vivax* CHMI.

Despite these promising results with PvDBP, *P. vivax* infections in Duffy-negative individuals stressed the possibility that there might be alternative invasion routes to DBP that might be mediated by other parasite proteins. If that possibility is confirmed, it would be necessary to include other antigens apart from PvDBP to constitute a multi-component vaccine that would inhibit the major potential invasion pathways. For instance, reticulocyte-binding proteins could represent new candidates of blood-stage antigens. Recently, it has been demonstrated that PvRBP2b is the ligand for the reticulocyte-specific receptor CD71 (Gruszczyk et al., 2018). Indeed, monoclonal antibodies against PvRBP2b blocked reticulocyte binding and *P. vivax* invasion (Gruszczyk et al., 2018) and naturally acquired humoral responses against PvRBP2b have been associated with protection against vivax malaria (França et al., 2017).

In this era where the concept of malaria elimination has a huge popularity, vaccine strategies against the sexual blood stages have been promoted since they would directly tackle the transmission of the infection. However, their effect in the prevention of symptoms in an infected vaccinee would be minimal. The transmission-blocking vaccine (TBV) candidate Pvs25 entered clinical Phase I to assess its safety and immunogenicity as a recombinant protein adjuvanted with montanide ISA 51. In parallel, the TBV candidate for *P. falciparum* Pfs25 was also tested. However, the trial was interrupted due to unexpected reactogenicity (Wu et al., 2008). Interestingly, a trial on non-human

primates aimed at improving Pfs25 immunogenicity by using encapsulation in synthetic vaccine particles (SVP) of approximately 100nm and potent TLR-based adjuvants. SVP formulations rendered substantial antigen-specific CD4+ T-cells (Thompson et al., 2018).

A recent report has explored the functionality of a chimeric PvMSP1 protein, an erythrocytic-stage antigen, genetically conjugated to the TBV candidate Pvs25 in a murine model (McCaffery et al., 2019). In this study, it was shown that immunization with this multi-stage chimeric protein elicited antibodies that recognized both erythrocytic and sexual stages and blocked the transmission of *P. vivax* isolates indirect membrane-feeding assays. This represents a potential strategy to control malaria transmission while targeting the stages responsible for clinical manifestations.

Even though we still lack a lot of understanding in immune responses to malaria and there are many obstacles to overcome in the development of vaccines, there are still many potential approaches to be explored. In a recent publication, Beeson and collaborators have extensively reviewed those challenges and have clearly summarized the potential strategies to generate highly efficacious and long-lasting vaccines in malaria. Among them, targeting key epitopes, maximization of activity of functional antibodies, maximization of T-cell function, optimization of adjuvants and dosing as well as the use of alternative vaccine platforms are on the front line of approaches to be pursued (Beeson et al., 2019).

1.2 Extracellular Vesicles

Extracellular vesicles (EVs) are small membranous vesicles that can originate from any type of cell and are present in every body fluid. EVs are very heterogenous, but still two main subsets have been defined: exosomes, which have an endocytic origin and a smaller size (50-150nm) and microvesicles (MVs), which bleb directly from the plasma membrane and have a larger size (usually 50-500nm) (Yáñez-Mó et al., 2015). EVs play a plethora of different roles. Seminal studies of Johnstone *et al* (Johnstone, Adam, Hammonds, & Turbide, 1987) and Harding *et al* (Harding et al., 1983) on *Rex* attributed a cargo-disposal function to these vesicles. Years later, works from Raposo *et al* showed how B-cell-derived exosomes have an antigen-presenting capacity (Raposo et al., 1996). Valadi *et al* showed for the first time that mRNA and microRNA present on EVs could be transferred and translated in target cells (Valadi et al., 2007), raising awareness about their enormous potential in inter-cellular communication. Reports in this last decade have pointed out the dual role of EVs to contribute either to immune defenses or immune evasion during cancer or infections, opening novel avenues to use EV-based therapeutic approaches to modulate the immune system (Théry, Ostrowski, & Segura, 2009).

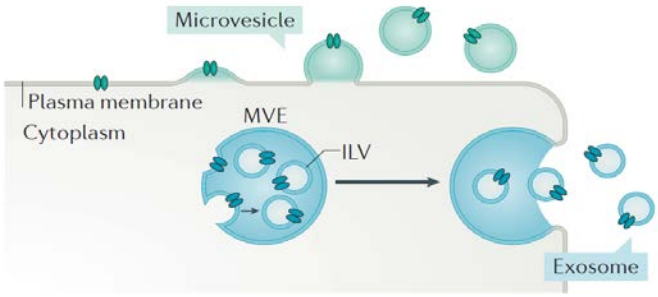


Figure 1.9 **Biogenesis of extracellular vesicles.** Source of figure: Van Niel, D'Angelo, and Raposo, 2018. Reprinted with permission.

1.2.1 *EVs Biogenesis and Composition*

Most of the studies on EVs have specialized in their potential roles rather than studying their origin. Currently, the EV field is confounded by the lack of gold-standard methods to identify and isolate the different subtypes of vesicles. Therefore, we normally deal with heterogenous EV populations where we do not know which specific subpopulation(s) is(are) responsible for a given effect (Tkach, Kowal, & Théry, 2018).

Intraluminal vesicles (ILVs) included in multivesicular bodies (MVEs) are released to the extracellular milieu once MVEs are fused to the plasma membrane. ILVs are then recognized as exosomes. However, MVs are generated as an outward budding of the plasma membrane (see Figure 1.9). Processes involved in the formation of exosomes and MVs include differential elements and common mechanisms. In both cases, vesicle cargo would be clustered to microdomains of the limiting membrane of MVEs for exosomes, and the plasma membrane for MVs.

There are many membrane proteins cycling between the plasma membrane and the endosomal compartment. In differentiated cells where the recycling is impaired such as reticulocytes, we can find a large enrichment of plasma membrane components in exosomes (Blanc & Vidal, 2010). Additional machinery is normally required for the budding and the abscission of membranes. Different subunits of the endosomal sorting complex required for transport (ESCRT) (Hurley, 2008; Tamai et al., 2010) as well as accessory proteins such as Alix are usually involved (Zimmermann et al., 2005) (see Figure 1.10). However, ESCRT-independent mechanisms have been described during EV biogenesis (Stuffers, Sem Wegner, Stenmark, & Brech, 2009). In the case of exosomes, ceramide-enriched domains have been related to the spontaneous negative curvature on the membranes (Goñi & Alonso, 2009). Additionally, several members of the tetraspanin family such as CD9 have been associated with the

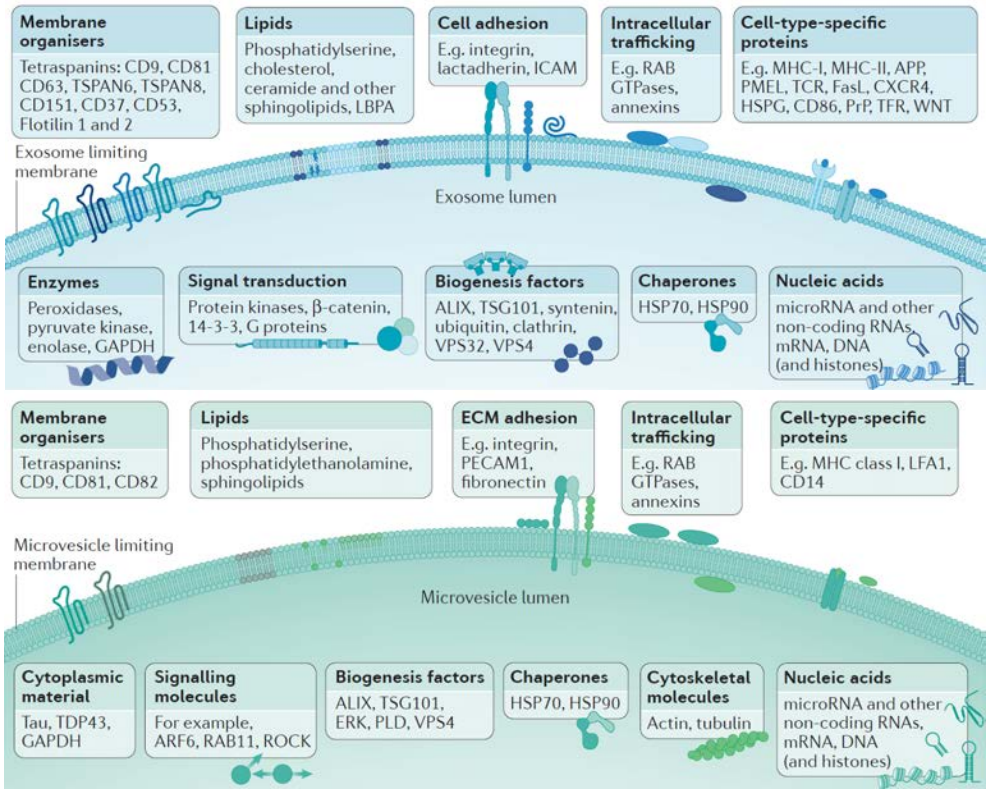


Figure 1.10 **Composition of extracellular vesicles.** Upper part of the figure represents the typical components of an exosome and the bottom part the ones of a microvesicle. Source of figure: Van Niel, D'Angelo, and Raposo, 2018. Reprinted with permission.

generation of dynamic domains and the direct sorting of cargo to exosomes (Buschow et al., 2009).

Apart from the mentioned mechanisms, targeting of selective membrane or cytosolic components to exosomes seems to rely on lipid rafts, sphingolipid-rich microdomains, and chaperones. Lipid rafts are enriched in GPI-anchored proteins such as CD55 or flotillins (Chatterjee, Smith, Hanada, Stevens, & Mayor, 2001). Chaperones like HSP70 contribute in the sorting of cytosolic proteins (Géminard, de Gassart, Blanc, & Vidal, 2004). However, evidence suggests that cargo-sorting mechanisms to EVs are dependent on multiple intrinsic factors (e.g., cell type, differentiation stage) as well as environmental conditions (Wolf & Casadevall, 2014).

The machinery of biogenesis of MVs has not been studied in depth. However, as we have described before, many mechanisms are known to be common to the ones involved in exosome biogenesis. Still, some unique flipping processes on certain lipids from the budding membrane that will originate MVs have been

reported (Al-Nedawi et al., 2008). Components of the cytoskeleton and their regulators have been shown to be important for MV biogenesis as well (B. Li, Antonyak, Zhang, & Cerione, 2012). Given the several common mechanisms in the biogenesis of exosomes and MVs, it is complicated to determine the origin of produced EVs and therefore, one should be careful when using this inconclusive nomenclature.

Tetraspanins, ESCRT machinery components, proteins involved in cell adhesion, proteins mediating intracellular trafficking, chaperones and metabolic and signaling enzymes are some of the proteins that have been reported in many types of EVs (Colombo, Raposo, & Théry, 2014). Apart from this general EV components, depending on the cell type from which they originate, EVs will contain specific components that would be accountable for specific functions. For instance, EVs originated from antigen-presenting cells such as DCs and B-cells contain MHC class I and II molecules (Clayton et al., 2019). Some tumor-derived EVs display metalloproteinases that promote metastasis (You et al., 2015).

In terms of lipids, EV membranes are enriched in cholesterol, sphingolipids, ceramides and phosphatidylserine compared to plasma membranes (Lai & Lim, 2019). However, the lipidic composition is less known in MVs (Raposo & Stoorvogel, 2013). The nucleic acids carried in EVs include RNAs, mRNAs and non-coding RNAs (including microRNAs) (Nolte'T Hoen et al., 2012; Valadi et al., 2007) and DNA (Thakur et al., 2014). miRNA with specific motifs have been shown to be specifically sorted to exosomes (Villarroya-Beltri et al., 2013) but the mechanisms of RNA loading into EVs are still unclear (Janas, Janas, Sapoń, & Janas, 2015). Some reports have claimed that EVs could include miRNA biogenesis and silencing machinery (Silva & Melo, 2015), which has been challenged in a recent work on the re-assessesment of exosome composition (Jeppesen et al., 2019). Of note, this report by Jeppesen *et al* has also shown that DNA and histones are secreted through an amphisome-dependent mechanism and are not carried on exosomes. This study has questioned as well whether glycolytic enzymes, chaperones or cytoskeletal proteins are truly associated to exosomes (Jeppesen et al., 2019).

EV composition is a constant matter of debate. In the first place, researchers might be dealing with different EV subsets since those subsets are not clearly defined. Therefore, populations of small EVs, including exosomes and other membranous particles, and larger EVs, including MVs, are urgently needed to be re-defined. For that, EV community requires novel studies addressing EV heterogeneity and looking for techniques of isolation and characterization able to distinguish those subpopulations.

1.2.2 *EVs Isolation and Characterization*

1.2.2.1 EVs Isolation

Differential centrifugation (dUC) has been the first method used to isolate EVs and is still the most widely used technique (Raposo et al., 1996; Théry, Amigorena, Raposo, & Clayton, 2006). In order to get small EVs (containing exosomes), culture supernatant or a body fluid must be previously centrifuged at ∼2,000×g and later, at ∼10,000×g to get rid of dead cells and cell debris, respectively. In some cases, this last centrifugation could be exchanged for (or combined with) a filtration through a 0.22μm filter to ensure the elimination of all particles above 200nm (Lässer, Eldh, & Lötvall, 2012). Eventually, the pre-cleared supernatant would be ultracentrifuged at ∼100,000×g for 1-2h to obtain a pellet of small EVs. The pellet should be washed with PBS and ultracentrifuged again (Théry et al., 2006).

Some contaminants (e.g., protein aggregates) might co-precipitate with the small EVs when using dUC. To prevent this, dUC coupled to fractionation over density gradients are becoming more and more broadly used. This high-resolution methodology does not only enable to separate EVs from non-vesicular components, but also to further characterize EV subsets (Aalberts et al., 2012). Several studies have used this technique to compare the composition of highly purified EV populations to provide a better understanding of EV subtypes (Jeppesen et al., 2019; Kowal et al., 2016). Although dUC with gradient is a powerful methodology to explore the features and functions of specific EVs' subpopulations, it is a labor-intensive and time-consuming technique that would yield purer, but very scarce EV material.

Immunoaffinity-based isolation methods are also gaining popularity for EV isolation, with even some ready-to-use kits already available (Pedersen, Kierulf, & Neurauter, 2017). Normally, they are based on antibodies against surface markers of the tetraspanin family (CD63, CD9, CD81) that can be immobilized in different carriers (e.g., magnetic beads, ELISA plates, microfluidic devices) (F. Yang, Liao, Tian, & Li, 2017; Yoo et al., 2012). One of the main drawbacks for these methods is the lack of a pan-EV marker. Comparative proteomic analysis over different immune-isolated CD63+, CD9+ and CD81+ small EVs has robustly shown how each isolated population has a different composition and that none of them represents the whole population of EVs (Kowal et al., 2016). Another important limitation is the contamination of the EV sample with isolating molecules. Without cleaning the EV sample from antibodies and their carriers, accurate functional assays would not be possible. Still, this methodology could be useful to explore specific EV populations with biomarker or therapeutic potential in the future, as well as for identifying molecular EV cargo.

Size exclusion chromatography (SEC) is also getting popular for EV enrichment since it is a scalable time- and cost-effective technique that provides high purity and yield of EVs (Monguió-Tortajada et al., 2019). Boing *et al* described the efficiency of SEC to enrich EVs from platelet-free supernatant of platelet concentrates and separate them from soluble contaminants (Boing et al., 2014). SEC has been also adopted in EV isolation from urine (Lozano-Ramos et al., 2015), plasma (de Menezes-Neto et al., 2015) and cell culture supernatant (Monguió-Tortajada et al., 2017). The isolation of EVs by SEC is based on a difference in size. The column used in SEC contains a stationary matrix made of sepharose beads where EVs cannot enter and would have to elute through the void volume fluid instead. Bigger vesicles would elute faster than smaller ones. However, smaller particles, such as soluble proteins, can enter the sepharose beads and would elute much later due to their increased path.

So far, we have a wide variety of methods to isolate EVs. Each one with its advantages and drawbacks, which makes it difficult to establish a "gold-standard" technique. Different EV isolation methods will yield different EV populations with different properties. For the analysis of EV composition, differential centrifugation with gradients have been proven to render pure small EV populations and enable to finely distinguish the proteins and RNAs associated to the vesicles (Jeppesen et al., 2019; Kowal et al., 2016). Of note, most of those studies have been performed over cell culture supernatants, likely with large volumes of starting material. To what extent these studies can be extrapolated to situations with limited starting volume, for instance to isolate EVs from body fluids of patients of any condition, remains to be assessed. Regarding functional EV studies, it is not clear if methods that alter the biophysical properties of EVs by addition of chemicals or application of strong forces during ultracentrifugation (UC) might alter the function of the vesicles. Some works have already described how EVs differ in functionality only due to the choice of EV isolation technique (Mol, Goumans, Doevendans, Sluijter, & Vader, 2017). Therefore, for the best choice of EV purification method, among other considerations, one should always bear in mind the objective of the study of EVs.

1.2.2.2 EVs Characterization

Fine characterization of EVs is crucial to determine the nature of isolated EVs and unravel their potential biological functions and translationality into biomedical interventions. Both biochemical and physical technologies are currently used for EV analysis.

One of the most important properties to be measured in EVs is their size. Nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) and electron microscopy (EM) are the most frequently used techniques to measure

the size of EVs (Hartjes, Mytnyk, Jenster, van Steijn, & van Royen, 2019). NTA measures the size of nanoparticles in a liquid suspension by tracking individual particles scattering light under laser illumination and undergoing Brownian motion. A camera records the path of each particle and such path is used to determine its diameter. Since this technique tracks every individual particle in a known volume, both size distribution and particle concentration can be determined (Vestad et al., 2017). DLS is similar, but measures the bulk scattered light from particles in Brownian motion (Stetefeld, McKenna, & Patel, 2016). EM is the preferred method to visualize EVs despite the requirement of specialized equipment and personnel. Apart from the size, morphology and integrity of EVs can be assessed in a straightforward manner (Linares, Tan, Gounou, & Brisson, 2017).

Analysis of the protein composition is one of the ways to characterize EVs. The protein content of EVs has been determined using standard colorimetric assays, such as bicinchoninic acid (BCA) or Bradford assays, and further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein staining or immunoblotting for the detection of EV-related proteins (e.g., tetraspanins, annexins). Other analysis have employed as well immunosorbent assays using the affinity of antibodies against EV proteins in order to detect specific EVs (Duijvesz et al., 2015) and even microfluidics-based on-a-chip systems for both EV isolation and analysis (Guo, Tao, & Dawn, 2018).

Flow cytometry is a powerful technique to characterize single cells. However, most of the conventional cytometers are not able to resolve particles with a diameter below 500nm (Van Der Pol, Van Gemert, Sturk, Nieuwland, & Van Leeuwen, 2012). To overcome this limitation, highly sensitive flow cytometers are being developed to discriminate particles up to 100 nm in diameter (Stoner et al., 2016). However, most of the laboratories do not have this specialized equipment. One of the most used approaches to circumvent the lack of highly sensitive flow cytometry instruments is bead-based flow cytometry (Théry et al., 2006). In this approach beads of large size would trap the EVs through non-specific adsorption. The bead-coupled EVs could be recognized by antibodies against specific markers and analyzed following highly standardized cytometry procedures. This method does not enable the analysis of single EVs, but it is still very informative to characterize the surface components of EV populations.

Mass spectrometry (MS)-based proteomics has become a popular technique in the analysis of EVs since it enables a high-throughput molecular profiling of the vesicles (Pocsfalvi et al., 2015). Most of the works have been performed according a bottom-up strategy, where samples undergo enzymatic digestion prior to injection on the spectrometer (Abramowicz, Widlak, & Pietrowska, 2016). Currently, the preferred technique to identify proteins in EVs is the liquid chromatography tandem mass spectrometry (LC–MS/MS) (Kreimer

et al., 2015). This method has enabled the detection of hundreds and thousands of proteins associated to EV samples from specific cell types or body fluids and has an invaluable potential to contribute to the understanding of EV functions and the discovery of EV-associated biomarkers.

1.2.3 *EVs and Immune Responses*

EVs released by both immune cells and non-immune cells have relevant roles in the immune regulation. Mechanisms by which EVs might mediate the immune response are very varied. Multiple reports have described the action of EVs over the innate immune system (Groot Kormelink, Mol, de Jong, & Wauben, 2018). Other works have focused in the contribution of EVs to acquired immune responses (Robbins & Morelli, 2014). Interestingly, EVs have been shown to exert both immunostimulatory or immunosuppressive effects, thus having a tremendous potential for positive or negative vaccination approaches.

1.2.3.1 EVs in Antigen Presentation to T-cells

Raposo and colleagues showed for first time the presence of MHC class II molecules in EVs. The EVs derived from Eipstein-Barr Virus (EBV)-transformed B-cells contained peptide–MHC class II complexes that could present antigens to T-cells (Raposo et al., 1996). Later, the antigen-presenting capacity of EVs was studied on dendritic cell-derived EVs. Zitvogel *et al* showed how DC-derived EVs bearing tumor peptide–MHC class I complexes promoted antitumor cytotoxic T-cell responses in a mouse model (Zitvogel et al., 1998). These investigations prompted the study of EVs in T-cell activation.

EVs containing MHC class I and II molecules maintain the same topology of their parent cells, and therefore, these surface complexes can stimulate CD8+ or CD4+ T-cells. EVs derived from DCs loaded with peptides from EBV, cytomegalovirus and influenza have been shown to directly stimulate CD8+ T-cells *in vitro* (Admyre, Johansson, Paulie, & Gabrielsson, 2006). However, it should be noted that those were probably memory T-cells. Free EVs might be able to function as APCs for primed T-cells or antigen-specific T-cell clones, but given their weak stimulatory capacity, it seems very unlikely they would directly stimulate naive T-cell responses (Robbins & Morelli, 2014).

It has been demonstrated that the capacity of EVs to stimulate T-cells relies on the intervention of APCs, such as DCs (Théry et al., 2002) (see Figure 1.11). Integrins and adhesion molecules present in EVs might facilitate their binding to DCs. Indeed, the upregulated expression of intercellular adhesion molecule-1 (ICAM-1) in EVs released from LPS-maturated DCs has been associated with their increased stimulatory function (Segura, Nicco, et al., 2005). Other components typically found on EVs such as tetraspanins or phosphatidylserine

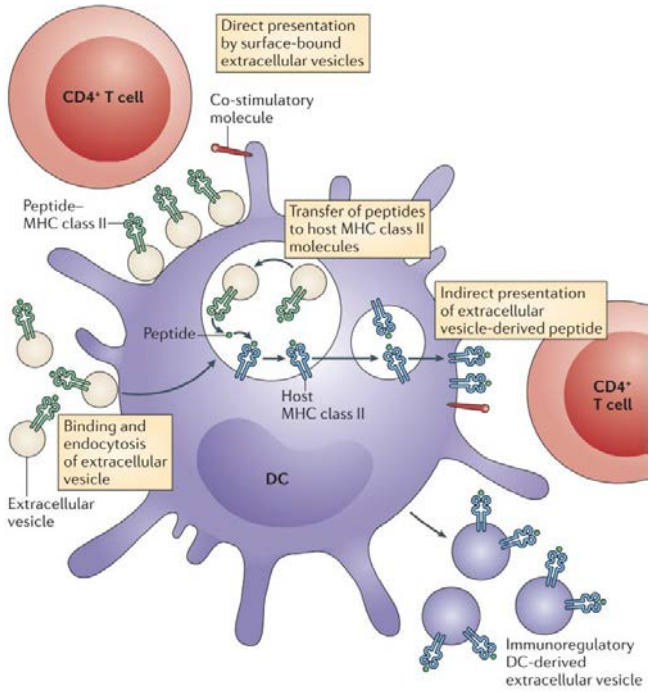


Figure 1.11 **Role of EVs in presentation to T-cells.** The presentation mechanisms depicted for MHC class II complexes can also occur for the MHC class I molecules, therefore regulation of CD8+ T-cells is also possible. Source of figure: Morelli et al., 2004. Reprinted with permission.

might contribute to their increased binding to DCs (Morelli et al., 2004).

Whereas some of the EVs might remain attached to the surface of the APCs, others might be internalized. Once EVs are internalized, peptide-MHC complexes carried on the EVs might be degraded and their peptides might be transferred to APCs' MHC class molecules to be indirectly presented to T-cells. Peptide-MHC complexes of EVs retained on the surface of the APCs might be directly presented to T-cells using the co-stimulatory molecules of the acceptor APCs. The passage of intact preformed peptide–MHC from one cell to other without further antigen processing for T-cell recognition is termed cross-dressing. Cross-dressing of peptide-MHC complexes already occurs between DCs, as demonstrated by *in vitro* experiments where DCs where shown to acquire peptide-MHC complexes from allogeneic DCs (Herrera et al., 2004). EVs relevance in cross-dressing was highlighted when their binding to DCs increased the stimulatory capacity, even for the priming of naive T-cells (Segura, Amigorena, & Théry, 2005; Théry et al., 2002).

1.2.3.2 EVs as Antigen Carriers

EVs can naturally carry antigens from the parental cells they derive from.

The cancer field pioneered the search of novel antigens in EVs derived from tumor cells (Wolfers et al., 2001). Years later, microbial components have been identified in EVs derived from infected cells during viral, parasitic, fungal and bacterial infections (reviewed in (Jeffrey Sean Schorey, Cheng, Singh, & Smith, 2015)). During infections, EVs contain microbial components that represent an alternative source of pathogen antigens. This might result on the promotion of both the innate and the acquired immune response.

Mycobacterial components were found in EVs released from *Mycobacterium avium*-infected macrophages (Bhatnagar & Schorey, 2007). These vesicles as well as EVs from *M. tuberculosis* and *M. bovis* BCG-infected macrophages could induce a proinflammatory response in resting macrophages (Bhatnagar, Shinagawa, Castellino, & Schorey, 2007). In addition, EVs derived from *Mycobacterium*-infected cells could activate antigen-specific CD4+ and CD8+ T-cells as well as induce the activation and maturation of bone marrow-derived DCs (Giri & Schorey, 2008).

With relevance for malaria field, the work by Martin-Jaular *et al* described parasite proteins in circulating EVs isolated from *P. yoelii*-infected mice and showed how exosomes derived from infected reticulocytes used in vaccination of naive mice could provide protection against a challenge with lethal strain of *P. yoelii* (Martin-Jaular et al., 2011). Other works in malaria field have described the presence of parasite proteins in EVs from *Pf*-infected RBCs (*Pf* iRBCs-EVs) (Mantel et al., 2013; Sampaio, Cheng, & Eriksson, 2017) and highlighted their proinflammatory properties (Mantel et al., 2013). However, parasite components present on EVs could also lead to the suppression of the immune response. In a recent study, it was shown that EVs from *P. berghei*-infected RBCs could suppress CD4+ T-cell responses due to the presence of virulence factors, histamine releasing factor and elongation factor 1 alpha, on those vesicles (Demarta-Gatsi et al., 2019). Further studies need to define how immune responses might change depending on the type and cargo of EVs released during malarial infections.

1.2.3.3 EVs in Vaccination Approaches

Given the potential of EVs to modulate immune responses, several works have investigated their role as vehicles for vaccine delivery. In the cancer field, EVs released from DCs that had been pulsed with tumor antigens or with tumor-derived EVs have been explore as vaccines to facilitate the recognition and killing of tumor cells (André et al., 2004; Liu et al., 2018). Indeed, several phase I or II clinical trials based on DC-derived EVs been performed to assess their potential in immunotherapy to treat different cancers (Besse et al., 2016; Dai et al., 2008; Morse et al., 2005). Although DC-derived EVs acted as potent stimulators for antigen-specific T-cell responses in preclinical

studies (Zeelenberg et al., 2008), those responses were not efficiently increased in patients. Instead, IFN-γ matured DC-derived EVs boosted activation of natural killer cells, which was later associated with longer survival (Besse et al., 2016). Despite the drawbacks, results from this first phase II clinical trial encourage further research on EVs for their use in cancer immunotherapy.

EV-based immunization strategies have been also pursued in infectious diseases. EVs derived from *Toxoplasma gondii* antigen-pulsed DCs contained parasite antigens and were used to effectively immunize naive mice against *T. gondii* infection (Aline, Bout, Amigorena, Roingeard, & Dimier-Poisson, 2004; Beauvillain, Ruiz, Guiton, Bout, & Dimier-Poisson, 2007). Similarly, EVs from antigen-loaded DCs were shown to mediate protective immunity against cutaneous leishmaniasis (Schnitzer, Berzel, Fajardo-Moser, Remer, & Moll, 2010). In addition, EVs isolated from *M. tuberculosis* culture filtrate protein-treated macrophages protected mice against *M. tuberculosis* infection (Cheng & Schorey, 2013). *In vivo* studies on Rab27a-deficient mice with diminished trafficking of mycobacterial components to EVs have demonstrated that EV release during *M. tuberculosis* infection significantly contributes to T-cell responses (Smith, Cheng, Bryant, & Schorey, 2017).

Altogether, these findings support the importance of EVs in antigen presentation and acquisition of adaptive immunity. Even if many mechanistic aspects of EVs role in immunity remain to be defined, their potential for the development of novel therapeutic approaches is now generally acknowledged.

1.2.4 *EVs in Malaria Infections*

EV release has been documented in multiple infections. In infected organisms, EVs have been shown to carry signals from both pathogens and hosts, enabling extracellular routes of communication among pathogens and between the pathogens and their hosts (Jeffrey Sean Schorey & Harding, 2016). This transfer of components has been hypothesized to be a major contributor in host-pathogen co-evolution (Barteneva, Maltsev, & Vorobjev, 2013).

EVs in infections might play opposing roles. In some contexts, EVs have been shown to promote immune responses that would protect the host (Aline et al., 2004; Bhatnagar et al., 2007; Martin-Jaular et al., 2011) while in other contexts, they seem to favor the survival and spread of the pathogens (Abrami et al., 2013; Cestari, Ansa-Addo, Deolindo, Inal, & Ramirez, 2012). Both phenomena might be occurring in the course of an infection depending on the type of pathogen, pathogen life-stage, and/or environmental specific conditions.

Production of EVs by parasites and parasitized-cells has been described in infections such as toxoplasmosis, trypanosomiasis, leishmaniasis and malaria, which affect millions of people worldwide. Indeed, the number of EV-related studies is rapidly growing for protozoan parasites such as malaria (Marcilla et al., 2014).

Importantly for the malaria field, the seminal electron microscopy study of Aikawa *et al* on *P. vivax*-infected RBCs revealed the presence of caveola-vesicle complexes throughout the erythrocyte plasmalemma back in 1975 (Aikawa et al., 1975). About two decades later, Olliaro and Castelli shown similar caveolae containing grape-like vesicular structures in *Plasmodium falciparum* parasites. These vesicles had a diameter ranging 80-100nm (Olliaro & Castelli, 1997). While these vesicular complexes seem to represent the origin of EVs in malaria parasites and malaria-parasitized RBCs, the mechanisms of biogenesis of EVs in these peculiar cells remain unknown.

In the last years multiple works have highlighted the relevance of EVs regarding malaria pathogenesis and inter-cellular communication studying natural malaria infections and using different experimental models (Sampaio et al., 2017).

Initial studies focused mainly on the association of microvesicles and malaria severity. Circulating MVs in children infected with *P. falciparum* malaria were shown to be associated with severe cerebral malaria (Combes et al., 2004). In this study, Combes *et al* described increased levels of endothelial-derived MVs during the infection. The same group found out that knocking out ABCA1 gene in a murine model diminished MV production and protected mice from cerebral malaria (Combes et al., 2005). ABCA1-/- mice had lower levels of TNF in serum and reduced platelet and leukocyte sequestration in the brain compared to control mice. Recently, a study of the fate of MVs in an *in vivo* model of cerebral malaria demonstrated that the presence of MVs in the brain was dependent on the infection (El-Assaad, Wheway, Hunt, Grau, & Combes, 2014). Therefore, these studies suggest that MV contribution to cerebral malaria require the presence or interaction of iRBCs with the endothelium for the development of neuropathology. Altogether, these studies remarked the potential pathogenic role of MVs in cerebral malaria.

EV release has been reported in several *Plasmodium* species that affect humans. Nantakomol *et al* quantified circulating red-cell derived MVs in field human samples from Thailand (Nantakomol et al., 2011). Most of the samples came from patients infected with *P. falciparum*, but there were also samples coming from *P. vivax* and *P. malariae* infections. In agreement with previous works, they observed an increased concentration of red cell-derived MVs in patients than in healthy donors. In this study the discriminated MVs coming from iRBCs based on the detection of parasitic ring-infected erythrocyte surface antigen (RESA). They showed a ten-fold increase of iRBCs compared to uninfected RBCs, which suggests an active release of MVs from iRBCs. The authors also pioneered the study of *P. falciparum* MVs release *in vitro* and showed that MV production augmented as the parasites matured.

Studies in Cameroon and India have reported as well increased levels of plasma-derived MVs originating from platelets, erythrocytes and endothelial

cells during active *P. falciparum* infections (Pankoui Mfonkeu et al., 2010; Sahu, Sahoo, Kar, Mohapatra, & Ranjit, 2013). Interestingly, in the study in Cameroon, platelet-derived EVs were particularly associated with severity in cerebral malaria (Pankoui Mfonkeu et al., 2010). In the study in India, the authors reported a positive correlation between levels of MVs and serum TNF (Sahu et al., 2013). During a field study of *P. vivax* infections in Brazil, Campos *et al* have also reported augmented levels of circulating MVs from platelets, erythrocytes and leukocytes. Remarkably, they found an association of the amount of platelet-derived EVs with the length of acute illness and fever at the time of collection (Campos et al., 2010). All these reports highlighted the potential contribution of host-derived MVs to malaria pathogenesis.

Couper *et al* conducted a crucial study to understand the role of MVs in pro-inflammatory responses during malaria infection (Couper et al., 2010). They showed that plasma-derived MVs from *P. berghei*-infected mice could induce remarkable immune responses via macrophage activation, promoting CD40 expression on their surface. These inflammatory MVs were derived from parasitized RBCs and contain parasite materials. Interestingly, the MV-mediated macrophage activation is MyD88 and TLR-4 dependent, which is distinct from other reported pathways. The authors already remarked the potential of this response to contribute to severe disease through systemic inflammation, but also to generate malaria adaptive immune responses.

The first report on malaria that focused on the study of EVs of smaller size was carried out on mice infected with *Plasmodium yoelii* 17X, a reticulocyte-prone parasite like *P. vivax* (Martin-Jaular et al., 2011). Martin-Jaular *et al* demonstrated the presence of exosome markers and parasite proteins in the circulating EVs of *P. yoelii*-infected animals. Immunizations of mice with circulating EVs from infections or with exosomes derived from *in vitro* cultures of *P. yoelii*-infected reticulocytes elicited IgG antibodies capable of recognizing *P. yoelii*-infected cells. Moreover, when exosomes derived from *P. yoelii*-infected reticulocytes were used in CpG-adjuvanted immunizations, they conferred a long-lasting protective immune response against lethal infections in close to 85% of the animals.

EV research has profited enormously from the continuous *in vitro* culture of *P. falciparum* blood-stages to investigate the role of *Pf*iRBCs-EVs (Sampaio et al., 2017). Unfortunately, since a continuous *in vitro* culture system has not been developed yet for *P. vivax* (Noulin et al., 2013), the study of the EVs produced by this parasite, as the rest of its biology, represents a challenging task.

Mantel *et al* provided a detailed proteomic composition and function analysis of *Pf*iRBCs-EVs (Mantel et al., 2013). Their proteomics analysis over *Pf*iRBCs-EVs revealed a particular increase of parasite proteins associated to host membranes and parasite proteins involved in invasion. Interestingly, in

a kinetic experiment from highly synchronized parasites, the authors demonstrated the peak of EV release in late parasite stages, before parasite egress. They observed that *Pf*iRBCs-EVs could be internalized by infected RBCs and could promote gametocyte production. In addition, the authors demonstrated that *Pf*iRBCs-EVs could stimulate the innate immune system. Concretely, they showed that *Pf*iRBCs-EVs induced upregulation of activation markers CD54, CD40 and CD86 in monocytes, activated the release of pro-inflammatory cytokines by macrophages and promoted neutrophil migration. Therefore, *Pf*iRBCs-EVs represent a major mediator for communication within the parasite population and between the parasite and the immune system (Mantel et al., 2013). In the same year, evidence from the study of Regev-Rudzki *et al* reinforced the crucial role of EVs in intercellular communication (Regev-Rudzki et al., 2013). Regev-Rudzki *et al* used two transgenic parasite strains, that contained plasmids with resistance genes for a concrete drug, blasticidin or WR99210. When these two lines were cultured individually, they could not survive the dual treatment. However, when the lines were cultured in a transwell system, parasites were able to survive and the next generation of parasites contained both drug resistant genes, thus demonstrating that the DNA transfer was mediated by EVs. In parallel to the EV-mediated gene transfer, they observed an increased gametogenesis *in vitro* (Regev-Rudzki et al., 2013), which is in agreement with the findings of Mantel *et al* (Mantel et al., 2013). Further studies by Mantel *et al* demonstrated that *Pf*iRBCs-EVs contained miRNA species and human Argonaute 2 protein that form a functional complex. The authors showed that these EVs were taken up by endothelial cells and modulated their barrier properties (Mantel et al., 2016).

In addition, it has been shown that *Pf*iRBCs-EVs derived from ring-stage parasite contained parasite genomic DNA that upon internalization by human monocytes could activate cytosolic immune sensors. The authors hypothesized that this EV-mediated DNA-sensing pathway might contribute to parasite virulence (Sisquella et al., 2017). However, it might also represent a host detection mechanism and trigger for anti-parasite immunity. Another work on EVs derived from early stage *P. falciparum* iRBCs showed that these vesicles present the parasite virulence factor PfEMP1 and induce transcriptional changes in human monocytes (Sampaio et al., 2018).

Recently, the development of liver-humanized mouse model able to sustain pre-erythrocytic stages of *P. vivax* has enabled a pioneering study of EVs during vivax liver infection. Remarkably, vivax proteins were identified in plasma-derived EVs of *P. vivax*-infected liver-humanized mice, thus opening new avenues in the search of biomarkers for vivax liver infection, including hypnozoites (Gualdrón-López et al., 2018).

Altogether, these reports highlight EVs as relevant communicators on parasite-host interactions during both human malaria infections and murine

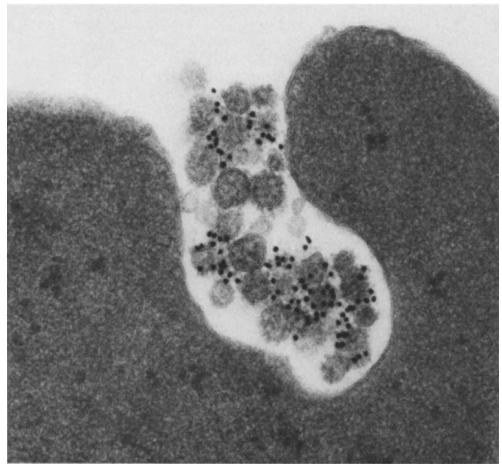


Figure 1.12 **Release of reticulocyte-derived exosomes visualized by electron microscopy.** Source of figure: Pan, Teng, Wu, Adam, and Johnstone, 1985.https://creativecommons.org/licenses/by-nc-sa/4.0/

models. Even though the study of EVs released *in vitro* from *P. vivax*-infected cells is limited, it would be feasible and relevant to investigate circulating EVs from *P. vivax* infections to understand their potential roles on the disease.

1.3 Reticulocyte-derived Exosomes: an EV-based Vaccine against Malaria

Reticulocytes were the object of study of two independent groups in the early 1980s (Harding et al., 1983; Pan & Johnstone, 1983). Both groups were interested on understanding how the transferrin receptor (TfR) was lost from reticulocytes during their maturation to erythrocytes. This curiosity led them to the discovery of nanovesicles of endocytic origin that were then termed exosomes (Johnstone et al., 1987). The release of these vesicles was clearly observed using immune-electron microscopy against the TfR (Pan, Teng, Wu, Adam, & Johnstone, 1985). They could observe endocytosis of the receptor, followed by budding into sac-like structures (currently known as multivesicular bodies). The small "buds" within the sac-like structures had approximately 50nm that kept bearing the label. Later, the sac-like structures fused with the plasma membrane and released the small intraluminal vesicles (exosomes) (see Figure 1.12).

1.3.1 *Rex as Cargo-disposal Mechanism in Reticulocyte Maturation*

Reticulocytes require the elimination of all membrane-bound organelles and ribosomes as well as extensive membrane remodeling to fulfill maturation to

erythrocytes (Ney, 2012). Exosome release largely contributes to the changes required in the reticulocyte membrane (Blanc & Vidal, 2010). In this process, reticulocyte cargo can be sorted to exosomes through ESCRT machinery, lectin clustering or association to lipid raft domains (Blanc & Vidal, 2010). Apart from TfR, components sorted into *Rex* include enzymes, adhesins, multiple salt, glucose and amino acid transporters as well as water channels such as aquaporin 1 (Johnstone et al., 1987; Vidal, 2010). More recently, MS-based proteomic analysis of *Rex* from phenylhydrazine-treated rats reinforced the view that *Rex* contain selective cargo (Carayon et al., 2011).

From these studies exosome release in reticulocytes has been traditionally considered a cargo-disposal mechanism to get rid of capacities that would be unnecessary and even pathological in mature erythrocytes (Vidal, 2010). However, given the huge potential that EVs have, it would be worthy to re-evaluate their role of *Rex* not only in erythropoiesis but also in reticulocyte-related pathologies such as vivax malaria.

1.3.2 *Rex in Reticulocyte-prone Malaria Infection*

Reticulocytes are the cells preferentially, if not exclusively, invaded by parasites such as *Plasmodium vivax* or the non-lethal *Plasmodium yoelii* 17X strain. Martin-Jaular *et al* studied exosomes in reticulocyte-prone infections in order to determine if in addition to their cargo-disposal role, *Rex* could contain parasite proteins and contribute to immune modulation (Martin-Jaular et al., 2011). In this study, the role of *Rex* in Balb/c mice infected with reticulocyte-prone *P. yoelii* 17X was explored.

Firstly, protein composition of circulating EVs from mice infected with *P. yoelii* 17X at two weeks post-infection, when reticulocytosis reached 60–90%, was analyzed. Flow cytometry analysis of several cell-specific markers revealed that most of the circulating EVs in these infected animals derived from reticulocytes. A MS-based proteomics analysis over these EVs identified several murine proteins previously identified in *Rex* such as aquaporin-1 or TfR. Moreover, parasite proteins were identified as well, including serine-repeat antigens, merozoite surface proteins 1 and 9, heat-shock proteins, and hypothetical proteins. Altogether these results suggested that circulating EVs in this reticulocyte-prone model contain parasite proteins likely derived from infected reticulocytes.

Immunization with circulating EVs isolated from mice infected with *P. yoelii* 17X elicited IgG antibodies capable of recognizing infected RBCs and attenuated the course of parasitemia of mice subsequently challenged with the lethal *P. yoelii* 17XL strain. In order to confirm that exosomes derived from infected reticulocytes are the ones responsible for the modulation of the course of infection, *Rex* were isolated from the supernatant of *in vitro* cultured *P. yoelii*

17X-infected reticulocytes. Remarkably, immunization with these exosomes rendered same results as those observed in immunizations with circulating EVs, but with higher survival times. Moreover, immunizations with *Rex* derived from *P. yoelii*-infected reticulocytes in combination with CpG-oligodeoxynucleotides conferred a long-lasting protective immune response against a lethal challenge with *P. yoelii* 17XL in close to 85% of the tested mice (Martin-Jaular et al., 2011).

In a more recent study, the mechanisms associated with protection elicited by CpG adjuvanted *Rex* from malaria infection were investigated (Martín-Jaular et al., 2016). A MS-proteomics analysis was performed over *Rex* derived from *P. yoelii*-infected reticulocytes to determine their protein composition. Parasite proteins as well as exosome markers and MHC class I molecules were detected.

Given the importance of the spleen in the control of malaria infection (Del Portillo et al., 2012; Engwerda et al., 2005), it was assessed if the spleen was required in the protective response. Protection mediated by immunization with CpG-adjuvanted *Rex* from malaria infection did not occur in splenectomized mice, but was restored after the transfer of splenocytes from immunized animals. Therefore, the spleen plays an essential role in the induction and/or effector functions required for this protective response. Immunization was associated with an increase of the proportion of memory CD4+ and CD8+ T-cells in the spleen, mainly with an effector memory phenotype. Importantly, T-cells elicited in the immunization did not express the exhaustion marker PD-1 whereas a high percentage of the T-cells from animals with self-resolved infection did express it. It might be possible that the presence of non-exhausted effector T-cells in the spleen could facilitate the mounting of protective responses in vaccinated animals. The transfer or depletion of specific T-cells subsets would be required to demonstrate whether those cells were responsible for the protection. In addition, to try to extrapolate these results to human *P. vivax* infections, human splenocytes were stimulated by plasma-derived EVs from vivax malaria patients and changes in spleen T-cell subsets were observed (Martín-Jaular et al., 2016).

These results encourage further studies on the involvement of *Pv*EVs, specifically *Rex* from infected reticulocytes, in the presentation of parasite antigens and the exploration of human *Rex* (*HuRex*) as a novel platform for vaccination against *P. vivax* malaria.

Chapter

2 Hypothesis and Objectives

This chapter presents the hypothesis of this thesis in Section 2.1 and the objectives addressed to explore it in Section 2.2.

2.1 Hypothesis

We hypothesize that EVs from vivax infections, particularly exosomes derived from *P. vivax*-infected reticulocytes, contain parasite proteins that serve as antigens and convey anti-malarial immune responses. We propose that these potential antigen-bearing vesicles are able to reach the human spleen to facilitate the presentation of antigens to immune cells. Therefore, reticulocyte-derived exosomes from vivax infections can be used in vaccine antigen discovery and human *Rex* represent a potential novel vaccine platform for eliciting immune responses against vivax malaria (see Figure 2.1).

2.2 Objectives

The main objectives of this thesis are: (i) the assessment of the potential of EVs from *P. vivax* infections as a source of antigens and as activators of immune responses, and (ii) the exploration of human reticulocyte-derived exosomes as a vaccine platform against *P. vivax* malaria.

The specific objectives addressed in this thesis are:

- Isolation and proteomic characterization of *Pv*EVs in order to discover potential antigens associated to these vesicles and gain insight on the

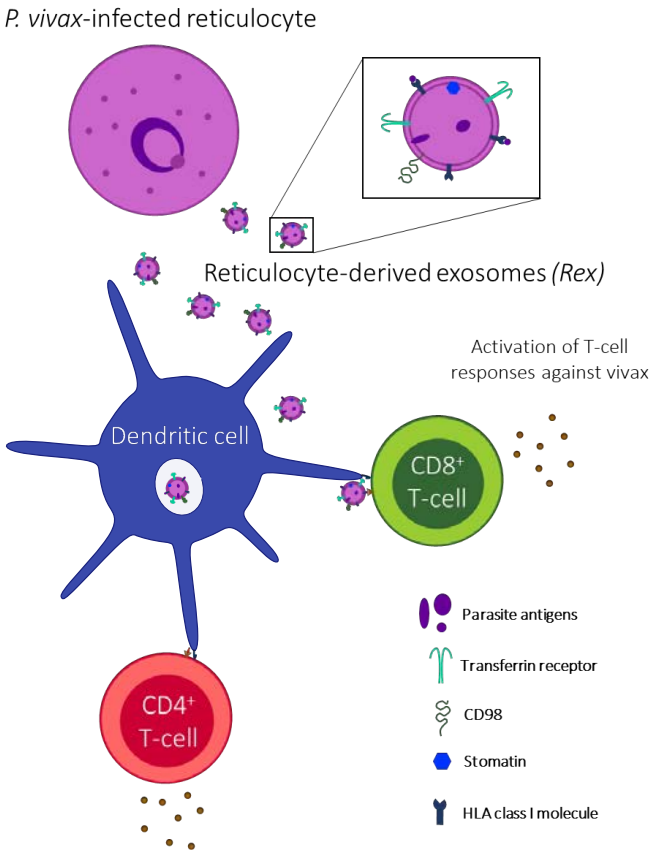


Figure 2.1 **Schematic representation of the hypothesis of this thesis.**

potential roles of *Pv*EVs during natural *P. vivax* infections. Findings on this matter will be presented in Chapter 4.

◇ Investigation of the interactions between immune cell populations from the human spleen and *Pv*EVs in order to assess the involvement of these vesicles in the initiation of splenic immune responses. Findings on this regard will be presented in Chapter 5.

◇ Implementation of an approach for assessing *in vitro* T-cell activation by EVs in order to evaluate the capacity of *Pv*EVs to stimulate T-cells from vivax exposed or naive individuals. This approach alongside some preliminary findings will be presented in Chapter 6.

◇ Isolation and proteomics analysis of *HuRex* and study of their interaction with antigen-presenting cells in order to explore their use as a vaccine platform. Findings on this subject will be presented in Chapter 7.

Chapter

3 Materials and Methods

This chapter describes the materials and methods used in the investigations performed during this thesis. Section 3.1 details the studied subjects and Section 3.2 describes the methodologies carried out.

3.1 Subject Details

Human studies were in accordance with the Good Clinical and Laboratory Practice Guidelines, the Declaration of Helsinki, and local rules and national regulations.

3.1.1 *Malaria Patients and Healthy Donors*

Samples from malaria patients with acute *P. vivax* infection were collected in two sites: Plasma samples from *P. vivax*-infected patients used in the studies described in Chapters 4 and 5 were collected at the Hospital of the Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (FMT-HVD) (Manaus, Amazonas, Brazil). Plasma and PBMCs from *P. vivax*-infected patients used in the experiments described in Chapter 6 were obtained at the E.S.E. Hospital San José de Tierralta (Tierralta, Córdoba, Colombia). Patients were tested for *Plasmodium* infection by thick smears. Patients recruited in Tierralta had parasitemias of 2500-5000 parasites per μL of blood. Under ten-year-old children and pregnant women were excluded from the study.

Plasma and/or PBMCs from healthy donors used in the studies described in Chapters 4,5 and 6 were collected at the Hospital Germans Trias i Pujol

(Badalona, Barcelona, Spain). Only healthy donors with no prior history of malaria were included in the study.

All studies were conducted with the expressed consent from the patients and donors and with approval of the local ethical committees of FMT-HVD, E.S.E. Hospital San José de Tierralta and Hospital Germans Trias i Pujol.

3.1.2 *Human Spleen Donors*

Human spleens used in the studies described in Chapter 5 were retrieved from deceased transplantation donors between 20-60 years old from the Transplant Program at the Hospital Germans Trias i Pujol (Badalona, Barcelona, Spain). Donation of these organs for biomedical research received written consent from family members and was in accordance with the protocol approved by the local ethical committee of the Hospital.

3.1.3 *Human Cord Blood Donors*

Human cord blood samples used in the studies described in Chapter 7 were obtained from the Blood and Tissue Bank of Barcelona (https://www.bancsang.net/). The protocol, including the informed consent forms, has been approved by the Clinical Research Ethics Committee of Vall d'Hebron University Hospital (Barcelona, Spain).

3.2 Method Details

3.2.1 *Blood Collection and Processing for Isolation of Plasma-derived EVs and PBMCs*

For studies described in Chapters 4 and 5, 3 mL of peripheral blood were collected by venipuncture in citrate pre-treated tubes from either *P. vivax* infected patients or healthy donors. Blood was centrifuged at 400×g for 10 min at RT. Plasma was collected and centrifuged at 2000×g for 10 min at 4ºC twice. Supernatant was recovered, aliquoted and frozen at -80ºC. Frozen plasma from patients were shipped to Germans Trias i Pujol Research Institute (IGTP) (Badalona, Barcelona) to continue the experiments.

For the studies of Chapter 6, requiring plasma-derived EVs and PBMCs, 10mL of peripheral blood from either vivax malaria patients or donors were collected as mentioned above, with minor modifications. Blood was centrifuged at 700×g for 15 min at RT. Plasma was collected and processed as previously described. Pelleted cells were further processed for PBMCs isolation as described in Section 3.2.17.

3.2.2 *Separation of Plasma-derived EVs by Size-exclusion Chromatography*

EVs were isolated from plasma samples of either *P. vivax*-infected patients or healthy donors by size-exclusion chromatography (SEC) under sterile conditions. Plasma was defrosted on ice, centrifuged twice at 2000×g for 10 min at 4ºC to pellet debris. Extensive passage of sterile and 0.22μm filtered 1X PBS was performed over commercial sepharose columns (Izon Science Ltd) or hand-made 10 mL-sepharose CL-2B (Sigma) columns, packaged as previously described in de Menezes-Neto et al., 2015. Once almost all the buffer eluted from the chromatography column, but preventing drying of the sepharose, 1mL of cleared plasma was loaded on the top of the column and 500 μL-fractions were immediately collected using sterile and 0.22 μm-filtered 1X PBS as elution buffer. Typically, 15 fractions were collected. Protein concentration of chromatographic fractions was measured by BCA assay (Thermo Scientific). Fractions were aliquoted and frozen at -80ºC until use.

Note that plasma-derived EVs used in MS-based proteomics studies described in Chapter 4, were obtained using 1X PBS prepared with LC-MS quality water (Sigma) and 1.5-mL Protein LoBind Tubes (Eppendorf) for the collection of fractions.

Plasma-derived EVs used in the studies described in Chapter 4 and 6 were obtained using commercial sepharose columns (Izon Science Ltd). Plasma-derived EVs used in the work presented in Chapter 5 were isolated using hand-made 10 mL-sepharose CL-2B (Sigma) columns.

3.2.3 *Purification of Human Reticulocytes*

Human cord blood samples used in the experiments described in Chapter 7 were processed similarly to a previous work (Russell et al., 2011). Briefly, 70 mL of cord blood were centrifuged at 1000×g for 15 min. After removal of plasma, the pelleted cells were washed and re-suspended at 50% HCT with RPMI-1640 medium (Sigma). Leukocytes and platelets were removed by filtration through columns of cellulose powder (Whatman), previously packed with 10 mL of cellulose, irradiated with ultraviolet light for sterilization and washed with a double volume of RPMI medium immediately before their use. Filtrated blood was washed twice with RPMI medium and adjusted to 50% hematocrit (HCT). 5 mL aliquots of blood at 50% HCT were carefully layered on 6 mL 70% Percoll (Healthcare). After centrifugation at 1200×g for 15 min at 20°C, concentrated reticulocytes in the Percoll interface were carefully collected and washed twice with RPMI medium. Schematic view of the process is depicted in Figure 3.1. Reticulocyte quantification was performed by examination of brilliant cresyl blue/Giemsa stained thin blood smears. Only samples with >20% reticulocytes and <2% leukocytes were used for *HuRex* production. We

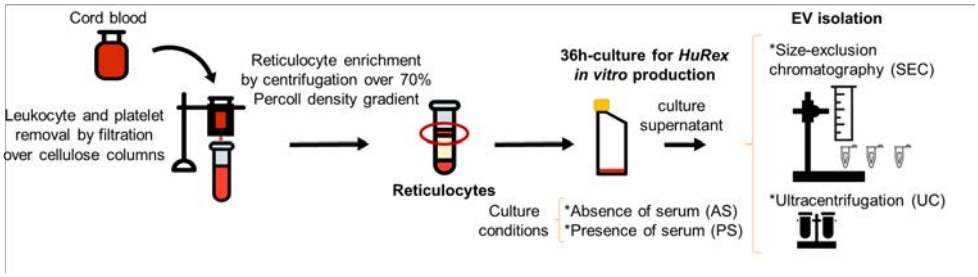


Figure 3.1 **Purification of human reticulocytes and *in vitro* production and isolation of *HuRex*.**

routinely obtained in the order of 10^8 reticulocytes per 70 mL of cord blood.

3.2.4 *In vitro Production and Isolation of HuRex*

Reticulocytes were cultured for 36 h at 37ºC and 1–3% HCT in RPMI medium, not supplemented or supplemented with 0.5% of human AB serum. Serum was previously depleted of exogenous EVs by ultracentrifugation at 100,000×g for 16h at 4ºC. Viability of reticulocytes after culture was assessed using Trypan Blue stain 0.4% (Sigma). Cell-free post-culture supernatants were collected by centrifugation at 1,300×g for 20 min at 20ºC. To isolate *Rex*, 2 mL of culture supernatants were isolated by SEC using hand-made 10 mL-sepharose CL-2B (Sigma) columns as previously described (see Section 3.2). Alternatively, 6–10 mL of culture supernatants were subjected to sequential centrifugation at 4ºC for 15,000×g for 45 min and UC at 100,000×g for 90 min (Théry et al., 2006), where EV pellets were resuspended in 200 μL of PBS. Protein content of SEC fractions and UC samples was determined by Nanodrop ND-1000 and BCA assay (Thermo Scientific).

3.2.5 *Bead-based Flow Cytometry of EVs*

EV-enriched SEC fractions were identified and molecularly characterized by bead-based flow cytometry (de Menezes-Neto et al., 2015; Monguió-Tortajada et al., 2019; Théry et al., 2006). Figure 3.2 depicts a graphical summary of the method. Each sample was diluted up to a volume of 45 μL and coupled to 5 μL of 1:10 pre-diluted solution of 4 μm-aldehyde/sulfate-latex beads (Invitrogen). PBS was added to the negative-control tube. Coupling incubation was performed for 15min at RT. Beads were then resuspended in 1 mL of bead-coupling buffer (BCB: PBS with 0.1% BSA and 0.01% NaN_3) and incubated overnight at RT on rotation. EV-coated beads were then centrifuged at 2,000×g for 10 min at RT and washed once with BCB prior to incubation with primary antibodies for 30 min at 4ºC. After washing with BCB, EV-coated beads were incubated for 30 min at 4ºC with secondary antibodies made in rabbit (R)

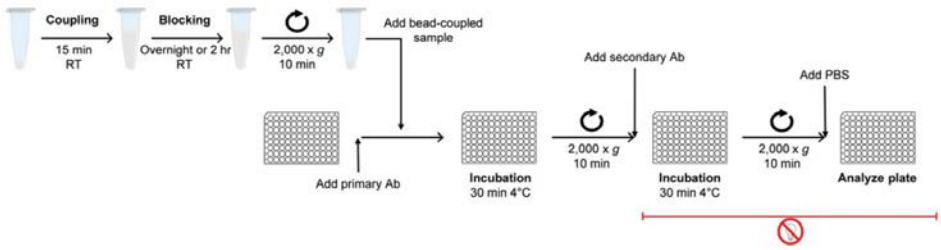


Figure 3.2 **Graphical summary of bead-based flow cytometry of EVs.** Source of the figure: Monguió-Tortajada et al., 2019. Reprinted with permission from John Wiley & Sons Lted.

or mouse (M). Table 3.1 shows the different antibodies and dilutions used to analyze the different EV samples by bead-based assay. Negative controls included coated beads incubated with secondary antibodies (CTRL B+2ary) and sample-coated beads only incubated with secondary antibodies (CTRL B+S+2ary). Labelled EV-beads were washed twice with BCB before being finally resuspended in PBS and subjected to flow cytometry (FacsVerse; BD). For each sample, 10^4 beads were acquired. FlowJo software was used to compare median fluorescence intensity (MFI) of EV-coated beads. Supplementary Figure A.1 shows the gating strategy followed for the analysis.

3.2.6 *Nanoparticle Tracking Analysis*

Size distribution and concentration of EVs were determined by NTA using a NanoSight LM10 instrument (Malvern Instruments Ltd) as described in de Menezes-Neto et al., 2015. Data were analyzed with NTA software (version 3.2 in studies described in Chapter 4 and version 3.1 in experiments shown in Chapter 7).

3.2.7 *Transmission Electron Microscopy*

TEM was performed over *HuRex* UC preparations negatively stained as previously detailed (Martin-Jaular et al., 2011). Briefly, 20 μL of an EV preparation were placed in a Cu-C 400 mesh grid and were treated with uranyl acetate and Reynolds lead citrate solutions for 5 and 1 min, respectively. Examination was performed on a Jeol JEM-1400 (Jeol Ltd, Tokyo, Japan) TEM equipped with a Gatan Ultrascan ES1000 CCD Camera. Size distribution from images was assessed by using the ImageJ software (NIH) and setting calibration at pixels/nanometer.

For immunogold labeling, samples were fixed with paraformaldehyde 4% (w/v) and glutaraldehyde 0.1% (v/v), placed on 200 mesh gold-grids, blocked with 1X PBS, 1% BSA solution (blocking solution) for 25 min and incubated with primary antibody anti-CD71 (Abcam, ab84036) at a 1:50 dilution in

EV sample	Primary antibodies			Secondary antibodies		
		reference	*dilution*		*reference*	*dilution*
Plasma-derived EVs	Mouse anti-CD81 (5A6)	hybridoma supernatant	1:10	Goat F(ab')2 Anti-Mouse IgG(H+L), Human ads, FITC	Southern Biotech, 1032-02	1:100
	Mouse anti-CD63 (TEA 3/10)	hybridoma supernatant	1:10			
	Mouse anti-CD9 (VJ1/20)	Immunostep, 9PU-01MG	1:500			
	Mouse anti-CD71 (MEM-189)	Abcam, ab1086	1:500			
	Mouse anti-HLA-ABC (W6/32)	Life Technologies, 14-9983-82	1:250			
	Rabbit anti-CD71	Abcam, ab84036	1:1,000	Goat anti-Rabbit IgG (H+L) Cross-Adsorbed, Alexa Fluor 488	Invitrogen, A11008	1:500
	Rabbit anti-CD5L	Abcam, ab45408	1:500			
Rex	Rabbit anti-CD71	Abcam, ab84036	1:1,000	Goat anti-Rabbit IgG (H+L) Cross-Adsorbed, Alexa Fluor 488	Invitrogen, A11008	1:100

Table 3.1 **Antibodies used in bead-based flow cytometry of EVs.**

blocking solution for 30 min. Later, grids were washed 4 times in 1X PBS, 1% BSA and incubated with secondary antibodies coupled to 10 nm gold particles at a 1:25 dilution in blocking solution for 30 min. Grids were washed 4 times with 1X PBS, 1% BSA and 5 times in deionized water and treated with uranyl acetate and Reynolds lead citrate solutions for 5 and 1 min, respectively, prior to TEM examination.

3.2.8 *Mass Spectrometry-based Proteomics*

For proteomic studies on plasma-derived EVs presented in Chapter 4, 200 μL aliquots of EV-enriched SEC fractions 7, 8 and 9 from *P. vivax* patients (N=10) or healthy donors (N=10) were pooled by individual (PV or HD).

EV-enriched pools were processed for MS proteomics analysis as detailed in Gualdrón-López et al., 2018. For prior EV solubilization and double protein digestion (Lys-C/trypsin) samples were processed as follows: EV pools were incubated at 70ºC with RIPA buffer [(50mM Tris pH 8, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 10mM MgCl2, 0.5mM DTT, 1:100 protease inhibitor cocktails (Thermo Scientific)] at an equal ratio (v/v). Samples were sonicated (Bioruptor, Diagenode) for 10 min every 30 s at the highest intensity. Samples were centrifuged at 16,000×g during 15min at 4ºC and supernatant was recovered. Proteins were precipitated with cold acetone at a 1/6 ratio (v/v) overnight at -20ºC and recovered after subsequent centrifugation at 16,000×g during 15min at 4ºC. Precipitated proteins were resuspended in 6M urea, reduced in 10mM DTT (Sigma) and alkylated with 55mM of iodoacetamide (Sigma). Samples were brought to a concentration of 2M urea and digested with a concentration of Lys-C (Wako) corresponding to 10% of the sample (μg) overnight at 37ºC. Samples were further diluted to 1M urea and digested with a concentration of trypsin (Promega) corresponding to 10% of the sample (μg) for 12 h. Samples were desalted on MicroSpin C18 columns (The Nest Group), evaporated to dryness and dissolved in 0.1% formic acid.

For proteomic studies on described in Chapter 7, *HuRex* were processed for MS proteomics analysis as detailed in de Menezes-Neto et al., 2015. Most of the samples were processed for protein digestion as follows: samples were reduced with 10mM DTT (Sigma), alkylated with 55mM of iodoacetamide (Sigma), precipitated with 10% TCA, washed with 100% acetone. Samples were brought to a concentration of 1.6M urea and digested with 1μg of trypsin (Promega) overnight at 37ºC. The reaction was stopped with 1% formic acid. Alternatively, to ensure a better protein coverage, some *HuRex* samples (CB30_PS_SEC; CB30_PS_UC; CB31_AS_SEC; CB31_AS_UC) were solubilized and doubly digested as previously described for plasma-derived EVs.

Finally, 1 μg of each sample was analyzed using liquid chromatography (nanoLCULTRA-EKSIGENT) followed by mass spectrometry in the Orbitrap Fusion Lumos (Thermo Fisher Scientific) in the case of plasma-derived EVs or

in an LTQ Orbitrap Velos (Thermo Fisher Scientific) for *HuRex*.

3.2.9 *Protein Identification and in silico Analysis*

Raw MS data files were analyzed with the search algorithm Mascot v2.5.1 (Matrix Science) on the Proteome Discoverer^{TM} Software (Thermo Scientific). For peptide identification in EVs from vivax infected patients and healthy donors in Chapter 4, we used a customized protein P. vivax database composed of all sequences from the *P. vivax* strains deposited at UniProt (strain Belem, strain Salvador I, Brazil I, India VII, IQ07, Mauritania I, North Korean) and the UniProt human database, downloaded on November 2016. In the analysis described in Chapter *HuRex* for *HuRex*, we made the search using the human sequences from the UniProt human database, downloaded on April 2017. In both searches, peptides were filtered based on a maximum false discovery rate (FDR) for peptides and proteins of 1% and minimum unique peptides per protein of 1.

In Chapter 4, human proteins were accepted if more than 2 unique peptides were identified and assigned with the category of Master protein. Assigned contaminants as *Bos taurus* entries, immunoglobulins, keratins and abundant proteins plasma reported in de Menezes-Neto et al., 2015 were removed (see Dataset B.1, Table 5). *P. vivax* proteins were accepted if at least one unique peptide was present and were assigned with the category of Master protein. Sequences of *P. vivax* proteins were retrieved from their corresponding UniProt entries and blasted in PlasmoDB (https://plasmodb.org/plasmo/) to identify and assign their corresponding gene name and description for the *P. vivax* Sal-I and P01 strains. False positive *P. vivax* proteins were filtered out from the final list of proteins when peptides were detected in healthy donors (see Supplementary Dataset B.2, Table 2).

To determine proteins associated to *HuRex* in Chapter 7, a protein was considered if it was identified by at least 2 unique peptides or by 1 unique peptide present in more than one donor. Keratins and keratin-associated proteins as well as potential serum contaminants were removed from the final list of proteins (see Supplementary Dataset B.4, Sheet 14). We considered as serum contaminants the classical plasma proteins described by Anderson and Anderson proteomics (Anderson & Anderson, 2002) as well as other protein components compiled in this work that were exclusively detected in *HuRex* preparations from serum-supplemented cultures. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD008545 (Deutsch et al., 2017; Jarnuczak & Vizcaíno, 2017).

Filtered proteins associated in *HuRex* and human proteins detected in plasma-derived EVs from *P. vivax* patients or healthy donors were compared to those reported in Vesiclepedia (http://www.microvesicles.org/), a database

of extracellular vesicle cargo, using FunRich tool (Pathan et al., 2015). In the case of *HuRex* all proteins were crossed against the proteins compiled in the version 3.1 of Vesiclepedia (Kalra et al., 2012). The proteins identified in plasma-derived EVs were exclusively crossed with the proteins described in plasma in version 4.1 of Vesiclepedia.

GO term-enrichment analysis at cellular component and molecular function level of the proteome of the *HuRex* or human proteins exclusively present in *P. vivax* patients was performed with Database for Annotation, Visualization and Integrated Discovery (David 6.8) (Huang, Sherman, & Lempicki, 2009).

We estimated relative protein abundance in *HuRex* calculating the normalized spectral abundance factor (NSAF) as described elsewhere (Paoletti et al., 2006). This protein quantification index was established considering all proteins identified prior to filtering with their corresponding number of peptide spectrum matches.

3.2.10 *Western Blot Analysis*

For experiments presented in Chapter 4, pools of EV-enriched SEC fractions of each vivax patient or healthy donor were concentrated with Amicon Ultra 0.5mL 10K (Millipore) following manufacturer's instructions prior to western blot analysis. Equal amounts of protein (5μg) were mixed with reducing loading buffer, heated at 95ºC for 5 min and separated on 8% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Amersham) that were incubated overnight in blocking buffer (1X PBS, 0.1% Tween-20, 5% milk powder). After extensive washings, blots were probed with primary antibodies: rabbit anti-PvMSP3 serum (Jiang, Barnwell, Meyer, & Galinski, 2013) at 1:500 dilution or rabbit anti-PvPHIST (Akinyi et al., 2012) at 1:500 dilution. After extensive washings, blots were incubated with secondary antibodies IRDye 680RD goat anti-rabbit (925-68021, Li-Cor Biosciences) at 1:20,000 dilution. Both primary and secondary antibodies were diluted in a buffer containing 1X PBS, 0.1% Tween-20, 1% milk powder and incubated for 1 hour at RT each. Signal was detected on Odyssey near-infrared system (Li-Cor Biosciences) using default settings, except for 700-laser intensity, which was set up at 5 for PHISTc detection, and at 3 for MSP3.1 detection. Images were edited using the software Image J (NIH).

For Western blot analysis on *HuRex* shown in Chapter 7, 10 μL aliquots of either a UC preparation or individual SEC fractions were used to detect human CD71. Samples were mixed with reducing loading buffer, heated and run on 10% SDS-PAGE gels. After extensive washings, blots were probed with rabbit polyclonal antibody anti-CD71 (Abcam, ab84036) at 1:250 dilution. A goat anti-rabbit IgG coupled to HRP (Sigma, A6154) was used at a dilution of 1:2,500. Revealing was performed using ECL Western Blotting Substrate (Pierce^{TM}) in ImageQuant LAS 4000 (GE Healthcare Life Sciences). Additionally, 20 μL

aliquots of UC preparations were analyzed to confirm the presence of HSP70, GAPDH and stomatin. Membranes were incubated with primary antibodies anti-HSP70 (Santa Cruz Biotechnology, W27 sc-24) at 1:250 dilution, anti-GAPDH (Sigma, G9545) at 1:500 dilution or anti-stomatin (Invitrogen, PA5-30019) at 1:250 dilution. Subsequently, membranes were washed and incubated with the Li-Cor IRDye-labeled secondary antibodies IRDye 800CW goat-anti-mouse (925-32210, Li-Cor Biosciences) at 1:15,000 dilution or IRDye 680RD goat anti-rabbit (925-68021, Li-Cor Biosciences) at 1:20,000 dilution. Blots were analyzed with the Odyssey near-infrared system (Li-Cor Biosciences) having the intensity of 700 channel set up at 5 and the one of 800 channel at 7. Images were edited using the software Image J (NIH).

3.2.11 *T-cell Epitope Prediction*

We predicted 8-11 mer HLA-I restricted T-cell epitopes within proteins associated to EVs from *P. vivax* infections using NetMHCpan 4.0 (Jurtz et al., 2017). We predicted HLA-restrictions of peptides for representative HLA-I alleles from twelve HLA-I supertypes (HLA-A*01:01, HLA-A*02:01, HLA-A*03:01, HLA-A*24:02, HLA-A*26:01, HLA-B*07:02, HLA-B*08:01, HLA-B*15:01, HLA-B*27:05, HLA-B*39:01, HLA-B*40:01, HLA-B*58:01)(Sidney, Peters, Frahm, Brander, & Sette, 2008) throughout the full length of MSP3.1 595 aa sequence and PHISTc 710 aa sequence downloaded from UniProt (A0A0J9TBC1 and A0A0J9T4K3 respective accession numbers). Following the algorithm's recommendation, we considered peptides whose % rank was lower than 0.5 as strong binders and peptides whose % rank was >0.5-≤ 2 as moderate binders.

3.2.12 *Processing of Human Spleens for Cell Isolation*

Peripheral blood was removed by perfusion with University of Wisconsin solution (Viaspan) before removal of organs from the deceased donors. Collected spleens were kept in this solution for their overnight storage at 4ºC before their processing. Typically, 5-10 g of spleen were cut in small pieces (2 mm^2 approximately) and the tissue was disrupted mechanically in the presence of complete RPMI medium [RPMI 1640 (Sigma) supplemented with 10% FBS (Gibco) and 50 U/mL penicillin – 50 μg/mL streptomycin (Gibco)]. Tissue suspension was passed twice through a 70 μm cell strainer washing with complete RPMI medium to obtain a spleen single-cell suspension (SSCS). The SSCS was let sit for 30 min to sediment large tissue particles and released nuclei acids. The supernatant was collected avoiding the precipitated components. The cleared SSCS was passed once more through 70 μm cell strainer and viability of cells was assessed by trypan blue (Sigma) exclusion. Immediately, SSCS was used for further separation of different cell populations (see Section 3.2), analysis by flow cytometry (see Section 3.2) and/or freezing in FBS at

10% DMSO (Sigma) and storage in liquid nitrogen.

3.2.13 *Separation of Spleen Cell Populations*

SSCS was processed to separate several spleen cell populations. Figure 5.4 depicts a graphical summary of the whole procedure. Firstly, splenocyte suspensions were diluted up to 5×10^7 cells/mL with complete RPMI and passed again through the 70 μm cell strainer immediately before it was layered over Ficoll-Histopaque 1077 (Sigma) suspension to isolate spleen mononuclear cells by density centrifugation. Two-thirds of the splenocyte suspensions were processed by conventional Ficoll density centrifugation and one-third was depleted of T lymphocytes using RosetteSep^{TM} CD3 Depletion Cocktail (Stemcell Technologies) combined to Ficoll density centrifugation following manufacturer's instructions. After centrifugation, whole mononuclear cells and T lymphocyte-depleted mononuclear cells were collected from the interphase and washed separately with complete RPMI medium. T-cells were isolated from 1×10^8 whole mononuclear cells by negative selection with Pan-T-cell Isolation Kit (Miltenyi Biotec) or with CD3+ positive selection magnetic beads (Miltenyi Biotec). T lymphocyte-depleted mononuclear cells were pooled with the cells from the CD3 negative fraction when CD3+ MACS separation was performed. T lymphocyte-depleted mononuclear cells were enriched in DCs using EasySep^{TM} Pan-DC Pre-Enrichment Kit (Stemcell Technologies). Both DC-enriched and non-enriched fractions as well as the previously obtained T-cells were analyzed by flow cytometry and were immediately used in interaction studies with EVs. Cell viability and count was assessed throughout the whole process with trypan blue (Sigma) staining on a hemocytometer chamber.

3.2.14 *Flow Cytometry Analysis of Spleen Cell Populations*

SSCS was processed both with or without red blood cell lysis by BD Pharm Lyse buffer following manufacturer instructions prior to staining. Briefly, SSCS or enriched cell populations were washed with PBS before staining with Fixable Viability Stain 575V (BD Biosciences) at 1:1,000 dilution for 15 min at RT. Cells were washed with PBS at 1% FBS (Gibco) and subsequently incubated for 15 min at RT with antibodies against surface markers shown in Table 3.2 diluted in cell separation buffer [1X MACS Quant buffer (Miltenyi Biotec) with 0.5% BSA (Sigma)]. Cells were further washed with cell separation buffer prior to acquisition in an LSR Fortessa flow cytometer (BD). For each sample, a minimum of 10^5 cells was acquired. Controls included unstained samples and single fluorochrome compensation beads. Results were analyzed using FlowJo software. Figure 5.1 shows the gating strategy followed for the analysis of human spleen cells phenotyping. Supplementary Figure A.2 details the gating strategy for the interaction assays of EVs with total splenocytes. Supplementary Figures A.3, A.4, A.5 and A.6 describe the gating strategy applied to define

separated populations from the spleen and to assess their interaction with EVs.

3.2.15 *Fluorescent Labelling of EVs for Interaction Assays with Human Splenocytes*

Pools of plasma-derived EVs from 10 *P. vivax* patients (*Pv*EVs) and from 10 healthy donors (*h*EVs) were labelled with PKH67 or PKH26 labeling mini kit (Sigma). EV staining was conducted as previously described (Martín-Jaular et al., 2016) with some modifications. Briefly, up to 50 μg of EVs diluted up to 1mL with Diluent C were gently mixed with 4 μL of dye in 1 mL of Diluent C and incubated for 5 min at RT. Labelled EVs were washed 5 times using Amicon Ultra-15 100-kDa filters units (Millipore) to remove the excess of dye. Washings were performed as follows: samples were centrifuged at 4000 $\times$ g for 10 min, washed twice with 1 mL of 1X PBS and washed three times more with 100 μL of 1X PBS. As a control, 1X PBS was labelled and washed in the same manner as EVs. Protein concentration of labelled-EVs was quantified by BCA assay (Thermo Scientific). PKH-labelled-EVs were further diluted to get final concentration of 3 μg/mL in EV-depleted culture medium. Medium was depleted from exogenous EVs as described in Section 3.2.4. Labelled-PBS controls were diluted in an equivalent manner.

3.2.16 *Interaction Assays of PvEVs and hEVs with Human Splenocytes*

Total splenocytes (SSCSs) or enriched spleen cell populations were incubated were seeded in 24-well plates at 1×10^6 splenocytes/well or in 96-well plates at 5×10^5 splenocytes/well using complete DMEM medium (Sigma) supplemented with EV-depleted 10% FBS (Gibco) and 50 U/mL penicillin – 50 μg/mL streptomycin (Gibco). 3 μg protein/mL of PKH26 or PKH67-labelled *Pv*EVs or *h*EVs were added and incubated at 37ºC for 3h. In parallel, labelled-PBS was incubated with the cells as a background control. After the 3h-incubation, total splenocytes or enriched cell populations were analyzed by flow cytometry as previously described in Section 3.2.14.

3.2.17 *Isolation of PBMCs*

Peripheral blood cells were re-suspended at 15-20% HCT with complete RPMI medium (previously detailed composition, see Section 3.2.12). 30mL of cells at 15-20% HCT were carefully layered over 15mL of Ficoll-Histopaque 1077 (Sigma). After centrifugation at 1,200 $\times$ g for 20 min at 20ºC and no brake, PBMCs were harvested from the interphase. PBMCs were washed twice with complete RPMI medium and cell viability and count was assessed with trypan blue (Sigma) staining on a hemocytometer chamber. PBMCs were immediately used, or frozen in cryovials with 1mL of FBS at 10% DMSO (Sigma) and kept in liquid nitrogen until use. Frozen PBMCs obtained from

Panel for RBCs					
antigen	*fluorochrome*	*clone*	*manufacturer*	*reference*	*dilution*
CD45	PerCP	5B1	Miltenyi biotec	130-113-120	1/100
		2D1	BD Biosciences	345809	1/50
CD235a	APC	REA175	Miltenyi biotec	130-100-270	1/100
CD71	PE	AC102	Miltenyi biotec	130-091-728	1/400

Panel for T-cells/NK cells					
antigen	*fluorochrome*	*clone*	*manufacturer*	*reference*	*dilution*
CD45	PerCP	5B1	Miltenyi biotec	130-113-120	1/100
		2D1	BD Biosciences	345809	1/50
CD3	FITC	REA613	Miltenyi biotec	130-113-138	1/400
		SK7	StemCell Technologies	60127FI.1	1/200
CD8	APC-Cy7	SK1	Biolegend	344714	1/200
			BD Biosciences	641400	1/400
CD4	APC	RPA-T4	Biolegend	300514	1/100
CD56	PE-Vio770	AF12-7H3	Miltenyi Biotec	130-098-132	1/100

Panel for monocytes/DCs/B-cells/neutrophils/macrophages					
antigen	*fluorochrome*	*clone*	*manufacturer*	*reference*	*dilution*
CD45	PerCP	5B1	Miltenyi biotec	130-113-120	1/100
		2D1	BD Biosciences	345809	1/50
CD14	PE-Cy7	M5E2	Biolegend	301813	1/100
	BV-785		Biolegend	301839	1/30
CD11c	BV-421	3.9	Biolegend	301627	1/100
	BV-711		Biolegend	301629	1/30
HLA-DR	APC-Cy7	L243	Biolegend	307617	1/100
			BD Biosciences	641393	1/200
CD19	PE	REA675	Miltenyi biotec	130-113-646	1/400
	BV-510	HIB19	Biolegend	302241	1/50
CD15	FITC	VIMC6	Miltenyi biotec	130-114-010	1/400
CD86	APC	REA968	Miltenyi biotec	130-116-264	1/100
CD163	PE-CF594	GHI/61	BD Biosciences	562670	1/200

Panel for hematopoietic stem cells					
antigen	*fluorochrome*	*clone*	*manufacturer*	*reference*	*dilution*
CD45	PerCP	5B1	Miltenyi biotec	130-113-120	1/100
		2D1	BD Biosciences	345809	1/50
CD34	FITC	AC136	Miltenyi biotec	130-081-001	1/100

Table 3.2 **Antibody panels used for phenotyping human spleen cells.**

patients were shipped to IGTP to continue the experiments.

3.2.18 *T-cell Activation by EVs*

For experimental optimization, fresh or frozen-thawed PBMCs were used to negatively isolate total T-cells with the Pan-T-cell Isolation Kit (Miltenyi Biotec) following manufacturer instructions. 100,000 T-cells were stimulated with 100 μL of anti-CD2/CD3/CD28 coated microbeads (Pan T-cell Activation Kit, Miltenyi Biotec) diluted in PBS to get a 1(bead):10 (cell) ratio, or with 100 μL of PBS, as a negative control, in a 96-well plate. Cells were incubated at 37ºC for 18h, 24h, 48h or 72h.

200,000 fresh or frozen-thawed PBMCs were stimulated with 100 μL of EV-enriched SEC fraction with the highest HLA-I expression isolated from heterologous plasma of a *P. vivax* patient (*Pv*EVs) or a healthy donor (*h*EVs) in a 96-well plate. As a negative control, 100 μL of EV-enriched fraction from their autologous plasma (auto-*h*EVs) were used. As positive controls, cells were stimulated with anti-CD2/CD3/CD28 coated microbeads (Pan T-cell Activation Kit, Miltenyi Biotec) at a 1(bead):10 (cell) ratio or with PHA-M (phytohemagglutinin-M)(Sigma) at a final concentration of 5 μg/mL. Cells were incubated at 37ºC for 48h.

To determine T-cell activation by flow cytometry, cells were washed with PBS and stained with Fixable Viability Stain 575V (BD Biosciences) at 1:1,000 dilution for 15 min at RT. Cells were washed with PBS at 1% FBS (Gibco) and incubated for 15 min at RT with antibodies against surface markers shown in Table 3.3 diluted in 1X MACS 0.5% BSA buffer. Cells were washed with 1X MACS 0.5% BSA buffer prior to acquisition. If intracellular staining against IFN-γ was performed, monensin (Biolegend) was added to cell cultures 4h before the staining. Cells were stained with Fixable Viability Stain 575V and surface markers as previously described and further stained against IFN-γ using Intrastain Kit (Dako) following manufacturer's instructions. For each sample, a minimum of 10^4 cells was acquired in an LSR Fortessa flow cytometer (BD). Controls included unstained and single-stained samples. Results were analyzed using FlowJo software. Supplementary Figure A.7 shows the gating strategy followed for the analysis.

3.2.19 *Monocyte-dependent or Direct T-cell Activation by EVs*

Thawed PBMCs were incubated with CD14+ positive selection magnetic beads (Miltenyi Biotec) to isolate CD14+ monocytes as detailed by the manufacturer. Total T-cells were isolated from the CD14 negative fraction using the Pan-T-cell Isolation Kit (Miltenyi Biotec) following manufacturer instructions. PBMCs, monocytes and T-cells were stained with Fixable Viability Stain 575V (BD Biosciences) and anti-CD3-FITC (Miltenyi Biotec, 130-113-139), anti-CD14-PE-Cy7 (Biolegend, 301813), anti-CD19-PE (Miltenyi Biotec, 130-

Panel for T-cell activation

antigen	*fluorochrome*	*clone*	*manufacturer*	*reference*	*dilution*
CD3	FITC	REA613	Miltenyi biotec	130-113-138	1/400
CD8	APC-Cy7	SK1	Biolegend	344714	1/200
CD4	APC	RPA-T4	Biolegend	300514	1/100
PD-1	PerCP	EH12.2H7	Biolegend	329937	1/100
CD69	PE	REA824	Miltenyi biotec	130-112-802	1/100
IFN-γ	PE-Cy7	4S.B3	Biolegend	502527	1/25

Table 3.3 **Antibodies used to analyze T-cell activation by flow cytometry.**

113-646) antibodies and analyzed by flow cytometry according to the procedure detailed in Section 3.2.14. Gating strategy of the analysis of the purification of these cell populations is presented in Supplementary Figure A.8.

For each cell donor,10,000 monocytes were incubated at 37ºC with 100 μL of EV-enriched SEC fraction with the highest HLA-I expression isolated from heterologous plasma of a *P. vivax* patient (*Pv*EVs) or a healthy donor (*h*EVs), or with PBS as a negative control in a 96-well plate. After 3 hours, 100,000 autologous T-cells were added. In parallel, 100,000 T-cells from the same cell donor were incubated with 100 μL of the *Pv*EVs or *h*EVs coming from the same donors that were used to prime monocytes. As negative controls, T-cells were incubated with PBS or auto-*h*EVs. As a positive control, T-cells were stimulated with anti-CD2/CD3/CD28 coated microbeads (Pan T-cell Activation Kit, Miltenyi Biotec) at a 1(bead):10 (cell) ratio. Cells were incubated at 37ºC for 18h. T-cell activation was analyzed by flow cytometry as detailed in Section 3.2.18.

Note that T-cell activation could be also performed by autologous stimulation with EVs from *P. vivax* patients or healthy donors following the approach depicted in Figure 6.2.

3.2.20 *HuRexDiI Generation*

For uptake experiments presented in Chapter 7, reticulocyte-enriched RBCs were labeled with DiI (Molecular Probes, V-22885) following manufacturer's instructions. Culture of stained cells and EV isolation was performed as for the unstained *HuRex* as described in Section 3.2.4. Fluorescence of *HuRexDiI* was determined by a Fluoroskan Ascent FL fluorimeter (Thermo Fisher Scientific).

3.2.21 *HuRex and VLP Capture by Mature Monocyte-derived DCs via Siglec-1*

2×10^5 LPS-matured monocyte-derived DCs were obtained as previously described (Izquierdo-Useros et al., 2009) and incubated with 50 μg *HuRexDiI* at 37ºC for 4 hours. Cells were washed and acquired with a BD FACSCalibur cytometer. Data were analyzed using FlowJo software. For microscopy analysis,

mature DCs (mDCs) were pulsed for 24 h with *HuRexDiI* and then with 150 μg of fluorescent viral-like particles containing Gag-enhanced green fluorescent (VLP*HIV-Gag-eGFP*) for 3 additional hours at 37ºC. Cells were washed, fixed, cytospun into coverslips, mounted with DAPI mounting media (Thermo Fisher Scientific) and analyzed with an Ultraview ERS Spinning Disk System (Perkin-Elmer) mounted on a Zeiss Axiovert 200 M inverted microscope. Volocity software (Perkin-Elmer) was used to analyze microscopy images. To determine whether Siglec-1 could represent a receptor for entrance of *HuRex*, mDCs were pre-incubated with 10 μg/mL of an anti-Siglec-1 monoclonal antibody (clone 7–239; Abcam) or a mouse anti-human IgG1 isotype control (BD) at RT for 15 min (Izquierdo-Useros et al., 2012) before addition of 50 μg *HuRexDiI* at 37ºC for 4 hours. Capture was analyzed by flow cytometry as described above.

Chapter

4 Results I: Characterization of EVs from *P. vivax* Infections: Implications in Antigen Discovery

Parasite proteins have been detected in EVs derived from both plasma and reticulocytes of mice infected with *P. yoelii*. Immunization with *Rex* from *P. yoelii* infection induced a protective immune response remarking the association of parasite antigens to these EVs as explained in Chapter 1 (see Section 1.3.2). Given that the lack of a continuous *in vitro* culture of *P. vivax* restricts the study of *Rex* derived from *P. vivax*-infected reticulocytes, we circumvented this limitation by exploring circulating *Pv*EVs, obtained directly from human patients. This chapter presents the findings on the proteomic characterization of *Pv*EVs in order to detect potential antigens associated to them and investigate their potential roles during *P. vivax* infections.

This chapter is structured as follows. Sections 4.1 and 4.2 present the isolation and proteomic characterization of *Pv*EVs, respectively. Section 4.3 describes the prediction of T-cell epitopes on parasite proteins associated to *Pv*EVs. Finally, Section 4.4 summarizes the main findings presented in this chapter.

4.1 Isolation of Circulating EVs from *P. vivax* Infections

Aiming to isolate circulating EVs from natural infections of *Plasmodium vivax*, we processed plasma from *P. vivax* infected patients by SEC. This is a user-friendly technique to enrich EVs from different biological fluids avoiding the co-isolation of contaminants. SEC is based on the fractionation of particles according to their size eluting in a mobile phase through a stationary matrix (de Menezes-Neto et al., 2015).

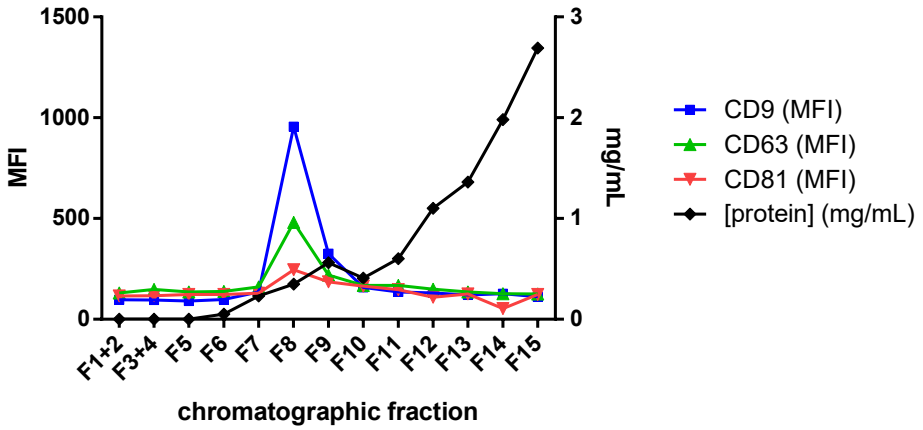


Figure 4.1 **Isolation of extracellular vesicles from plasma of a *P. vivax*-infected patient by size-exclusion chromatography.** Chromatographic fractions were assayed for protein concentration by BCA, and analyzed by bead-based flow cytometry for the detection of classical EV markers CD9, CD63 and CD81 to identify/characterize EV-enriched fractions. MFI, Median fluorescence intensity.

Plasma samples from individual *P. vivax* patients or from healthy donors were loaded on SEC columns. Eluted chromatographic fractions were analyzed for protein concentration and coupled to 4μm-latex beads to identify and characterize EV-enriched fractions by flow cytometry. We assessed first the presence of classical EV markers (CD9, CD63, CD81). The bulk of soluble plasma proteins eluted in the late chromatographic fractions while CD9, CD63 and CD81 signals were highest around fraction 8 (see Figure 4.1). Importantly, no apparent signal of these markers was observed in distal fractions containing abundant soluble proteins.

Secondly, we assessed the expression of CD5L, marker of plasma-derived vesicles (de Menezes-Neto et al., 2015) and transferrin receptor (CD71 or TfR), the major component of *Rex* (Harding et al., 1983; Pan & Johnstone, 1983; Vidal, 2010) on chromatographic fractions from vivax patients and healthy donors. CD5L and CD71 signals were highest between early fractions 8 and 10 (see Figure 4.2) confirming the association of these markers with circulating *Pv*EVs and *h*EVs.

Despite processing the same volume of plasma from patients or healthy donors, we realized that chromatographic fractions derived from patients tend to be more concentrated in terms of protein than those coming from healthy donors (see Figure 4.2). Moreover, we determined size and particle concentration of EV-enriched fractions by NTA and we observed that the concentration of particles in the size of EVs was higher in vivax patients than

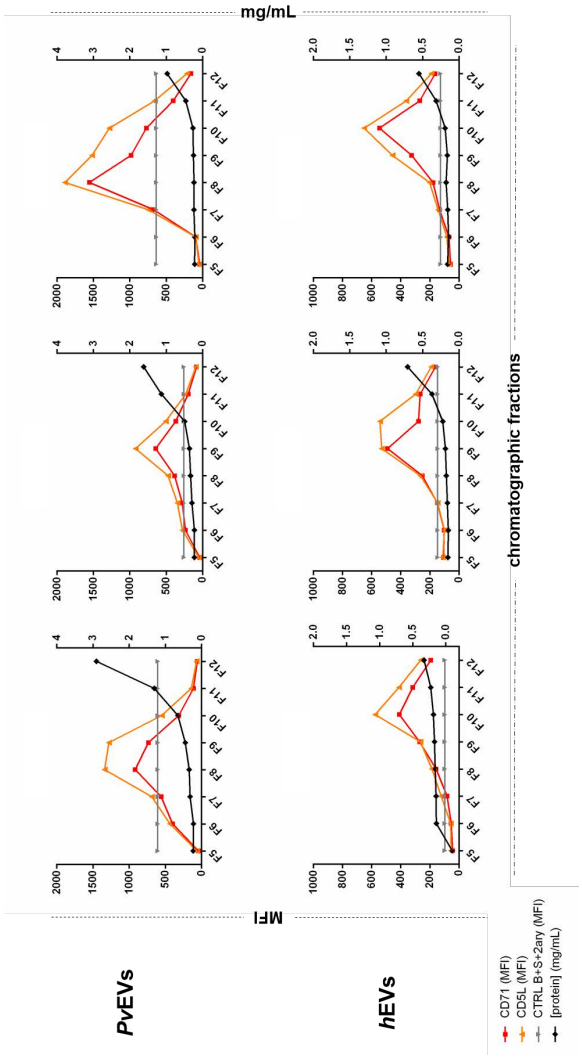


Figure 4.2 **Characterization of EVs from *P. vivax* infected patients and healthy donors.** SEC fractions of containing *Pv*EVs (upper part) and *h*EVs (bottom part) were analyzed by bead-based flow cytometry for the presence of EV-associated marker CD5L and transferrin receptor, CD71. Negative controls of EV-beads incubated with secondary antibodies (CTRL B+S+2ary) are also shown. MFI, Median fluorescence intensity. Protein concentration was assayed for each fraction. Representative analysis for SEC fractions from three individual PV patients and three individual HD are shown.

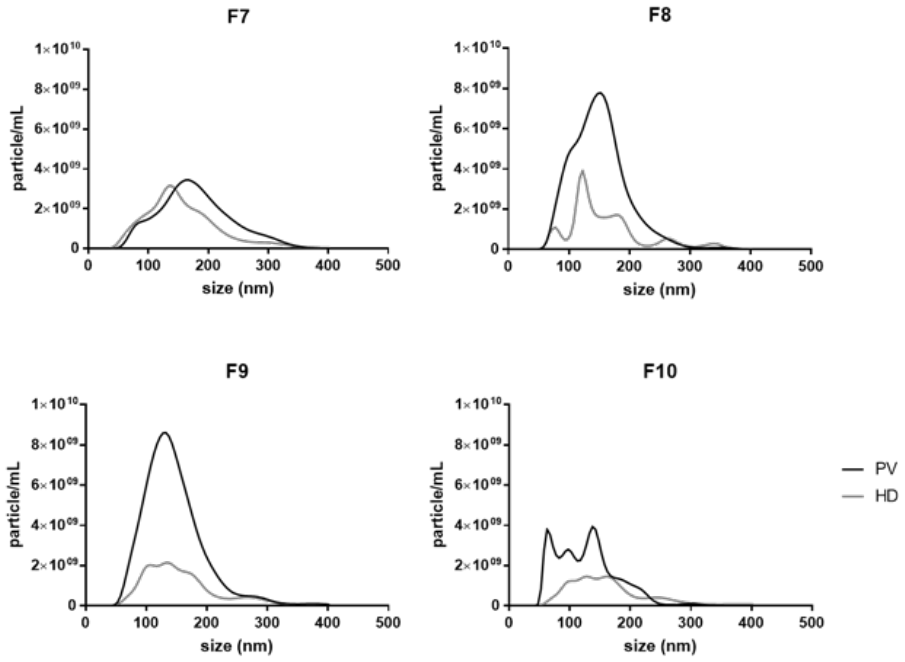


Figure 4.3 **Nanoparticle tracking analysis of plasma-derived extracellular vesicles from a *P. vivax* infected patient and a healthy donor.** Size *vs* concentration of particles from each SEC fraction (F7 to F10) using NanoSight LM10. Representative results for SEC fractions from an individual PV patient and an individual HD are shown. PV, *P. vivax*-infected patient. HD, healthy donor.

in healthy donors (see Figure 4.3). It is plausible that more EVs are being secreted to plasma milieu during acute vivax infections. These results are in agreement with previous observations on natural vivax infections which have shown that EVs are more abundant in plasma of vivax infected individuals compared to healthy volunteers (Campos et al., 2010).

4.2 Proteomic Characterization of EVs from *P. vivax* Infections

To identify *P. vivax* proteins associated with circulating EVs from natural vivax infections, EVs derived from plasma of ten different *P. vivax* patients and EVs from plasma of ten healthy donors were analyzed by mass spectrometry-based proteomics. Due to expected heterogeneity and limited amount of EVs, EV-enriched fractions (F7 to F9) from every vivax patient or healthy donor were pooled and processed for analysis. Overall, we identified 20 parasite proteins and

533 human proteins among the 20 MS-datasets (see Supplementary Datasets B.1 and B.2).

Parasite cargo of EVs from *P. vivax* infections

For the identification of *P. vivax* proteins associated to*Pv*EVs, we searched the MS raw data against a protein database composed of all the sequences from the several field *P. vivax* strains deposited at UniProt with an FDR $< 1\%$. After excluding false positive parasite proteins that appeared in healthy donors (see Supplementary Dataset B.2, Table 2), twenty *P. vivax* proteins were identified (see Table 4.1). As expected, the distribution of these proteins was diverse (see Supplementary Dataset B.2, Table 1). Notably, three vivax proteins were identified by at least two unique peptides: MSP3.1 (PVP01_1031700), PHISTc (PVP01_0119200) and GAPDH (PVP01_1244000) (see Table 4.1).

In order to validate the association of these parasite proteins with*Pv*EVs, we performed western blot analysis using available antibodies against *P. vivax* MSP3 (Jiang et al., 2013) and PHIST (Akinyi et al., 2012). *h*EVs were used as specificity control (see Figure 4.4). We detected specific bands corresponding to MSP3.1 and PHISTc proteins in several patients. MSP3.1 was detected in three patients with a variable and larger apparent molecular weight than the one expected (around 91kDa). This phenomenon has been already described in MSP3 proteins separated by SDS-PAGE (Galinski et al., 1999; Jiang et al., 2013). For PHISTc, differential bands were observed around 70-100kDa in several patients, which is in agreement with the variant expression of proteins from this family (Warncke, Vakonakis, & Beck, 2016). Both merozoite proteins and parasite proteins exported to the infected erythrocytes have been associated to EVs derived from malaria infected RBCs. These results will be discussed in Section 8.1.

Human cargo of EVs from *P. vivax* infections

Increasing evidence suggests that EVs in parasitic infections play a critical role in host-parasite interactions (Ofir-Birin & Regev-Rudzki, 2019). Besides parasite proteins, we believe that altered host cargo in circulating EVs also contributes to inter-cellular communication processes during infections. Therefore, we examined the human component of EVs from vivax infections.

Firstly, we made a global comparison of the 533 human proteins identified among all EV preparations coming from either vivax patients or healthy donors with the proteins of plasma-derived EVs compiled in Vesiclepedia. Vesiclepedia is a public repository composed of proteins, RNA, lipids and metabolites that have been detected in EVs from published and unpublished works (Pathan et al. 2019). This comparison showed that 444 proteins (83.3%) had been reported as plasma-EV cargo (see Figure 4.5A). Moreover, 24 out of 36 plasma-

Accesion	Gene name (Strain PVP01)	Description (Strain PVP01)	# Unique Peptides	# PSMs	# PV patients
A0A0J9TBC1	PVP01_1031700	*merozoite surface protein 3, MSP3.1*	4	8	5
A0A0J9T4K3	PVP01_0119200	*Plasmodium exported protein (PHISTc), unknown function*	2	2	2
A0A0J9WBG4	PVP01_1244000	*glyceraldehyde-3-phosphate dehydrogenase, putative, GAPDH*	2	2	1
A5K1L2	PVP01_1147600	*Plasmodium exported protein, unknown function*	1	1	1
A5K4L3	PVP01_0920600	*conserved Plasmodium protein, unknown function*	1	3	1
A0A0J9S584	PVP01_1430900	*conserved Plasmodium protein, unknown function*	1	1	1
A0A0J9SD62	PVP01_0815000	*conserved Plasmodium protein, unknown function*	1	1	1
A0A0J9T8C9	PVP01_1261300	*conserved Plasmodium protein, unknown function*	1	1	1
A0A0J9TMC1	PVP01_0113800	*conserved Plasmodium protein, unknown function*	1	1	1
A0A0J9TD53	PVP01_0943000	*conserved Plasmodium protein, unknown function*	1	1	1
A0A0J9TRX5	PVP01_1240000	*conserved Plasmodium protein, unknown function*	1	1	1
A0A0J9U0K3	PVP01_0703700	*dynein heavy chain, putative*	1	1	1
A0A0J9SCM5	PVP01_1111400	*guanylyl cyclase beta, putative*	1	1	1
A0A0J9TZP5	PVP01_0728900	*merozoite surface protein 1*	1	1	1
A0A0J9SKC9	PVP01_0003730	*PIR protein*	1	1	1
A0A0J9T2V4	PVP01_0002050	*PIR protein*	1	1	1
A0A0J9THR9	PVP01_0009490	*PIR protein*	1	1	1
A0A0J9VQS8	PVP01_0001040	*PIR protein*	1	1	1
A0A0J9SWK4	PVP01_0003060	*PIR protein*	1	1	1
A0A0J9VPJ6	PVP01_0000210	*Tryptophan-rich protein*	1	1	1

Table 4.1 ***P. vivax* proteins associated to *Pv*EVs identified by mass spectrometry-based proteomics.** Uniprot accession numbers, gene names and description according to strain PVP01 as well as number of unique peptides, number of PSMs and number of identifications in PV patients are shown for each protein.PSM, peptide-spectrum match.

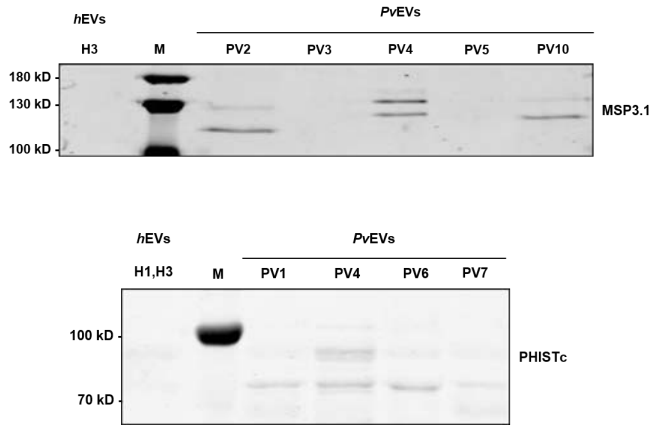


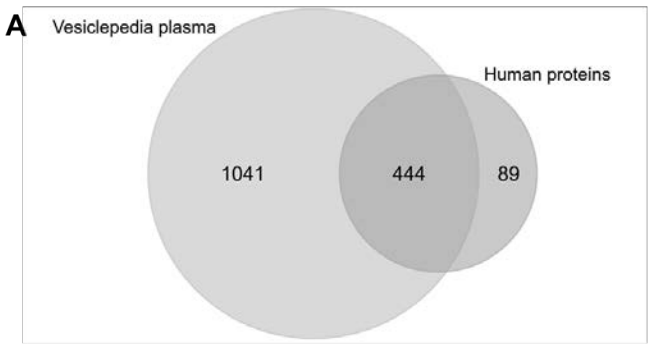
Figure 4.4 **Western blot analysis for detection of parasite proteins identified in *Pv*EVs.** EVs obtained from different patients (PV) and from healthy donors (HD) were analyzed. Anti-*P. vivax*MSP3.1 (upper membrane) and anti-PHISTc (bottom membrane) were used as primary antibodies. Molecular weight in kDaltons (kD) is shown on the left. M, molecular weight marker.

derived vesicle markers compiled in the study of de Menezes-Neto et al., 2015 were identified among these human proteins (see Figure 4.5B). These results reinforce the use of SEC as an efficient technique to isolate EVs from plasma.

Next, we compared the human protein cargo associated to EVs from *P. vivax* infections *versus* EVs from healthy donors. The analysis of the number of human proteins identified in each EV preparation showed that in average *Pv*EVs contain fewer human proteins than *h* EVs (see Figure 4.6A and see Supplementary Dataset B.1, Table 1). Looking into the intersection of the total human proteins identified in the two groups, we observed a core of 293 proteins. 33 proteins that were exclusively present in *Pv*EVs and 207 were only detected in *h*EVs (see Figure 4.6B). It is interesting to find a more restricted subset of human proteins in *Pv*EVs, despite the higher amount of EVs in the plasma of infected patients (see Figure 4.3).

To better understand the contribution of circulating EVs to the changes on the protein composition in plasma during vivax infection, we compared the human proteins exclusively detected in *Pv*EVs with the data of a recent study on the proteomics of the serum of vivax malaria patients (Ray et al., 2017). We found that 6 out of the 33 human proteins particularly associated to *Pv*EVs were also described in the serum of *P. vivax* patients with low and moderate parasitemia. These six proteins are the Leucine-rich alpha-2-glycoprotein (LRG1), Inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), Titin (TTN), Zinc-alpha-2glycoprotein (AZGP1), Complement factor I (CFI) and Serum amyloid A-2 protein (SAA2). This analysis suggests that EVs might help in the identification of surrogate markers of infection.

To gain insight into the processes that plasma-derived EVs might be



B

plasma-derived vesicle markers identified in human proteins
LGALS3BP
MSN
CD5L
ALDOA
YWHAZ
CLTC
PGK1
CFL1
GAPDH
YWHAE
YWHAG
GDI2
YWHAB
ANXA2
CD9
EEF1A1
PRDX1
RHOC
CD81
CD82
EZR
RDX
ANXA5*
ANXA6*

Figure 4.5 **Comparison of human proteins identified in *Pv*EVs and *h*EVs *versus* protein cargo previously reported in plasma-derived EVs.** (A) Human proteins were crossed against the proteins associated to plasma-derived EVs reported in Vesiclepedia. (B) Identified plasma-derived vesicle markers. Proteins listed according to their gene name. Proteins with an asterisk were exclusively identified in *h*EVs.

affecting during vivax infections, we performed a Gene Ontology (GO) term-enrichment analysis over the set of proteins exclusively present in *Pv*EVs. The most significantly enriched GO terms for the category of biological process corresponded to "inflammatory response", "leukocyte migrations" and "T-cell activation", which denotes that EVs are regulating the immune response during *P. vivax* infection (see Figure 4.7 and Supplementary Dataset B.3). Expectedly, GO terms such as "extracellular exosome", "extracellular space", "plasma membrane" and "extracellular region" were overrepresented at the cellular component category, which is in agreement with the EV nature of the preparations.

4.3 Prediction of Cytotoxic T-cell Epitopes of Parasite Proteins Associated to *Pv*EVs

CD8+ T-cell responses have been shown to be critical for protection against liver-stage malaria infection (W. R. Weiss et al. 1988; Epstein et al. 2011). Several works have tried to identify protective CD8+ T-cell epitopes for many *Plasmodium falciparum* liver-stage antigens (Heide, Vaughan, Sette, Jacobs, & Schulze zur Wiesch, 2019) and some *P. vivax* transmission/liver-stage antigens such as *P. vivax* circumsporozoite protein (Arévalo-Herrera et al., 2002) or *P.*

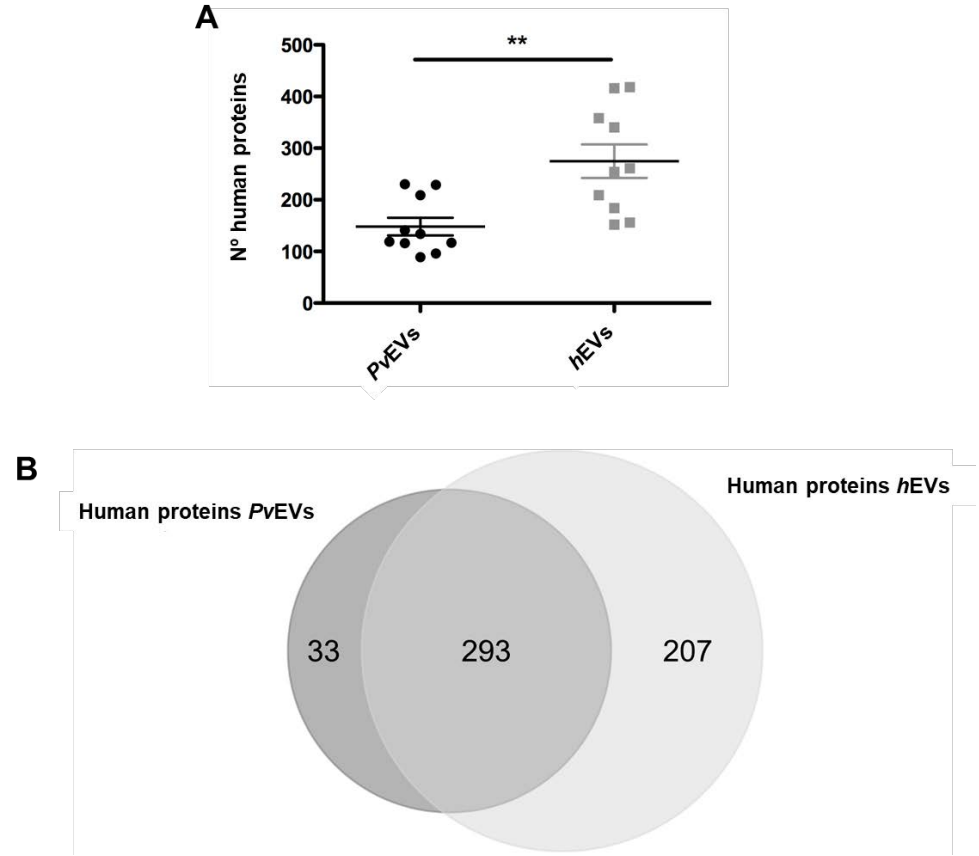


Figure 4.6 **Human proteins identified in EVs from *P. vivax* infections (*Pv*EVs) versus EVs from healthy donors (*h*EVs).** (A) Number of proteins identified in each preparation of *Pv*EVs and *h*EVs. T-test $p<0.005$ (**). (B) Venn diagram showing the intersection between total human proteins detected in *Pv*EVs and the ones detected in *h*EVs

vivax cell traversal protein of ookinetes and sporozoites (Bitencourt Chaves et al., 2017). However, CD8+ T-cell responses against blood-stage infection seem to be specifically associated with *P. vivax* (Burel et al., 2016; Junqueira et al., 2018) and to the best of our knowledge, there are no studies on potential cytotoxic T-cell epitopes for blood-stage vivax antigens. This unique defense mechanism must be related to the fact that *P. vivax* infects reticulocytes, which still retain HLA class I molecules.

Having demonstrated the association of parasite proteins to EVs from natural *P. vivax* infections, presumably derived from *P. vivax*-infected reticulocytes, we think that EVs might be a relevant source of antigens and might promote cytotoxic T-cell responses. We decided to predict HLA class I-T-cell epitopes from two of the parasite proteins detected with higher confidence in EVs

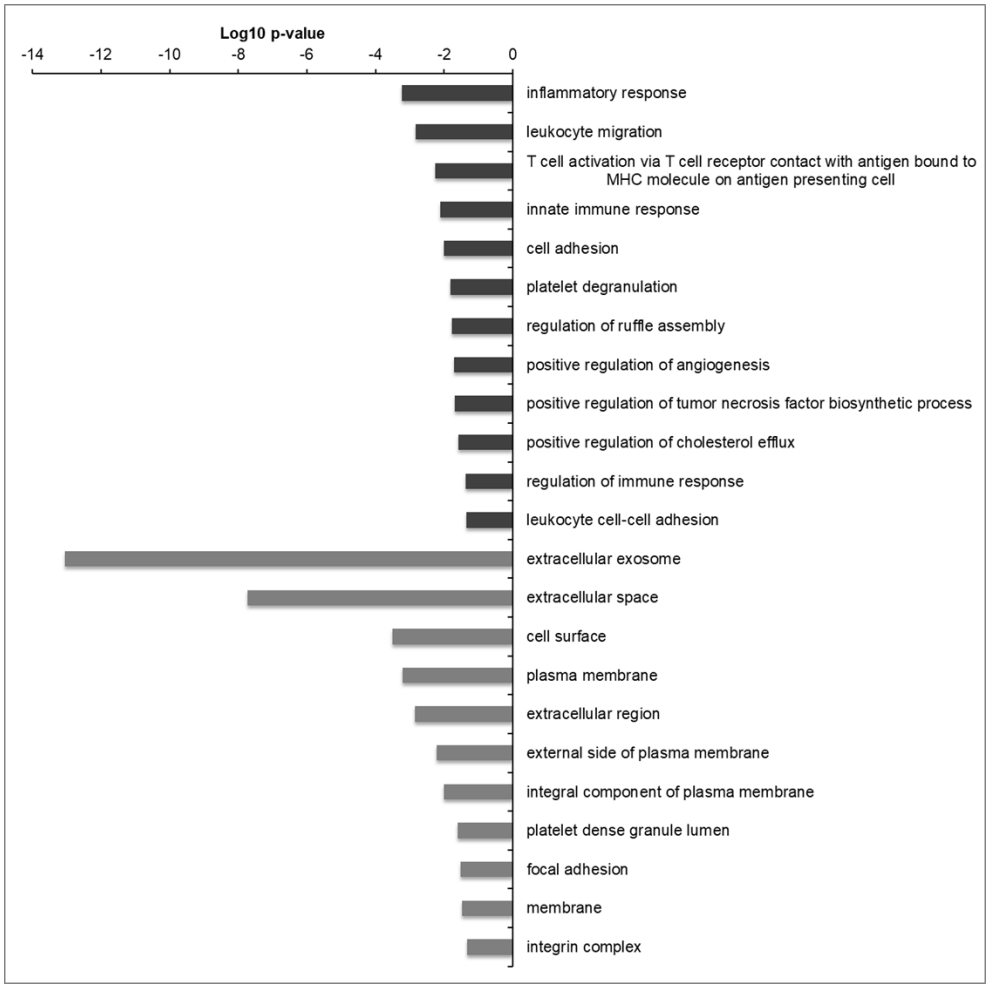


Figure 4.7 **Gene ontology (GO) enrichment analysis of the 33 proteins exclusively associated to *Pv*EVs.** Bar plot shows the more significant GO terms on the category Biological process (black bars) and Cellular component (gray bars). The most over-represented GO terms are shown (p<0.05).

from vivax infection: MSP3.1 and PHISTc. UniProt MSP3.1 595 aa sequence and PHISTc 710 aa sequence (A0A0J9TBC1 and A0A0J9T4K3, respective accession numbers) were analyzed with NetMHCpan (Jurtz et al., 2017) to predict 8-11mer peptides with high binding for twelve representative alleles from the HLA-A and HLA-B supertypes (Sidney et al., 2008). The algorithm calculated the potential binding of 2346 sequences from MSP3.1 and 2806 sequences from PHISTc to each allele and referred it in a rank. The rank threshold for strong binders is 0.5 and for moderate binders is 2. Sequences with higher rank values are not likely to bind. We calculated the median rank of

the twelve HLA alleles for each sequence and ordered the sequences according to it. The 15 sequences with lowest median rank (highest median binding) for each protein are shown in Table 4.2. As expected, there is variable degree of predicted binding of the same peptide sequence to each HLA molecule due to high polymorphism of HLA alleles. Still, we could detect sequences with strong and moderate binding to one or multiple HLA molecules. Comparing the median rank for the sequences of both proteins, lower values can be found for PHISTc sequences, which suggests that this protein might have more potential cytotoxic T-epitopes than MSP3.

4.4 Summary

In this chapter we have demonstrated that circulating EVs from natural *P. vivax* infections contain parasite proteins. Also, we have shown that *P. vivax* infection considerably alters the number and populations of plasma-derived EVs which suggests that EVs might be contributing to several processes of inter-cellular communication. *P. vivax* proteins associated to those EVs could serve as potential antigens. Indeed, we could predict several sequences in two of the identified parasite proteins that could represent promising cytotoxic T-cell epitopes. Altogether this data encourages us to continue investigating the potential contribution of EVs from *P. vivax* infections in the regulation of the immune response.

Protein	First position	Sequence	Binding (Rank)										Median Rank
			A*01:01	A*02:01	A*03:01	A*24:02	A*26:01	B*07:02	B*08:01	B*15:01	B*27:05	B*39:01	B*40:01	B*58:01	
PHISTc	595	VLFNQILSF	2.88	0.76	1.15	0.39	0.57	4.11	1.13	0.04	2.33	2.82	3.34	0.92	1.14
PHISTc	610	KMQEYIMQY	0.54	2.20	0.05	1.59	0.68	10.07	5.69	0.02	2.25	6.98	3.92	0.37	1.90
PHISTc	614	YIMQYSQYL	4.63	0.06	10.74	1.93	0.65	1.66	0.29	2.46	9.39	0.96	6.50	4.76	2.19
PHISTc	626	LLLPTPIRM	3.52	0.13	2.70	3.41	3.70	4.47	2.83	0.81	5.52	2.08	5.22	1.73	3.12
PHISTc	46	FADFNLASL	0.99	1.17	32.55	5.34	2.68	1.68	1.90	8.32	16.60	0.29	4.26	3.67	3.18
PHISTc	375	HRVIDENMPY	1.17	16.18	2.48	20.16	0.04	14.03	22.39	0.35	0.23	2.83	9.41	3.89	3.36
PHISTc	305	RHNQHGRVF	12.98	29.53	12.14	2.06	12.24	2.12	3.34	1.55	3.84	0.65	2.85	4.36	3.59
PHISTc	619	SQYLQKTLL	17.81	3.39	9.94	4.28	9.38	4.10	1.12	0.66	2.73	0.29	1.16	7.60	3.74
PHISTc	382	MPYPPNGPF	4.51	20.57	5.82	5.18	0.60	0.02	0.80	2.31	9.09	0.74	3.81	4.03	3.92
PHISTc	376	RVIDENMPY	0.66	6.69	0.23	6.58	0.09	3.98	14.78	0.01	3.91	7.19	5.99	0.24	3.95
PHISTc	398	IRSEQIAAM	12.32	12.94	11.84	8.00	4.15	3.97	1.99	3.02	0.27	0.04	3.46	8.31	4.06
PHISTc	628	LPTPIRMKY	1.53	23.62	4.42	10.25	0.85	0.64	4.76	2.83	8.28	4.00	6.58	2.07	4.21
PHISTc	52	ASLPYKVLF	3.13	11.89	4.92	0.71	2.79	5.26	6.71	0.77	4.20	7.46	5.05	0.06	4.56
PHISTc	397	SIRSEQIAAM	15.57	7.07	13.31	20.26	1.19	1.28	1.60	2.09	1.10	0.48	8.87	16.48	4.58
PHISTc	589	TVKEMFVLF	5.11	12.04	8.29	0.55	0.16	4.29	1.54	0.54	13.16	10.26	10.48	1.74	4.70
MSP3.1	583	LQTDVNATF	6.14	4.12	13.68	1.00	2.06	6.37	9.82	0.04	2.72	0.49	1.20	1.20	2.39
MSP3.1	201	IADHVVNAY	0.02	9.75	3.16	9.33	0.74	4.69	11.08	0.51	7.02	3.12	6.65	0.45	3.93
MSP3.1	405	ATKADGGETL	5.58	8.70	15.26	13.82	3.56	3.50	9.65	3.09	7.69	1.60	2.75	2.42	4.57
MSP3.1	332	KALENVKAI	29.16	4.36	17.70	5.36	8.77	2.31	1.69	3.80	12.90	3.05	4.79	0.60	4.57
MSP3.1	582	NLQTDVNATF	6.38	4.10	20.82	2.19	1.76	10.90	5.76	0.69	9.19	1.92	2.94	5.71	4.91
MSP3.1	2	HFAGIPLLV	5.44	1.87	5.29	0.63	3.31	10.13	6.86	10.89	4.59	2.07	7.00	5.30	5.30
MSP3.1	227	REIEKLSKI	21.29	7.01	16.74	5.01	5.08	7.41	3.29	3.04	5.85	2.73	0.13	5.60	5.34
MSP3.1	1	KHFAGIPLL	20.13	5.52	9.01	1.05	10.78	5.55	9.21	5.25	0.96	0.05	1.52	2.95	5.38
MSP3.1	434	ARDKAAFEL	5.62	6.12	9.77	4.87	7.04	3.89	9.11	11.43	0.17	0.03	1.41	6.77	5.87
MSP3.1	279	KLKQESTKL	18.19	2.10	5.48	7.27	10.69	2.29	0.74	0.66	14.34	11.07	6.85	5.13	6.17
MSP3.1	424	AVNASKEAM	6.89	13.68	8.11	18.39	2.41	0.89	3.16	0.93	19.31	5.56	7.62	3.33	6.22
MSP3.1	4	AGIPLLVLF	13.14	11.81	10.79	0.74	3.49	7.77	11.04	1.43	4.35	8.13	4.81	0.52	6.29
MSP3.1	22	VVSNENVNL	10.28	2.59	10.12	6.00	4.09	4.17	7.20	4.29	12.97	6.58	10.12	2.50	6.29
MSP3.1	71	LQKSDEASF	17.21	19.88	21.04	4.39	6.12	9.89	6.64	0.02	11.34	6.35	3.98	4.01	6.50
MSP3.1	433	KARDKAAFEL	6.79	13.76	18.40	11.35	17.73	1.29	1.11	7.07	1.13	1.16	6.42	1.78	6.61

Table 4.2 **Sequences of potential cytotoxic T-cell epitopes predicted for *P. vivax* MSP3.1 and PHISTc proteins.** NetMHCpan 4.0 % rank for each HLA allele is shown as well as the median rank for the twelve alleles. The 15 sequences with the lowest median rank are shown for each protein. Rank values below 0.5 (in green) were considered strong binders and rank values higher than 0.5 up to 2 (in blue) were considered moderate binders.

Chapter

5 Results II: Interaction of EVs from *P. vivax* Infections with Human Splenocytes

The spleen is a crucial organ in the control of malaria due to its clearance capacity of parasitized red blood cells and its immunological reactivity, as described in Chapter 1 (see Section 1.1.4.3). However, the understanding of the human spleen and its potential interactions with human malaria parasites is still limited. Therefore, in this chapter, we study the potential interactions of human splenocytes, particularly immune cells, with *Pv*EVs.

The structure of this chapter is the following. Section 5.1 describes the phenotyping of cell populations obtained from human spleens. Section 5.2 presents our assessment of the *in vitro* interaction of human splenocytes with *Pv*EVs and *h*EVs. Finally, Section 5.3 gives a brief summary of the findings presented in this chapter.

5.1 Phenotyping of Cell Populations from the Human Spleen

There are multiple reports on human spleen cells focused on leukocyte populations (Carpenter et al., 2018; Colovai et al., 2004; Langeveld & Gamadia, 2006; Mcilroy et al., 2001) but not on the cells from the red cell lineage. Therefore, before assessing any interaction of *Pv*EVs and *h*EVs with human spleen cells, we would like to have a comprehensive view of the major cell populations present in the human spleen. Of note, we avoided enzymatic digestions of the splenic tissue to avoid alterations of cell surface markers necessary for phenotyping. Instead, we generated a spleen single-cell suspension by mechanical tissue disruption followed by extensive filtration. By using a combination of antibodies (see Chapter 3, Section 3.2.14) and the gate strategy shown in the

Figure 5.1, we characterized the main leukocyte populations as well as mature and immature RBCs.

Our results show that erythrocytes (CD45-CD235a+CD71-) represent the major cell population of the human spleen, accounting for approximately 69.5% of the total cells. Reticulocytes (CD45-CD235a+CD71+) correspond to 0.3% of the total cells (see Figure 5.2A). The leukocyte population (CD45+) is composed by 30.5% of B-cells (CD19+), 16.6% of CD4+ T-cells (CD3+CD4+) and 10.2% of CD8+ T-cells (CD3+CD8+). Natural killer (NK) cells (CD3-CD56+) and natural killer T (NKT) cells (CD3+CD56+) were accounted for 6.2% and 1.7% of the leukocytes, respectively (see Figure 5.2B). The abundance of innate phagocytic cells was very heterogeneous. Neutrophils (CD45+CD15+) are the most abundant, accounting for 15.9% of the leukocytes, although a high variability was observed between different donors. Monocytes (CD45+CD19-CD14+) and DCs (CD45+CD19-CD14-CD11c+) were also present in frequencies of 2% and 0.3%, respectively. Surprisingly, macrophages defined as CD45+CD19-CD14medCD163+ cells were expected to be abundant, but were found in very low proportion (see Figure 5.2B). We also estimated the resident population of hematopoietic stem cells (HSCs) (CD45medCD34+). We found that only 0.02% of cells showed the typical HSC cell surface markers and morphology (see Figure 5.2B).

5.2 *In vitro* Interaction of EVs from *P. vivax* Infections with Human Splenocytes

Interaction of *Pv*EVs and *h*EVs with total spleen cells

Initially, we explored the capacity of total spleen cells to interact *in vitro* with *Pv*Evs and *h*EVs. *Pv*EVs and *h*EVs were purified by SEC and EV-enriched fractions were selected based on the expression of CD71 measured by bead-based flow cytometry as previously described in section 4.1. We labeled *Pv*EVs and *h*EVs with a lipophilic dye (PKH) and after their incubation with total splenocytes, we compared the proportion of different cell populations positively stained with the PKH by flow cytometry as a measurement of the interaction between EVs and cells. We found that in this condition, neither T-cells nor NK/NKT-cells were able to interact with EVs from any source above the background levels (see Figure 5.3). By contrast, an important proportion of phagocytic cells, monocytes and DCs, were found positively stained with PKH indicating interaction with *Pv*Evs and *h*EVs (see Figure 5.3). Interestingly, we observed a higher proportion of PKH67+ monocytes and PKH67+DCs when incubated with *Pv*EVs compared to *h*EVs (see Figure 5.3). This tendency was statistically significant in DCs, but not in monocytes (see Figure 5.3). It

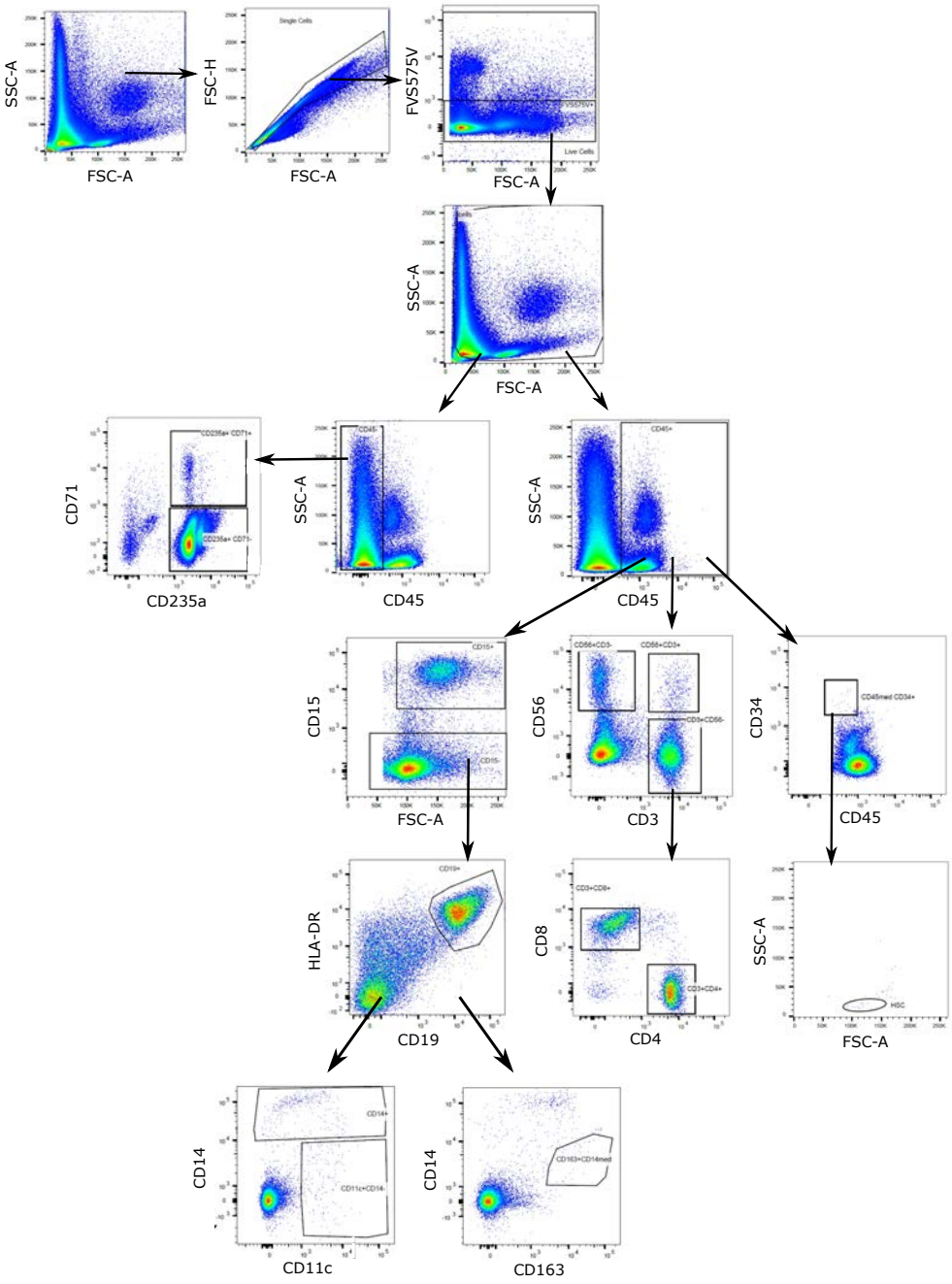


Figure 5.1 **Flow cytometry gate strategy for human spleen cells phenotyping.** Total spleen cells obtained in suspension after mechanical tissue disruption were assessed for viability and phenotyped against surface markers to distinguish cell populations. Representative analysis from one human spleen is shown.

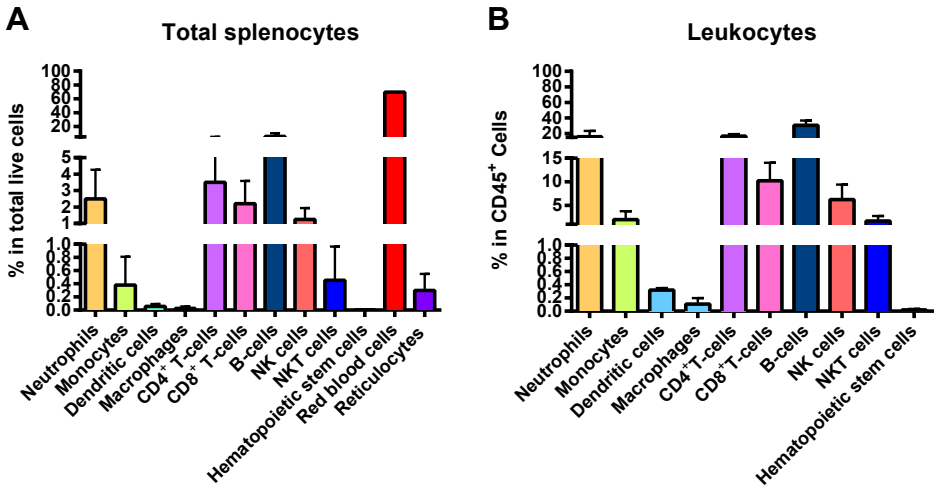


Figure 5.2 **Distribution of cell populations in the human spleen.** (A) Frequencies of different cell populations in total splenocytes. (B) Frequencies of different leukocyte populations in the CD45+ cells. Plots represent the mean and SD of five different spleen donors analyzed independently.

is worth mentioning that the low number of events quantified for the DCs population may be misleading for the significance of this result.

Interaction of *Pv*EVs and *h*EVs with isolated spleen cell populations

The spleen has a structural and functional architecture, where red and white pulp as well as the perifollicular zone are populated by distinctive cells. Indeed, the white pulp has defined areas with differently enriched types of immune cells. We expect that in physiological conditions, circulating EVs would encounter the different cell types in a site-specific manner. Therefore, monitoring the interactions of EVs with particular spleen immune cells could be closer to the *in vivo* scenario than assessing their interaction with total splenocytes.

In order to address the interaction of *Pv*EVs and *h*EVs with specific human immune spleen cells, we have settled a pipeline of cell separation steps involving density centrifugations followed by immunomagnetic cell separation using specific cell surface markers to enrich low abundant spleen dendritic cells and to isolate spleen resident T-cells (see Figure 5.4). The depletion of CD3+ T-cells during Ficoll density centrifugation in this pipeline is intended to remove this large fraction of leukocytes to facilitate the enrichment of low abundant myeloid cells. It could have been useful to deplete B-cells instead of T-cells, since they are more abundant in the spleen. However, we do not have notice of

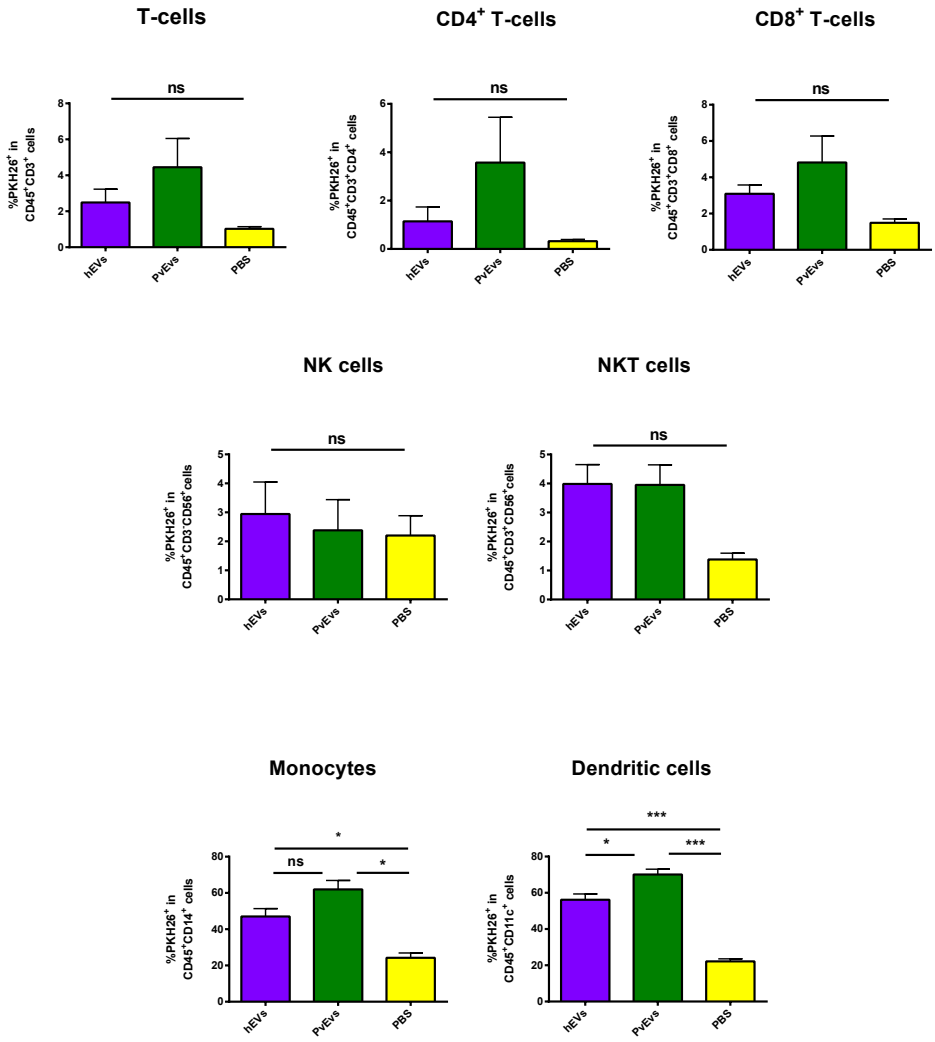


Figure 5.3 ***In vitro* interaction of *Pv*EVs and *h*EVs with total human spleen cells.** Total spleen cells were incubated with PKH26 labelled *Pv*EVs, *h*EVs or PBS for 3h. After incubation, cells were washed and stained against surface markers. Frequency of PKH26+ cells was determined by flow cytometry. Quantification of three technical replicates is shown. Data shows a representative experiment of two different spleen donors analyzed independently. Statistical significance was assessed with One-way ANOVA analysis of variances and Bonferroni multi comparisons test. *** $p<0.0001$, ** $p<0.001$, * $p<0.05$

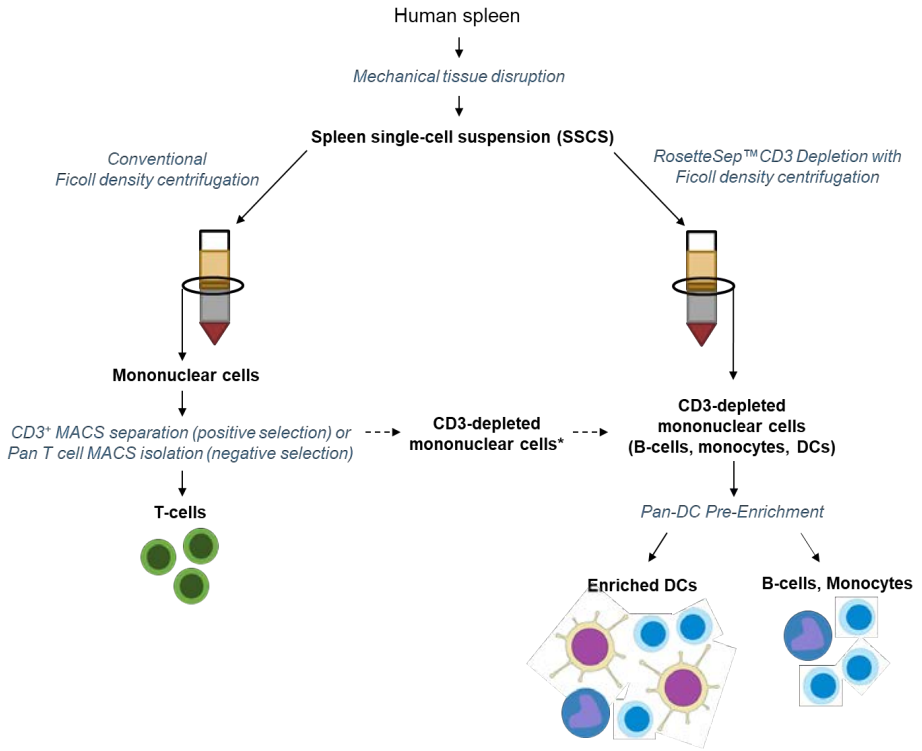


Figure 5.4 **Graphical summary of strategy for separation of different cell populations from human spleens.** *From CD3 negative fractions when CD3+ MACS separation was carried out.

such a straightforward technology for the depletion of B-cells. We observed that our strategy allowed a five-fold enrichment of splenic DCs. Despite this, only 1% of DCs (CD45+CD14-CD11c+) represent the DC-enriched fractions (see Figure 5.5A). Our phenotypic analysis showed that the purity of these cells was largely compromised by a prominent contamination of B-cells (60%) and by contributions of monocytes (0.8%) and neutrophils (0,2%) (see Figure 5.5A). We conducted interactions assays of the PKH67-labelled *Pv*EVs and *h*EVs with the cells from the DC-enriched fraction as well as with the cells retained in the DC-negative fraction of the negative selection step, that mainly corresponded to B-cells and monocytes. We observed that both types of EVs interacted with phagocytic populations and B-cells above the background levels (see Figure 5.5B). There was a tendency to an increased DCs interaction with*Pv* EVs when compared to *h*EVs, it was not statistically significant. Interestingly, we found an increased proportion of B-cells (2.3-fold increase) and monocytes (1.4-fold increase) interacting with*Pv*EVs than with *h*EVs (see Figure 5.5B)). This was also observed in B-cells and monocytes contaminating the DC-enriched fractions (data not shown).

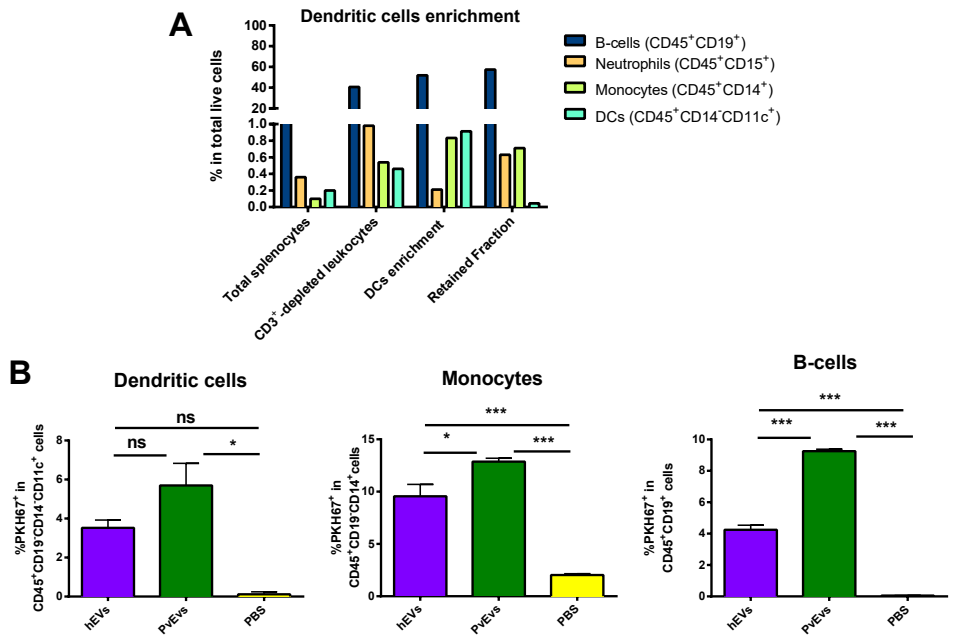


Figure 5.5 ***Pv*EVs and *h*EVs *in vitro* interaction with spleen phagocytic cells and B-cells.** (A) Two-step purification of dendritic cells. Enriched cells were stained with fluorescent conjugated antibodies against surface markers and analyzed by flow cytometry. (B) *Pv*EVs and *h*EVs *in vitro* interaction with spleen DCs, monocytes or B-cells. Frequencies of PKH67+ cells analyzed by flow cytometry are shown. Quantification of three technical replicates is shown. Data is representative of one experiment performed with one spleen donor. Statistical significance was assessed with One-way ANOVA analysis of variances and Bonferroni multi comparisons test. *** $p<0.0001$, ** $p<0.001$, * $p<0.05$.

In regard to the T-lymphocyte population, our purification procedure allows us to obtain nearly 70% enrichment of CD3+ cells, composed by 37% of CD4+ T-cells and 22% of CD8+ T-cells (see Figure 5.6A), was obtained using either negative T-cell selection or positive CD3 selection (data not shown). When we questioned the interaction between purified T-cells and EVs, we observed a four- and three-fold increased proportion of CD4+T-cells and CD8+T-cells, respectively, interacting with PKH67 labeled *Pv*EVs when compared to EVs derived from healthy donors. Importantly, the same result was obtained using negatively isolated T-cells, confirming that the activation state that could have been induced by the anti-CD3 antibodies during its positive selection was not affecting their uptake capacity (data not shown).

We would like to investigate whether EVs carrying parasite proteins might promote splenic immune responses that could contribute to the control of *P. vivax* infection. However, due to several biological and technical limitations that will be discussed in Chapter 8 (see Section 8.2.3), we will assess *Pv*EVs immunogenicity with peripheral blood immune cells.

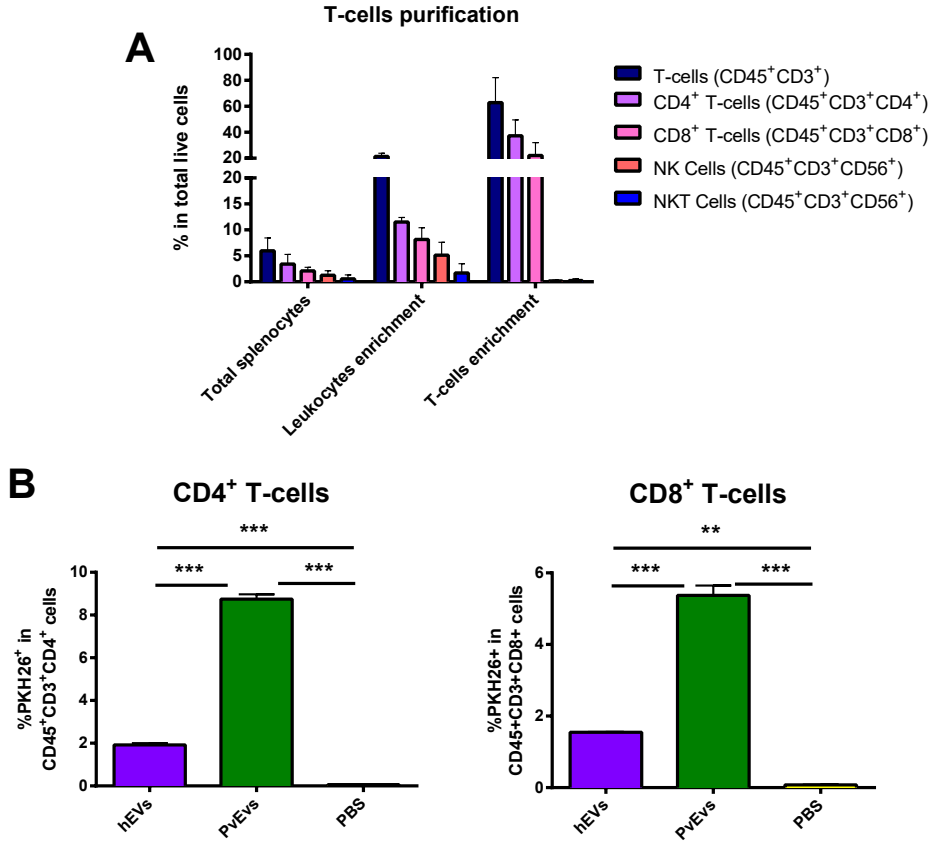


Figure 5.6 ***Pv*EVs and *h*EVs *in vitro* interaction with spleen T-cells.** (A) Two-step purification of T-cells. Enriched cells were stained with fluorescent conjugated antibodies against surface markers and analyzed by flow cytometry. (B) *Pv*EVs or *h*EVs *in vitro* interaction with spleen T-cells. Frequencies of PKH67+ cells analyzed by flow cytometry are shown. Quantification of three technical replicates is shown. Data is representative of one experiment performed with one spleen donor. Statistical significance was assessed with One-way ANOVA analysis of variances and Bonferroni multi comparisons test. ***$p<0.0001$, **$p<0.001$, *$p<0.05$.

5.3 Summary

In this chapter we have provided a comprehensive view of the human spleen by phenotyping its major populations by flow cytometry. We have assessed the interaction of *Pv*EVs *versus* *h*EVs and we have observed that both types of EVs could interact with DCs. Remarkably, there was a statistically significant increased interaction of monocytes, B-cells and T-cells with *Pv*EVs compared to *h*EVs. The biological relevance of these findings awaits further investigation.

Chapter

6 Results III: Immunogenicity of EVs from *P. vivax* Infections

EVs have been shown to modulate antigen presentation to T-cells as well as transport and spread antigens, thus making them attractive to develop vaccines as described in Chapter 1 (see Section 1.2.3). This chapter presents the findings related to the investigation of *Pv*EVs involvement in the activation of T-cell responses. These responses could represent a potential defense mechanism against *P.vivax* infections.

This chapter is structured as follows. Section 6.1 presents the assessment of the expression of HLA class I molecules in circulating EVs. Section 6.2 describes the implementation of an experimental approach in order to study *Pv*EVs ability to induce responses in T-cells from malaria exposed individuals. Section 6.3 presents our exploration of the capacity of *Pv*EVs to stimulate malaria naive T-cells. Finally, Section 6.4 presents a brief summary of the findings described in this chapter.

6.1 Evaluation of HLA class I Expression on Circulating EVs

EVs from vivax patients and healthy donors were purified in sterile conditions by SEC, as previously described, to be used in immunological assays. Importantly, previous work from Junqueira *et al* described that *P. vivax*-infected reticulocytes present malaria antigens to CD8+ T-cells via HLA class I molecules (Junqueira et al., 2018). Therefore, we assessed the expression of HLA class I molecules in circulating EVs since they might facilitate the recognition of potentially HLA-I-bound antigens. We measured its expression with a pan class HLA-I antibody by bead-based flow cytometry. In parallel, we

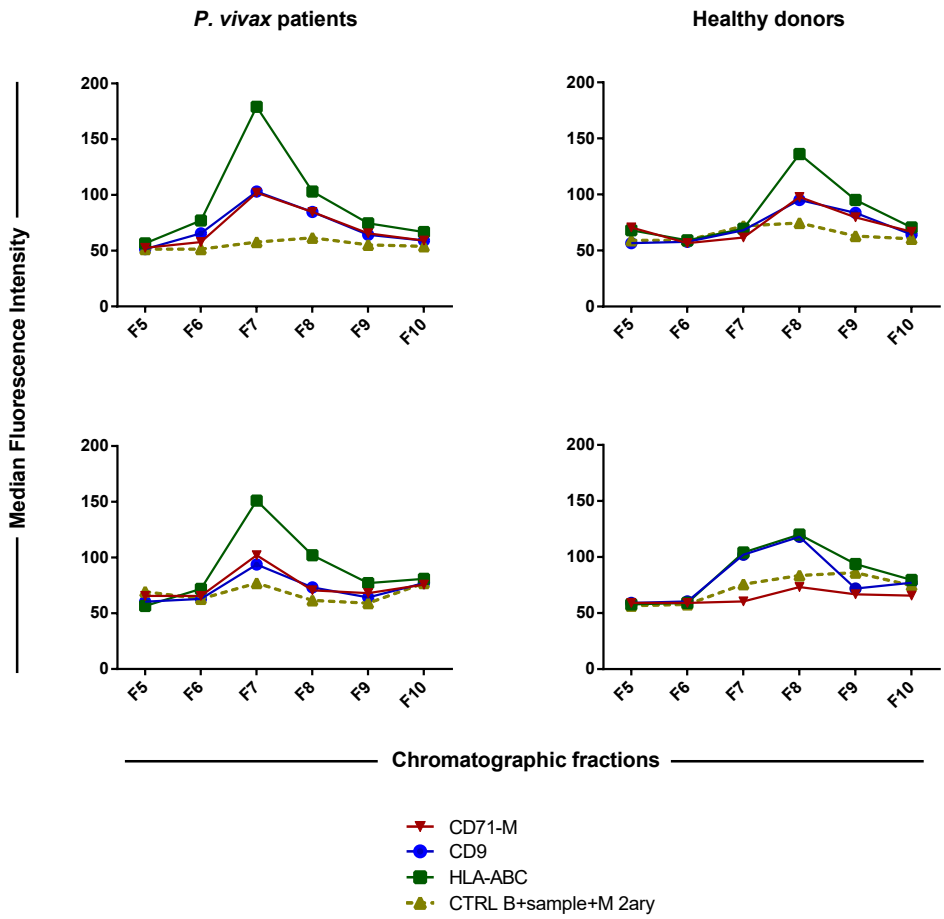


Figure 6.1 **HLA class I, CD71 and CD9 expression on circulating EVs from *P. vivax* patients and healthy donors.** SEC fractions collected after processing plasma from *P. vivax* patients (left part) or healthy donors (right part) were analyzed by bead-based flow cytometry for the presence of HLA class I molecules, CD71 and CD9. Negative controls of EV-beads incubated with secondary antibodies (CTRL B+S+ M 2ary) are also shown. Representative analysis for SEC fractions from two individual PV patients and two individual HD are shown.

assessed the expression of transferrin receptor (CD71), the major component of *Rex*, and CD9, a classical EV marker on chromatographic fractions from vivax patients and healthy donors (see Figure 6.1). Interestingly, the signal of the three markers is coincidental in the same EV-enriched fractions. It is possible that at least part of the HLA-I+ CD71+ EVs could derive from *P. vivax*-infected reticulocytes. Specific markers would be required to confirm it. SEC fractions enriched in EVs expressing HLA-I, CD71 and CD9 and potentially carrying parasite antigens were used in subsequent immunological assays.

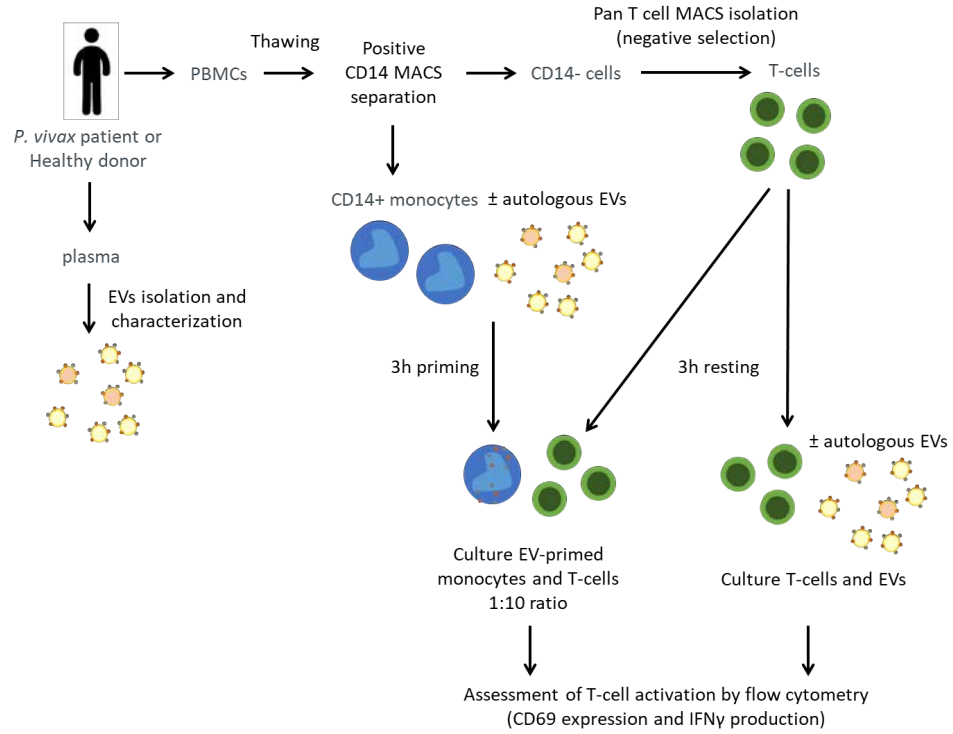


Figure 6.2 **Monocyte-dependent or direct T-cell responses by autologous EVs.** Schematic representation of an experimental approach to determine T-cell activation by autologous circulating EVs from *P. vivax* patients and healthy donors, in a monocyte dependent or direct manner.

6.2 Towards the Assessment of T-cell Responses by *Pv*EVs in Malaria Exposed Individuals

The following procedures should be performed to investigate if*Pv*EVs contain malaria antigens that can be presented to T-cells. Whole peripheral blood from acute vivax patients or healthy donors needs to be collected. Plasma to purify EVs as well as autologous PBMCs have to be isolated. Then, T-cells from malaria-exposed individuals would be stimulated with autologous EVs. As a control, cells from healthy donors need to be stimulated with their autologous EVs. Besides informing us about the stimulatory capacity of *Pv*EVs, these experiments will help us evaluate whether *Pv*EVs carry potential antigens that are targets of natural immunity (see Figure 6.2).

Note that the presence of HLA class I molecules on the EVs suggests that CD8+ T-cell responses could be directly stimulated. However, it is more likely that the presence of APCs is required for an efficient antigen presentation. DCs are considered the most efficient APCs, but they are very infrequent

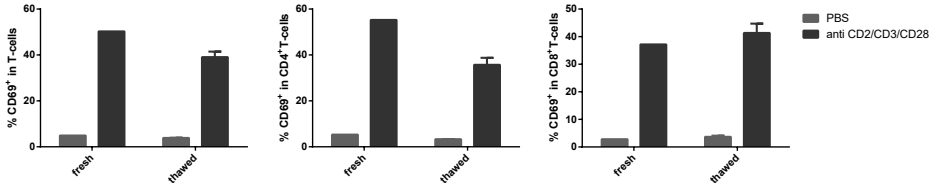


Figure 6.3 **T-cell activation of fresh or thawed T-cells.** Percentage of CD69+ T-cells was assessed by flow cytometry. CD2/CD3/CD28 coated microbeads were used to stimulate negatively isolated fresh or thawed T-cells at a 1(bead):10 (cell) ratio for 18h. As a negative control, cells were incubated with PBS. Data represents fresh T-cells from one donor and thawed T-cells from three different donors.

in circulation and very sensitive to freezing and thawing procedures. Taking into account that we could only obtain 10mL of whole blood from each donor and that their PBMCs were shipped frozen from malaria endemic regions, we decided not to use DCs as APCs. Instead we would use monocytes that even though do not have such a high antigen-presenting capacity, are more abundant (Jakubzick, Randolph, & Henson, 2017). Therefore, we would assess whether T-cells from malaria exposed individuals get activated by EVs carrying parasite proteins in a dependent or independent monocyte manner (see Figure 6.2)

Before collecting samples from malaria exposed individuals, we confirmed that T-cells negatively isolated from thawed PBMCs would be functional to perform these experiments. We isolated T-cells from either fresh PBMCs or thawed PBMCs from healthy donors and stimulated them with anti-CD2/CD3/CD28 coated beads. After 18h of stimulation, we checked the expression of early activation marker CD69 on T-cells by flow cytometry. Both fresh and thawed T-cells were activated by anti-CD2/CD3/CD28, thus indicating that T-cell activation capacity was unaffected by cryopreservation (see Figure 6.3).

Next, we evaluated the feasibility of isolating sufficient number of pure monocytes and T-cells from 10 mL of whole peripheral blood, a restricted quantity of initial sample. We obtained PBMCs from healthy donors, froze them and thawed them as it would be carried out with PBMCs from malaria exposed individuals. After thawing, we had a recovery of 59.9%±6.5 of HD PBMCs with 94.08%±2.47 viability. Monocyte purification was performed by positive isolation with magnetic CD14 microbeads and T-cells were negatively isolated from the CD14- population. Percentage of T-cells (CD3+ cells), B-cells (CD19+ cells) and monocytes (CD14+ cells) in total thawed PBMCs and in purified populations was assessed by flow cytometry using the gating strategy represented in Supplementary Figure A.8. There was an 8-fold enrichment of isolated monocytes compared to total PBMCs, reaching 88% purity in average (see Figure 6.4). We obtained an average purity of 86% of purified T-cells,

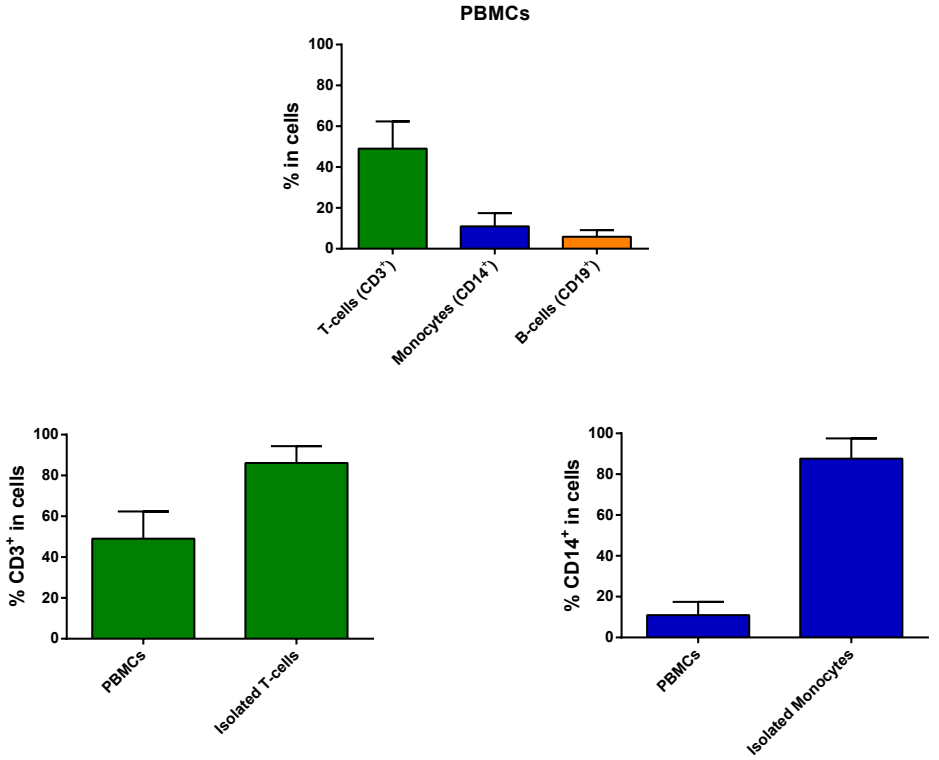


Figure 6.4 **Isolation of monocytes and T-cells from thawed PBMCs.** The percentages of CD3+ (T-cells), CD14+ (monocytes) or CD19+ (B-cells) were analyzed by flow cytometry. Upper part shows the frequency of cells in total PBMCs. Bottom part shows the enrichment of T-cells (left) and monocytes (right) after purification. Data represents six independent purifications from different donors.

representing a 1.6-fold enrichment compared to whole PBMCs (see Figure 6.4). From a starting number of 6.23 x 10^6 ± 1.96 x 10^6 PBMCs, we were able to obtain 7.77 x 10^5 ± 5.52 x 10^5 purified monocytes and 1.70 x 10^6 ± 8.07 x 10^5 isolated T-cells.

Altogether, these results indicate that our experimental approach to assess direct or monocyte-dependent T-cell responses by EVs is feasible. Unfortunately, we have not been able to carry out the stimulation of T-cells from malaria-exposed individuals because the viability of PBMCs shipped from the malaria endemic region was extremely low (data not shown). Currently, we are working on the collection of samples from a new cohort of acute vivax patients and looking for the safest way to ship these samples.

6.3 Stimulation of Malaria Naive T-cells by *Pv*EVs

The impossibility to assess T-cell responses by *Pv*EVs in malaria exposed individuals at the present moment prompted us to explore the capacity of EVs from vivax infections to stimulate malaria naive T-cells. Chances to directly activate naive T-cells *in vitro* by EVs are low given the poor antigen-presentation capacity of EVs. However, the presence of antigen presenting cells could enable the presentation of antigens to naive T-cells (Théry et al., 2002). In order to explore the stimulation of malaria naive T-cells by *Pv*EVs and to reinforce that our strategy to determine T-cell activation by EVs in a monocyte-dependent (or independent) manner is viable, we performed the following experiment: we cultured purified T-cells from malaria naive donors with heterologous EVs isolated from different *P. vivax*-infected patients (*Pv*EVs) or from healthy donors (*h*EVs) as a control. Given the expectable requirement of APCs, in parallel, we co-cultured T-cells with autologous monocytes primed with EVs. After 18h of stimulation, we determined T-cell activation by the expression of the activation marker CD69 and the production of IFN-γ by flow cytometry following the gating strategy presented in the Supplementary Figure A.7.

Expectably, no differences were observed in the CD69 expression and IFN-γ production on malaria naive T-cells directly stimulated with heterologous EVs (*Pv*EVs or *h*EVs) compared to the negative controls (PBS or auto-*h*EVs). Only the positive control, T-cells stimulated with anti-CD2/CD3/CD28 coated beads produced a significant increase in both the expression of CD69 and IFN-γ production. This confirms the activation capacity of the T-cells used (see Figure 6.5, left). However, we could not observe any difference on the CD69 or IFN-γ signal between the T-cells co-cultured with EV-primed monocytes (with *Pv* EVs or *h* EVs) and the T-cells co-cultured with unprimed monocytes (PBS) (see Figure 6.5, right). Given that the CD69 expression and IFN-γ production on T-cells stimulated by EVs are comparable to the ones observed on the negative controls, even in the presence of monocytes, either malaria naive T-cells cannot be stimulated by EVs *in vitro* or this approach is not optimized enough to study the expectable discrete activation of malaria naive T-cells.

One of the potential limitations of this approach might be the rapid assessment of T-cell responsiveness since we measured CD69 expression and IFN-γ production only 18h post-stimulation. This time was enough to detect an increased activation in T-cells stimulated with anti-CD2/CD3/CD28 beads (see Figures 6.3 and 6.5). However, this potent and fast stimulation of T-cells is likely not to be observed upon antigen stimulation. Positive responses to antigen-mediated stimulation might require much longer time, especially when T-cells are naive for the antigen. In order to establish a later readout, we stimulated T-cells with anti-CD2/CD3/CD28 beads and analyzed the kinetics of CD69 expression during T-cell activation. The expression of the early activation

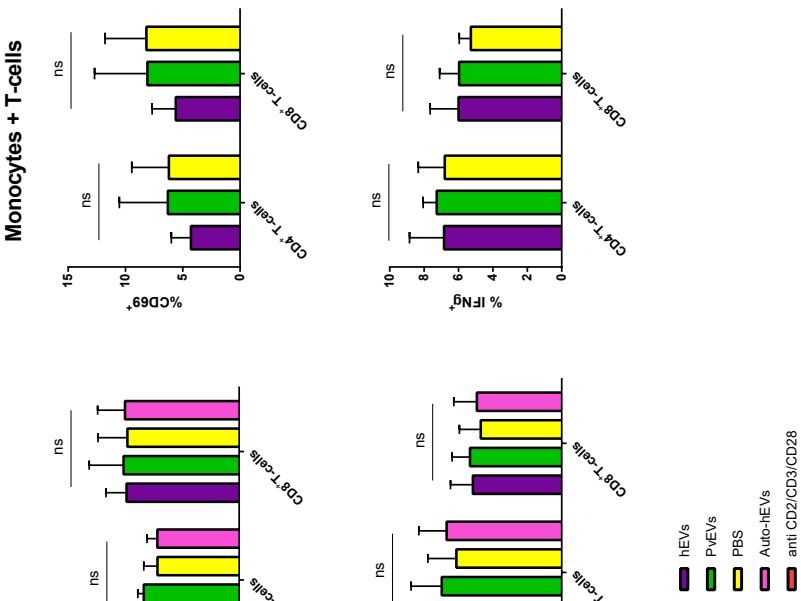


Figure 6.5 **Culture of malaria naive T-cells with EVs in the presence or absence of monocytes..** The percentage of CD69+ or IFN-γ+ T-cells was analyzed by flow cytometry. T-cells were cultured for 18h in the presence of heterologous *h*EVs or *Pv*EVs. As negative controls, cells were cultured with autologous EVs (auto-*h*EVs) or PBS. As a positive control, T-cells were stimulated with anti-CD2/CD3/CD28 beads (on the left and middle). In parallel, monocytes were primed for 3h with the same heterologous *h*EVs or *Pv*EVs, or with PBS as a negative control, and cultured with autologous T-cells (on the right). Data represents three different malaria naive cell donors in triplicate. Kruskal-Wallis and Dunn's multiple comparisons test were used for statistical analysis. ** $p<0.001$, * $p<0.05$.

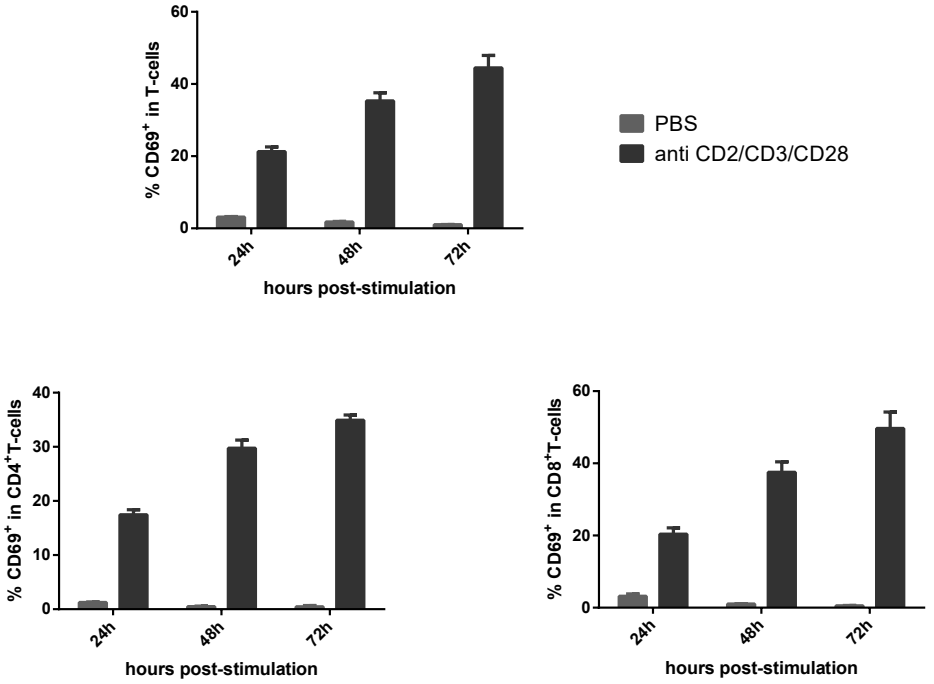


Figure 6.6 **Kinetics of CD69 expression during T-cell activation.** Percentage of CD69+ T-cells was assessed by flow cytometry. CD2/CD3/CD28 coated microbeads were used to stimulate PBMCs at a 1(bead):10 (cell) ratio for 24h, 48h or 72h. As a negative control, PBMCs were incubated with PBS. Data represents measurements from one cell donor in triplicate.

marker CD69 consistently increased till 72h post-stimulation (see Figure 6.6). There was a 1.7-fold increase and a 2.1-fold increase of CD69+T-cells at 48h and 72h post-stimulation, respectively, compared to the levels at 24h post-stimulation. The increase on CD69 expression was comparable in CD4+ T-cells and in CD8+ T-cells (see Figure 6.6). Viability of cells as revealed by staining with Fixable Viability Stain 575V was 82.8 ± 0.6 % at 24h, 80.7 ± 4.7 % at 48h and 76.7 ± 4.7 % at 72h post-stimulation. We decided to analyze the T-cell activation by EVs at 48h post-stimulation in the next experiments.

Despite the priming of monocytes with the EVs before co-culture with T-cells might seem a viable approach to assess the involvement of EVs in antigen presentation, we do not know if other cellular interactions might be required in this process. Therefore, we will assess T-cell activation on total peripheral blood mononuclear cells stimulated by EVs in the upcoming experiments.

Thawed or fresh PBMCs from malaria naive donors were stimulated with heterologous EVs isolated from different *P. vivax* infected patients (*Pv*EVs). Stimulation with heterologous *h*EVs and autologous *h*EVs (auto-*h*EVs) were

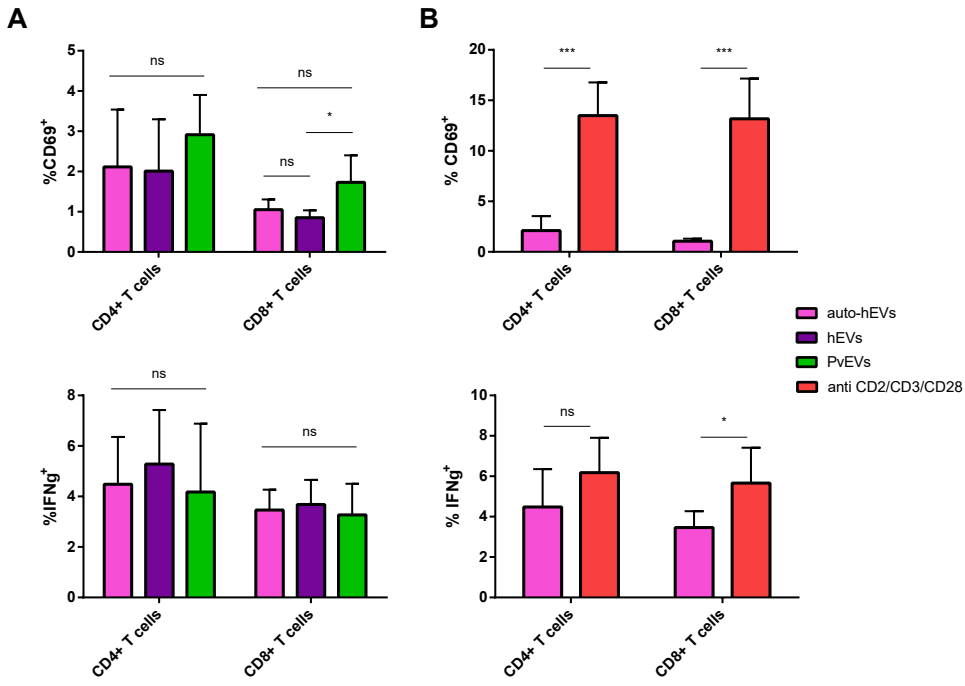


Figure 6.7 ***In vitro* expression of CD69 and IFN-γ production by T-cells upon stimulation of thawed PBMCs with EVs.** The percentage of CD69+ or IFN-γ+ T-cells was analyzed by flow cytometry. (A) Thawed PBMCs from three different malaria naive donors were cultured for 48h in the presence of autologous EVs (auto-*h*EVs), heterologous *h*EVs or *Pv*EVs. (B) As a positive control, PBMCs were stimulated with anti-CD2/CD3/CD28 beads. All conditions were performed in duplicate. Kruskal-Wallis and Dunn's multiple comparisons test were used in A and two-tailored Mann-Whitney was used in B for statistical analysis. ***p<0.0001, *p<0.05.

used as negative controls. T-cell activation was determined after 48h of stimulation measuring expression of CD69 and production of IFN-γ by flow cytometry as previously described. Stimulation of PBMCs with anti-CD2/CD3/CD28 beads or PHA was used as a positive control for T-cell activation.

Upon stimulation of thawed or fresh PBMCs with anti-CD2/CD3/CD28 beads (see Figure 6.7B) or PHA (see Figure 6.8B), we observed a significant increase in the expression of CD69 on T-cells compared to the negative control with auto-*h*EVs. IFN-γ production was significantly increased on thawed CD8+ T-cells stimulated with anti-CD2/CD3/CD28 beads (see Figure 6.7B) compared to auto-*h*EVs, and in fresh T-cells treated with PHA compared to cells stimulated with beads (see Figure 6.8B). Altogether these results confirm that these T-cells are functional. Moreover, we observed a significant increase in the expression of CD69 in thawed CD8+ T-cells stimulated with *Pv*EVs compared to *h*EVs (see Figure 6.7A) that was reproduced in fresh CD8+ T-cells stimu-

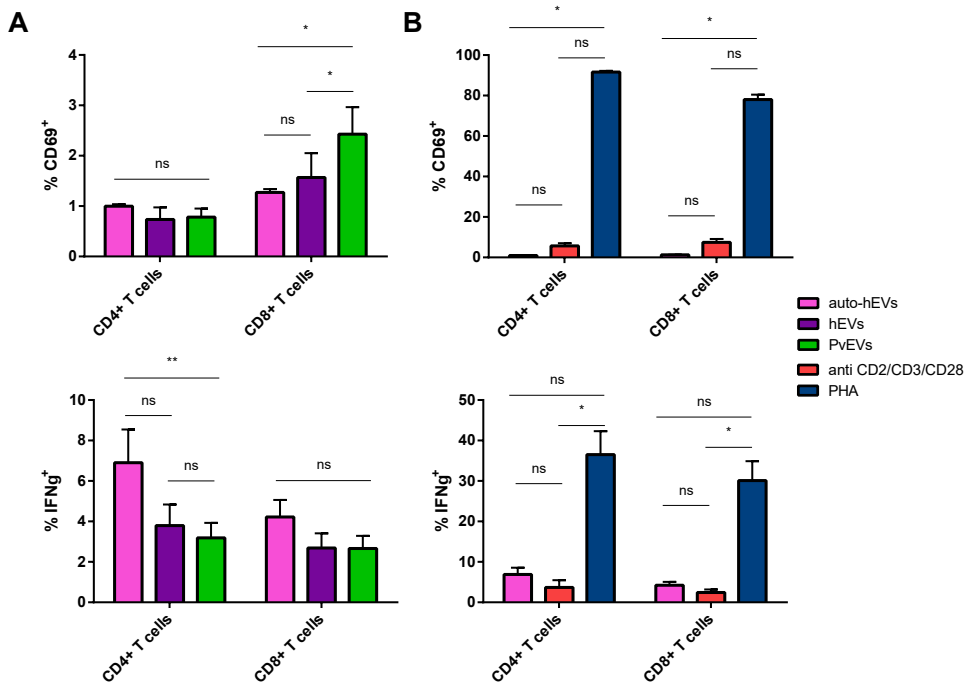


Figure 6.8 ***In vitro* expression of CD69 and IFN-γ production by T-cells upon stimulation of fresh PBMCs with EVs.** Percentage of CD69+ or IFN-γ+ T-cells was analyzed by flow cytometry. (A) Fresh PBMCs from a malaria naive donor were cultured for 48h in the presence of autologous EVs (auto-*h*EVs), heterologous *h*EVs from three different healthy donors or *Pv*EVs from three different vivax patients. (B) As a positive control, PBMCs were stimulated with anti-CD2/CD3/CD28 beads or PHA. All conditions were performed in triplicate. Kruskal-Wallis and Dunn's multiple comparisons test were used for statistical analysis. ** $p<0.001$, *$p<0.05$

lated with *Pv*EVs (see Figure 6.8A). There were no statistical differences on the IFN-γ production on T-cells stimulated with different EVs when using thawed PBMCs. However, there was a decrease in IFN-γ production on fresh CD4+ T-cells cultured with *Pv*EVs compared to those treated with auto-*h*EVs. These inconclusive results need further experimentation to be clarified. Yet, there is a reproducible upregulation of CD69 on CD8+ T-cells when PBMCs were stimulated with EVs from vivax infections. Further experiments will be conducted to confirm whether *Pv*EVs might activate CD8+ T-cell immune responses.

6.4 Summary

In this chapter, we have detected the presence of HLA class I molecules on circulating EVs from *P. vivax* patients and healthy donors, which enables EVs to potentially carry HLA-I-bound antigens. We have set up an approach to

evaluate whether T-cells from malaria exposed individuals get activated by EVs carrying parasite proteins in a dependent or independent APC manner. Future experiments using this approach will be used to evaluate cellular responses of malaria exposed individuals against potential antigens carried in EVs. Finally, we have also explored the stimulation capacity of EVs from vivax infections over malaria naive T-cells. Preliminary results indicate that CD8+ T-cells responses could be activated, but further experimentation is required to corroborate it.

Chapter

7 Results IV: *HuRex*: A Potential Vaccine Platform against *P. vivax* Malaria

Protection elicited by CpG-adjuvanted immunization with *Rex* from *P. yoelii* infection showed evidence that *Rex* might represent a novel platform for vaccination against human reticulocyte-prone malarial infections such as *P. vivax* as described in Chapter 1 (see Section 1.3.2). This chapter presents the findings of our investigation of *HuRex* composition and interaction with APCs in order to study their potential as a vaccine platform.

This chapter is structured as follows. Section 7.1 presents the isolation methodology and mass spectrometry-based proteomics of *HuRex* from cultures of human reticulocyte-enriched cord blood. Section 7.2 describes the *in vitro* interaction of *HuRex* with dendritic cells. Finally, Section 7.3 summarizes the main findings presented in this chapter.

7.1 Isolation and Characterization of Human Reticulocyte-derived Exosomes

Enrichment of human reticulocytes and *in vitro* production and characterization of human reticulocyte-derived exosomes

Enriched reticulocyte samples were obtained from human blood of umbilical cords, a source with higher percentage of reticulocytes than adult humans' blood and with no major proteomic differences between them (Wilson et al., 2016). After removal of leukocytes and concentration on Percoll gradients, percentages of reticulocytes ranged between 20 to 60% among different donors (see Figure 7.1). Reticulocytes were subsequently cultured *in vitro* for 36 h at 1–3% HCT. Of note, our cultures contain significant percentages of mature RBCs, yet these cells lack endocytic machinery and it has been clearly established

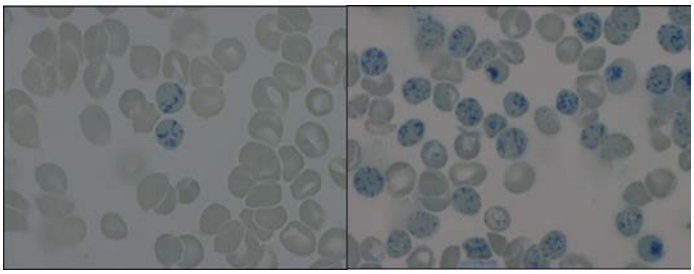


Figure 7.1 **Reticulocyte enrichment from cord blood.** Brilliant cresyl blue-stained thin smears of human cord blood before (left) and after (right) reticulocyte enrichment.

that they do not secrete *bona fide* exosomes (Blanc et al., 2009; Johnstone et al., 1987). Furthermore, we discarded reticulocyte-enriched preparations that contained over 2% of contaminating leukocytes to minimize the presence of leukocyte-derived vesicles in the cultures. We refrained from using CD71 affinity bead purification as there is a large heterogeneous population of reticulocytes from CD71negative to CD71high (Malleret et al., 2013). *HuRex* were produced *in vitro* in the presence or absence of serum, previously depleted of EVs, as there is evidence suggesting that the protein cargo varies significantly in the absence of serum (J. Li et al., 2015). Before and after culture, cell viability was assessed by microscopy using Trypan Blue stain. We demonstrated 95.6 $\pm$ 2.1% of cell viability after 36 hours of culture independently of serum supplementation, thus excluding a major proteomic confounding due to apoptotic vesicles in culture supernatants. It has also been emphasized that another factor affecting the quality of EV preparations is the method of purification (Abramowicz, Widlak, and Pietrowska 2016); therefore, we purified *HuRex* by either UC (Théry et al., 2006) or SEC (de Menezes-Neto et al., 2015). The use of sequential centrifugation removing most apoptotic bodies (1,300 g pellet) and microvesicles (15,000 g pellet) (Théry et al., 2006) and the use of SEC also removing these types of vesicles (de Menezes-Neto et al., 2015), further emphasizes that we are mostly isolating exosomes derived from reticulocytes (Lötvall et al., 2014). However, we cannot exclude the possibility that a small number of these proteins are wrongly assigned to *Rex* due to intrinsic variability of human samples and the lack of a unique method for isolation of EVs.

HuRex were first subjected to NTA. Results demonstrated that *HuRex* were homogeneous with a median size of 127 nm and that particle concentration was in the range of 10^8 particles/μL. Particle concentration in SEC fractions was always lower than in UC preparations (see 7.2A). We determined the presence of the major component of *Rex*: the transferrin receptor, CD71 (Harding et al., 1983; Pan & Johnstone, 1983; Vidal, 2010) by bead-based flow cytometry. Higher MFI values for UC compared to those for SEC were always observed

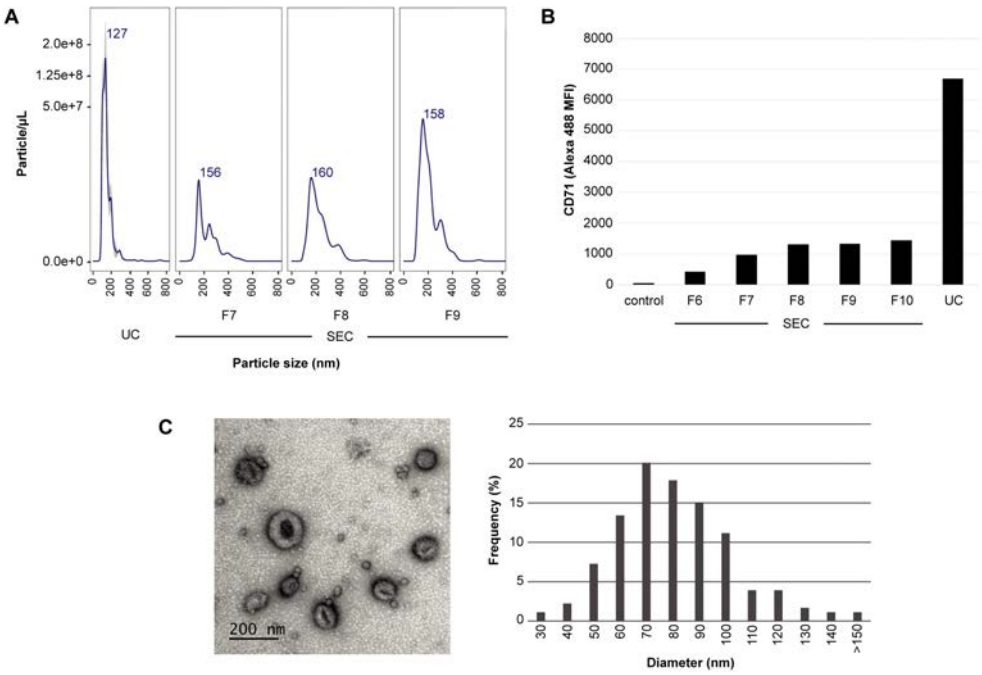
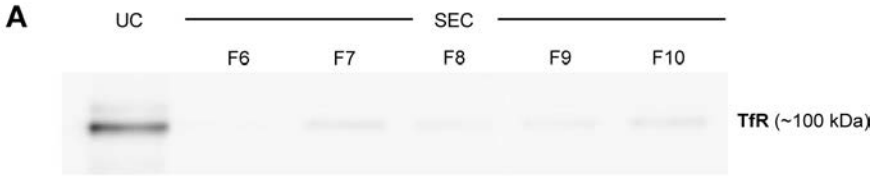


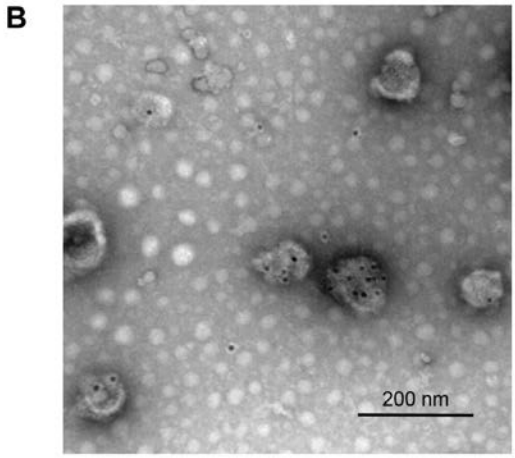
Figure 7.2 **Isolation and characterization of exosomes from human cord blood reticulocytes.** (A) NTA profiles of *HuRex* from ultracentrifugation (UC) and size exclusion chromatography (SEC) fractions. Concentration is shown in particle/μL. (B) Flow cytometry analysis of transferrin receptor, CD71, in *HuRex*. MFI, Median Fluorescence Intensity. (C) Electron microscopy. Representative TEM image on the left. Bar represents 200 nm. Size distribution from TEM images quantified by ImageJ on the right. nm, nanometers.

(see Figure 7.2B). To confirm vesicle integrity, *HuRex* preparations obtained by UC were analyzed by transmission electron microscopy (TEM) by means of negative staining (see Figure 7.2C). As expected from fixation and dehydration (van der Pol et al., 2014), size distribution of TEM images revealed smaller vesicle mean size (70 nm), than the size recorded by NTA (see Figure 7.2C).

Detection of the transferrin receptor on human reticulocyte-derived exosomes

The transferrin receptor is completely and selectively removed in EVs during the terminal differentiation of reticulocytes into erythrocytes (Harding et al., 1983; Pan & Johnstone, 1983; Vidal, 2010). We first analyzed the presence of this protein by immunoblot in *HuRex* purified by UC and SEC (see Figure 7.3A). As expected from concentration of particles/μL (see Figure 7.2A), a higher signal was observed in the UC preparation as compared to SEC fractions. Next, immunoelectron microscopy demonstrated that TfR is associated with EVs corroborating their reticulocyte origin (see Figure 7.3B).





C

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  1 MMDQARSAFS NLFGGEPLSY TRFSLARQVD GDNSHVEMKL AVDEEENADN NTKANVTKPK RCSGSICYGT IAVIVFFLIG FMIGYLGYCK GVEPKTECER
101 LAGTESPVRE EPGEDFPAAR RLYWDDLKRK LSEKLDSTDF TGTIKLLNEN SYVPREAGSQ KDENLALYVE NQFREFKLSK VWRDQHFVKI QVKDSAQNSV
201 IIVDKNGRLV YLVENPGGYV AYSKAATVTG KLVHANFGTK KDFEDLYTPV NGSIVIVRAG KITFAEKVAN AESLNAIGVL IYMDQTKFPI VNAELSFFGH
301 AHLGTGDPYT PGFPSFNHTQ FPPSRSSGLP NIPVQTISRA AAEKLFGNME GDCPSDWKTD STCRMVTSES KNVKLTVSNV LKEIKILNIF GVIKGFVEPD
401 HYVVVGAQRD AWGPGAAKSG VGTALLLKLA QMFSDMVLKD GFQPSRSIIF ASWSAGDFGS VGATEWLEGY LSSLHLKAFT YINLDKAVLG TSNFKVSASP
501 LLYTLIEKTM QNVKHPVTGQ FLYQDSNWAS KVEKLTLDNA AFPFLAYSGI PAVSFCFCED TDYPYLGTTM DTYKELIERI PELNKVARAA AEVAGQFVIK
601 LTHDVELNLD YERYNSQLLS FVRDLNQYRA DIKEMGLSLQ WLYSARGDFF RATSRLTTDF GNAEKTDRFV MKKLNDRVMR VEYHFLSPYV SPKESPFRHV
701 FWGSGSHTLP ALLENLKLRK QNNGAFNETL FRNQLALATW TIQGAANALS GDVWDIDNEF
```

Figure 7.3 **Detection of the transferrin receptor (TfR) in *HuRex*.** (A) Immunoblot in *HuRex* purified by UC and SEC fractions. (B) Immunogold labelling of UC-*HuRex* using a secondary antibody conjugated to 10 nm-gold spheres. Scale bar represents 200 nm. (C) Protein coverage by unique peptides (grey boxes) identified by MS. The peptide sequence YTRFSLARQV, corresponding to the binding domain of TfR for hsc71, is boxed in black. UniProtKB – P02786 TfR sequence is shown.

Proteomic analysis of human reticulocyte-derived exosomes

Characterization by mass spectrometry, LC-MS/MS, was performed over*HuRex* from six different cord blood donors to determine their molecular composition. As research on the molecular cargo of EVs is largely confounded by the lack of a "gold-standard" methodology (Abramowicz, Widlak, and Pietrowska 2016), we applied several approaches for obtaining *HuRex* prior

to MS. *HuRex* preparations were obtained in absence of serum (AS) from three donors and isolated by means of SEC (n = 3) and UC (n = 3). *HuRex* preparations in the presence of serum were obtained from three other donors and purified by SEC (n = 3) and UC (n = 1). In total, 367 different proteins were identified in the 10 MS-dataset according to UniProt accessions, although protein numbers from each cord blood were different from each other due to the intrinsic variability of such samples (see Supplementary Dataset B.4).

Most of the proteins identified by SEC are a subset of the ones identified by UC (not shown). Thus, after removal of leukocytes, the use of SEC and culturing of human reticulocytes from cord blood in the presence of serum, depleted of EVs, seems a robust method for furthering studies of *HuRex*. To further verify the reticulocyte origin of EVs (Harding et al., 1983; Pan & Johnstone, 1983; Vidal, 2010), we first demonstrated the presence of TfR in all MS-datasets (see Supplementary Dataset B.4). In addition, 45 unique peptides, including the sequence YTRFSLARQV previously shown to interact with hsc70 (Géminard, Nault, Johnstone, & Vidal, 2001), and covering 64.08% of this receptor, were identified (see Figure 7.3C). Further western blotting analysis on these vesicles confirmed the detection of the raft-associated protein stomatin, previously associated to*HuRex* (Gassart, Ge, & Fe, 2003) and verified the identification of newly detected proteins such as HSP70 and GAPDH (see Figure 7.4A).

Proteins were assigned different subcellular locations and percentages according to Gene Ontology (GO) annotation obtained from UniProt: Extracellular region (25.0%), Cytosol (15.9%), Plasma membrane (14.9%), Nucleus (13.5%), Cytoskeleton (5.9%), Lysosome (5.7%), Endosome (5.7%), Golgi apparatus (4.5%), Endoplasmic reticulum (3.9%), Mitochondrion (3.4%), "Not retrieved" (1.1%) and Peroxisome (0.5%) (see Figure 7.4B). Of note, most of the proteins identified in this work were already listed in Vesiclepedia, a public data repository for extracellular vesicle cargo (Kalra et al. 2012) (see Figure 7.4C). Moreover, in agreement with these data, over-representation GO analysis of cellular component revealed that the bulk of proteins identified in *HuRex* correspond to extracellular exosome (see Figure 7.5 and see Supplementary Dataset B.5). According to the idea that*HuRex* remove adhesins (Blanc and Vidal 2010; Vidal 2010), GO analysis of molecular function revealed protein binding as a major function of *HuRex* (see Figure 7.5 and see Supplementary Dataset B.5).

Biochemical analysis of EVs obtained from sheep reticulocytes originally demonstrated that in addition to TfR several membrane enzymes and transporters were also selectively removed by EVs (Johnstone et al., 1987). Accordingly, we identified several of such and other membrane proteins, namely: Na+/K+ transporting ATPase (ATP1A1, ATP1B3), calcium-transporting ATPase (ATP2B4), neutral amino acid transporters (SLC1A5, SLC43A1, SLC7A5), and glucose transporters 1, 2, 3 and 4 (SLC2A1, SLC2A2, SLC2A3, SLC2A4). A notable exception was acetylcholinesterase which was reported

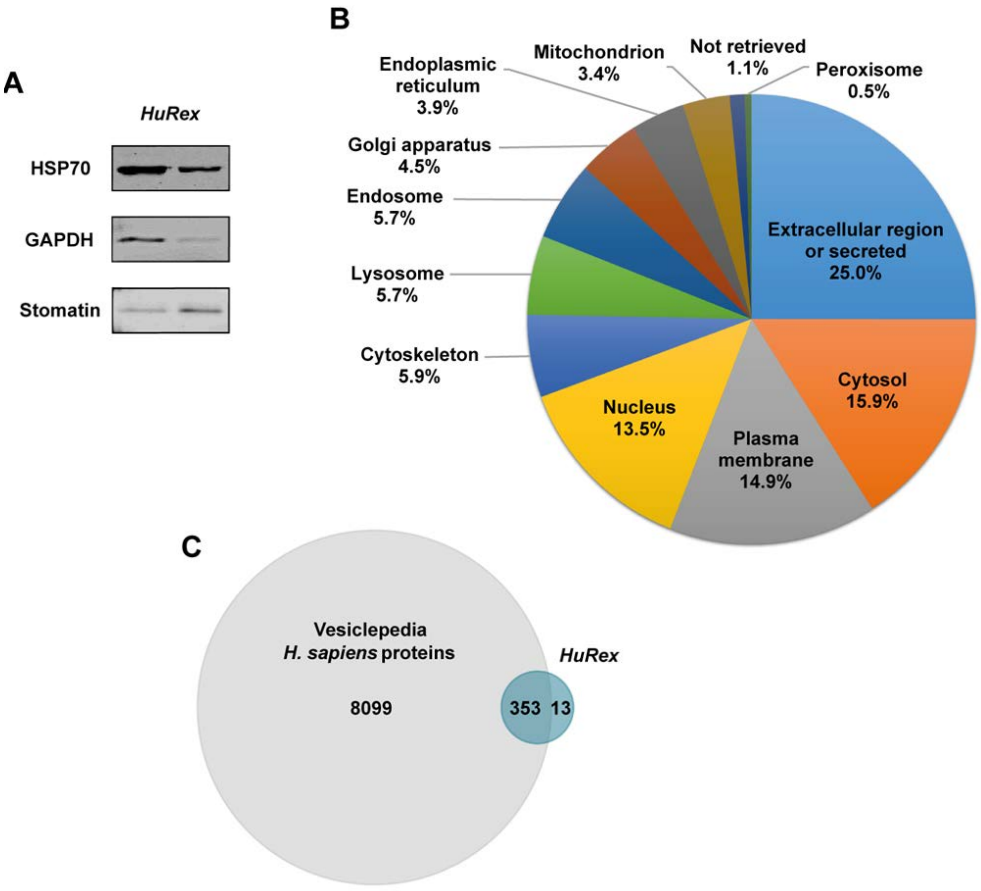

Figure 7.4 **Proteome of human reticulocyte-derived exosomes identified by LC-MS/MS.** (A) Western blot validation for HSP70, GAPDH and stomatin on UC-*HuRex*. Samples were purified from two cord blood donors, each one loaded in a different lane. (B) Distribution of the proteome of *HuRex* in subcellular location categories retrieved from UniProt database according to Gene Ontology (GO) annotation. (C) Venn diagram showing the overlap of proteins detected in *HuRex* and those reported in Vesiclepedia, a database of extracellular vesicle cargo.

to have high activity in the original description of exosomes (Johnstone et al., 1987). Other MS studies of human reticulocytes and *Rex* from other species (Carayon et al., 2011; Gautier et al., 2016) have also failed to detect this enzyme. Without other alternative method confirming its absence, we cannot rule out this is due to technical issues. Several other plasma membrane proteins previously not reported in *HuRex* were identified in our analysis. These include several integrins alpha and beta (ITGA2B, ITGA4, ITGAM, ITGB1, ITGB2, ITGB3) and transporters (SLC6A9, SLC7A1) among others (see Supplementary Dataset B.4).

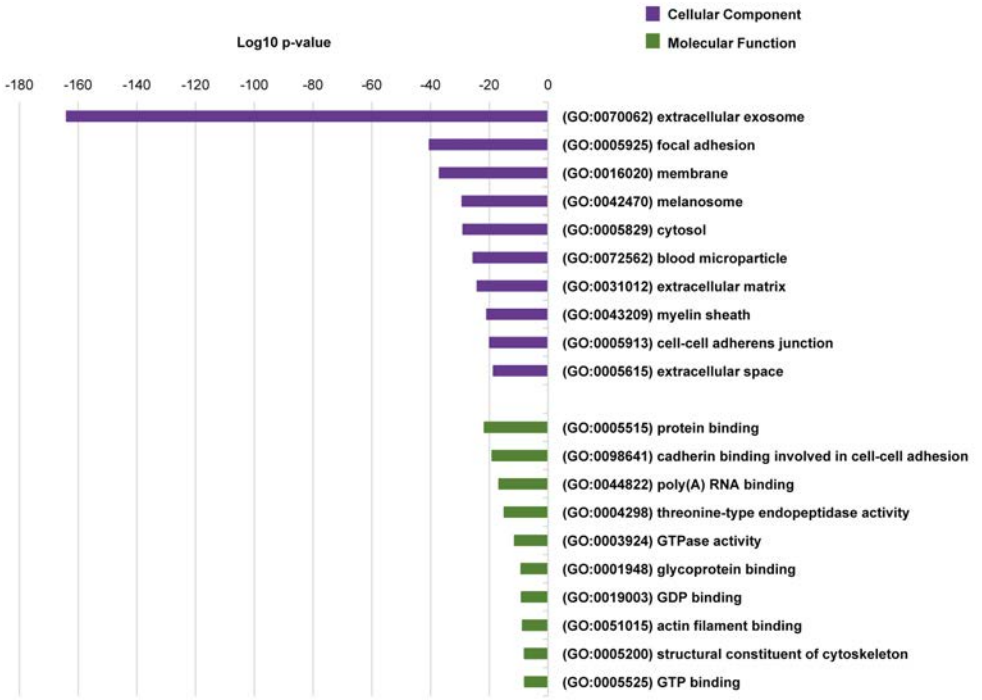

Figure 7.5 **GO enrichment analysis of proteome of *HuRex*.** GO term-enrichment at cellular component and molecular function level performed with Database for Annotation, Visualization and Integrated Discovery (David 6.8). The most over-represented GO terms are shown.

Previous functional enzymatic analyses of *Rex* revealed little or no activity of the cytosolic enzymes lactate dehydrogenase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase (Johnstone et al. 1987). All these enzymes were identified in our MS analysis,*albeit* with different coverage and numbers of unique peptides (see Supplementary Dataset B.4). Of note, glyceraldehyde-3-phosphate is inactive when binding to band 3 (Tsai, Murthy, and Steck 1982) but this explanation for the lack of activity was discarded as band 3 was not acknowledged to be present in *Rex* (Johnstone et al., 1987). Yet, our studies identified band 3 suggesting this alternative explanation. Other cytosolic enzymes and proteins associated with *HuRex* are worth mentioning. Several Rab GTPases were identified, in particular Rab7a, Rab11b, and Rab14. In contrast, we failed to identify Rab27, which is known to play a main role in EV biogenesis in other cells (Blanc & Vidal, 2017). Similarly to acetylcholinesterase, further experimentation would be required to demonstrate its absence or by the contrary, prove its association to*HuRex*. We also detected five S100 proteins, calcium-modulated proteins pertaining to a vertebrate multigene family which in humans contain

24 members (Donato et al., 2013). Notoriously, we identified S100-A9 with a coverage of 89.47%, a protein that has been recently found in plasma-derived EVs associated to chronic lymphocytic leukemia (Prieto et al., 2017). We have identified several histones, including histone H4 previously shown to be largely exported to the cytoplasm (Gautier et al., 2016). Last, we and others (Gautier et al., 2016) failed to identified Tsg101, a major player in the biogenesis of EVs in other cells (Colombo et al., 2014).

We crossed *HuRex* proteome with reported red cell MS proteomes from human reticulocytes (Chu et al., 2017; Gautier et al., 2016; Wilson et al., 2016) and mature RBCs (Bryk & Wiśniewski, 2017; D'Alessandro, Dzieciatkowska, Nemkov, & Hansen, 2017; Pasini et al., 2006; Wilson et al., 2016). For that we first compared the cell proteomes (see Figure 7.6) and we defined a reticulocyte core proteome of 587 proteins and a mature RBC core proteome consisting of 1055 proteins. The intersection of*HuRex* proteins with the core proteomes of reticulocytes and matured RBCs (see Figure 7.6 and see Supplementary Dataset B.6) showed that many of the proteins identified in *HuRex* are also detected in these works, most of them associated to plasma membrane and cytosol (see Supplementary Dataset B.6). Moreover, when we performed a direct comparison of the proteins identified in *HuRex* with the mentioned human reticulocyte proteomes (Chu et al., 2017; Gautier et al., 2016; Wilson et al., 2016), around 70% of the proteins described in *HuRex* have been detected in their cell of origin (see Figure 7.6).

Even though in this first MS proteomic description of *HuRex* we focused on protein identification, we calculated the normalized spectral abundance factor (NSAF) (Paoletti et al., 2006) (one of the most common protein quantification indexes used in label-free quantification proteomics based on the spectral counting) (Nahnsen, Bielow, Reinert, & Kohlbacher, 2013) to estimate relative protein abundance. We observed that previously detected proteins in *HuRex* such as TfR and stomatin, as well as newly identified like S100A9 and HSPA8, were found among the 50 most abundant proteins (see Figure 7.7 and Supplementary Dataset B.7).

7.2 Interaction of *HuRex* with Dendritic Cells

MHC class-I molecules are involved in antigen presentation where professional antigen presenting cells such as dendritic cells are usually required to process and present antigens to T-cells (Chaput & Théry, 2011). As we detected HLA class-I antigens in *HuRex* and our own previous results demonstrated the presence of MHC class-I molecules in EVs from rodent malaria infections (Martín-Jaular et al., 2016; Martin-Jaular et al., 2011), we decided to run a functional assay to determine if *HuRex* could be uptaken by dendritic cells.

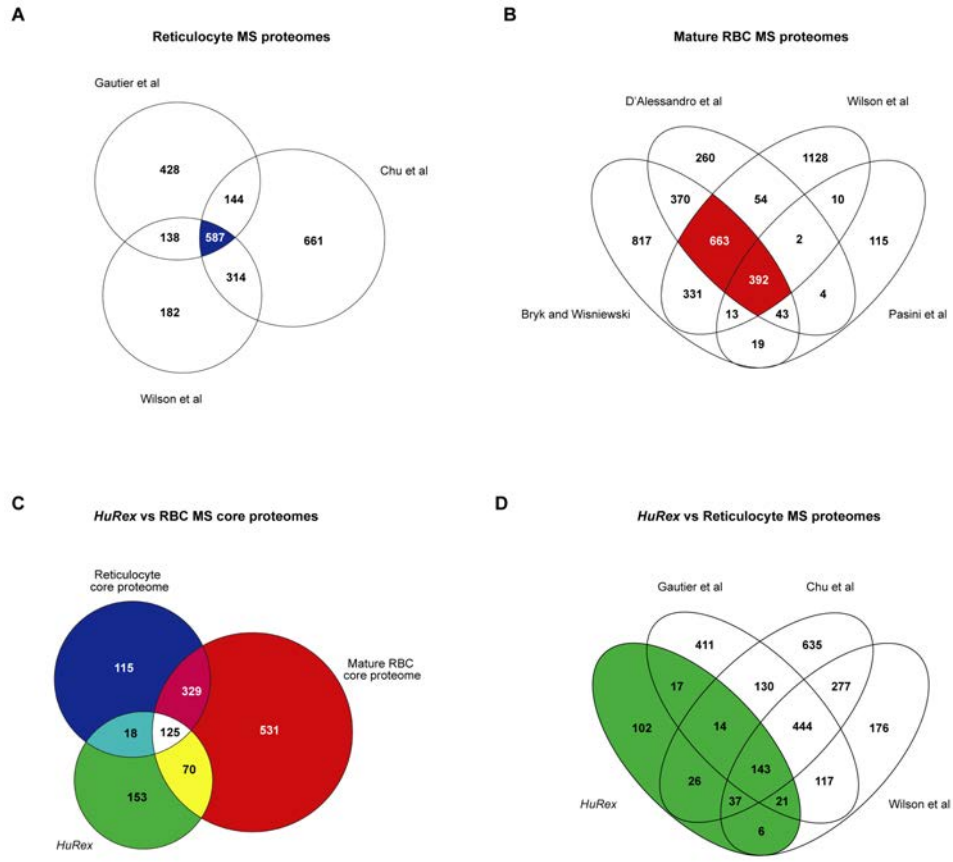

Figure 7.6 **Comparative analysis of *HuRex* with human red cell MS proteomes.** (A) Intersection of human reticulocyte MS proteomes. Core proteome consists of 587 proteins marked in blue. (B) Intersection of human mature RBC MS proteomes. Core proteome consists of 1055 proteins marked in red. (C) Intersection of *HuRex* proteome with the reticulocyte core proteome and the mature RBC core proteome. Those proteins common to both core proteomes (white), to reticulocytes (cyan), to mature RBCs (yellow) and those exclusively related to *HuRex* are listed in the Supplementary DataSet B.6 (D) Intersection of *HuRex* proteome with human reticulocyte MS proteomes. All comparisons have been performed using gene name annotation.

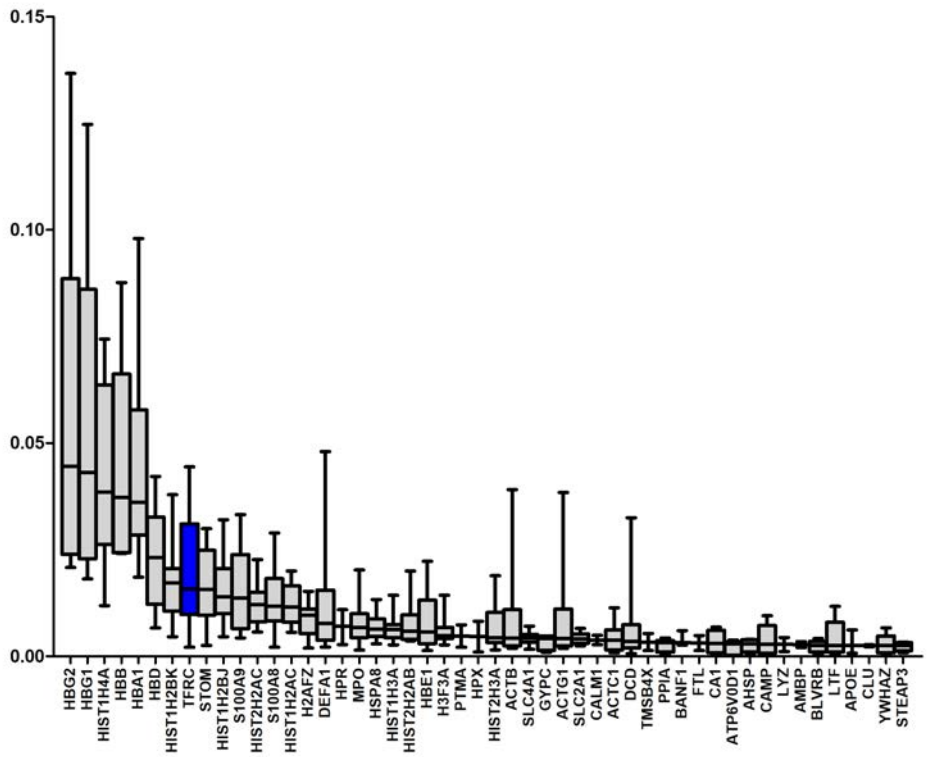

Figure 7.7 **The 50 most abundant proteins in *HuRex* by NSAF.** Proteins, annotated by Gene ID, with the highest NSAF median.

This assay had previously demonstrated that the sialic acid-binding Ig-like lectin 1, Siglec-1, is responsible for exosome capture on mature DCs and follows the same trafficking route as HIV-1 particles (Izquierdo-Useros et al., 2012; Izquierdo-Useros et al., 2009). Flow cytometry analysis revealed that 19 ± 8% of mature monocyte-derived dendritic cells actively uptake *HuRex* labeled with DiI (*HuRexDiI*) (see Figure 7.8A).

We next assessed if *HuRexDiI* were retained within the same sub-cellular compartment as fluorescent HIV-1 VLP. As shown in Figure 7.8B , mDCs exposed to*HuRexDiI* and subsequently exposed to HIV-1 VLP retained both types of vesicles within the same sack-like compartment, as analyzed by confocal microscopy.

Last, pretreatment of mDCs with a monoclonal antibody against Siglec-1 significantly inhibited *HuRexDiI* capture (see Figure 7.8C). Thus, *HuRex* are efficiently captured by mDCs via Siglec-1.

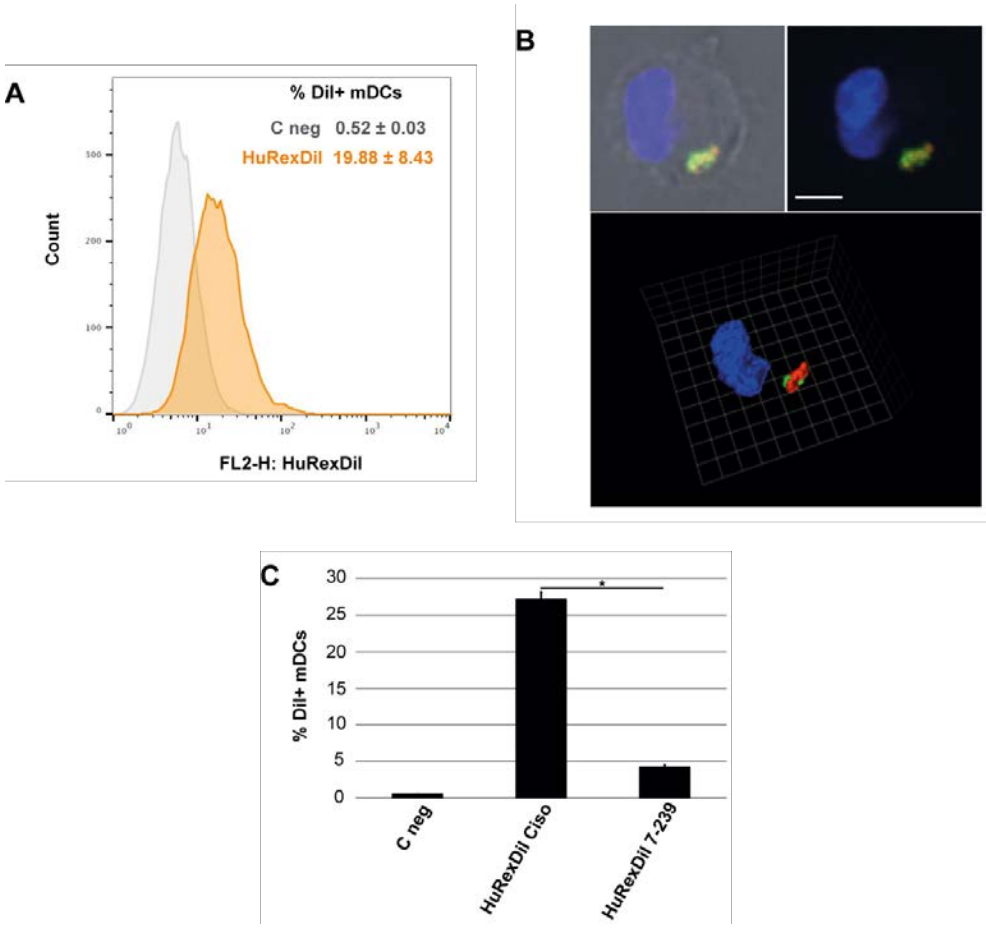

Figure 7.8 **Siglec-1 dependent capture of *HuRex* by mature dendritic cells.** (A) Flow cytometry analysis of *HuRexDiI* capture by mDCs. (B) Confocal microscopy co-localization of *HuRexDiI* (in red) and VLP*HIV-Gag-eGFP* (in green) in mDCs (nuclei stained with DAPI). Top: z-plane showing fluorescence and bright field (scale bar 5 μm); Bottom: 3D reconstruction of z-planes (reference scale unit 2,48 μm). (C) Inhibition of *HuRexDiI* capture by mDCs by blocking of Siglec-1 with α-Siglec-1 mAb. mDCs not treated with antibodies nor with *HuRexDiI* were incubated in parallel (C neg). Data show mean values and SD from 2 donors. T-test $^*p<0.05$. mDCs, mature DCs.

7.3 Summary

In this chapter we have presented the first mass spectrometry-based proteomic analysis of human *Rex* , consisting of 367 proteins. These data represent a valuable resource to better understand erythropoiesis and drive novel therapeutic and diagnostic strategies for red cell diseases. Noticeably, we identified HLA class-I molecules. The presence of these key molecules for antigen presentation in *Rex* suggests that they might facilitate the detection of vivax components

by the immune system during the reticulocyte-prone *P. vivax* infection. Interestingly, we found out that dendritic cells, professional antigen presenting cells, uptake *HuRex* and might contribute to the presentation of potential antigens carried in these EVs.

Chapter

8 Discussion and Future Work

The control of malaria infections would benefit from the development of highly-effective vaccines. This requires much further work, especially in the field of *P. vivax* vaccines. A better understanding of the unique *P. vivax* biology and the mechanisms of protective immunity against this parasite is urgently needed to develop such vaccines. Additionally, antigen discovery, functional testing and the use of novel vaccine platforms, among other strategies, should be substantially expanded to accelerate vaccine development against this neglected parasite.

EVs during infections have been shown to carry pathogen antigens and elicit immune responses. Remarkably, reticulocyte-derived exosomes from a reticulocyte-prone murine malaria model contain parasite proteins and confer long-lasting protective immune responses when used in CpG-adjuvanted immunizations.

We hypothesized that the use of EVs, particularly reticulocyte-derived exosomes, in antigen discovery and as vaccine platform to convey immune responses could provide novel insights to be harnessed in vaccine development against vivax malaria.

8.1 EVs from *P. vivax* Infections: Potential Source of Antigens and Beyond

EVs serve as couriers enabling communication among parasites and between the parasites and their hosts during malaria infections. Initial studies focused on the pathogenic role of microparticles in natural *P. falciparum* infections. Later studies on *P. vivax* infections showed increased levels of circulating EVs in vivax infected individuals compared to healthy volunteers (Campos

et al. 2010). Still, most of the works intended to gather understanding on EV-mediated parasite-host interactions have used *in vivo* malaria models and EVs derived from *in vitro* cultured *P. falciparum*-infected RBCs. Studies on EVs from vivax infections are limited mainly due to the lack of a continuous *in vitro* culture system for *P.vivax* (Noulin et al., 2013).

In this thesis, we aimed at isolating and assessing the proteomic composition and immune function of circulating EVs from *P. vivax*-infected patients. Note that the composition of EVs, and consequently their potential functions, is likely to change depending on intrinsic and environmental factors during infections. EV samples collected from human malaria infections will reflect that heterogeneity much better than EVs derived from *in vitro* cultures. While working with such variable samples is challenging, any findings derived from malaria patients are highly informative to understand the role of EVs in natural human infections.

8.1.1 *Isolation and Proteomic Characterization of PvEVs*

Studying EVs, particularly from complex body fluids as plasma, represents a daunting task. Nowadays the field of EVs is confounded by the lack of gold-standard technologies to isolate pure EVs and the absence of specific markers to distinguish EV subpopulations. Recent works applying high-resolution density gradient fractionation and immunoaffinity capture for isolation of EVs have highlighted the huge EV heterogeneity and the significant contamination of non-vesicular components in EV preparations (Jeppesen et al., 2019; Kowal et al., 2016). These studies represent a warning for the EV field and urge to find more precise ways to purify and characterize EVs. Apart from the use of high-resolution density gradients and immune-isolation procedures, the EV field is moving forward in the use of technologies that enable the analysis of single EVs to expand our understanding on EVs.

The quantity of sample material available from vivax patients is limited. Therefore, we looked for an EV separation technique that enabled us to get the best ratio in purity and amount. Previous work using limited amount of plasma from healthy donors demonstrated that SEC is a stand-alone methodology that enables the enrichment of circulating EV populations in early fractions, where the most abundant plasma proteins have been largely excluded (de Menezes-Neto et al., 2015). In addition, SEC separates EVs based on their size, thus enabling the separation of circulating EVs that derive from different cell origins, similarly to the *in vivo* scenario. Therefore, we decided to apply SEC for the isolation of EVs secreted in the bloodstream of acute vivax patients. As demonstrated by the molecular characterization (see Figure 4.1 and Figure 4.2) and the size (see Figure 4.3) of the EV-enriched SEC fractions, SEC enabled an effective EV separation. These results are in agreement with previous works

using this technique to enrich circulating EVs from healthy volunteers (de Menezes-Neto et al., 2015) and from liver-humanized mice (Gualdrón-López et al., 2018).

We conducted a MS-based proteomic analysis over SEC-isolated EVs from vivax patients and healthy donors. We detected 20 parasite proteins associated to *Pv*EVs (see Table 4.1), and corroborated the identification of the two proteins detected with the highest number of unique peptides, MSP3.1 (PVP01_1031700) and PHISTc (PVP01_0119200), by western blot (see Figure 4.4). Parasite proteins present an expected heterogenous distribution between the different *P. vivax* patients (see Supplementary Dataset B.2). A high number of factors including the different degrees of parasitemia and the heterogeneity of *P. vivax* parasites (Cornejo, Fisher, & Escalante, 2014) might contribute to variability of the proteins identified per patient. The number of parasite proteins identified is considerably reduced compared to the numbers of proteins described in previous proteomes of EVs derived from malaria infected RBCs (Abdi et al., 2017; Mantel et al., 2013; Martín-Jaular et al., 2016). Note that these works have been performed over EVs isolated from the supernatants of *in vitro* cultured infected RBCs, while we are characterizing the population of EVs in the bloodstream during natural infections. It is likely that the EVs derived from infected cells represent a small proportion of the total EVs in circulation, thus limiting the identification of parasite proteins associated to them.

Proteomes of EVs derived from malaria infected RBCs have been shown to be enriched in merozoite proteins such as MSP-1 (Abdi et al., 2017; Mantel et al., 2013; Martín-Jaular et al., 2016) and exported parasite proteins such as *P. falciparum* PTP2, member of the PHISTc subfamily (Regev-Rudzki et al., 2013). Despite the mentioned limitations, we have detected merozoite proteins MSP-1 (PVP01_0728900) and MSP-3.1 in *Pv*EVs. Interestingly, *P. vivax* MSP1 as well as some members of the *P. vivax* MSP3 family are being investigated as potential vaccine candidates (França et al., 2017; Mourão et al., 2012). We also have found a PHISTc protein associated to *Pv*EVs [PHISTc (PVP01_0119200)], ortholog of *P. cynomolgi* PHIST/CVC-8195, that had been visualized in the caveolae-vesicle complexes of *P. cynomolgi*- infected erythrocytes (Akinyi et al., 2012). PHISTc has been shown to be highly immunogenic evaluating humoral responses to *P. vivax* infection (Lu et al., 2014; Wang et al., 2016). Given that cytotoxic T-cell responses might represent a control mechanism for blood-stage vivax infection (Burel et al., 2016; Junqueira et al., 2018), we directed our research to predict T-cell epitopes within MSP-3.1 and PHISTc to have an overview of the peptides of these proteins that could be recognized via HLA class I molecules (see Table 4.2). Lower values of median rank (higher median binding) can be found for the predicted sequences of PHISTc than for the ones of MSP3.1, which suggests PHISTc might have more potential cytotoxic T-epitopes than MSP3. Expectably, this analysis showed one of the greatest

obstacles to design T-cell based vaccination approaches: HLA polymorphism.

8.1.2 *Other Parasite Components and Human Cargo Associated to PvEVs*

Apart from parasite proteins, EVs derived from *P. falciparum*-infected RBCs have been shown to contain parasitic nucleic acids able to regulate the immune response (Sisquella et al., 2017). Whether similar nucleic acids are present in EVs from vivax infections is yet to be determined.

Besides parasite components, human cargo on *Pv*EVs is likely to influence the functions of circulating EVs. Remarkably, when we compared the human proteins detected in *Pv*EVs with the ones described in *h*EVs, we found a significant decrease in the plasma-derived EVs of infected patients (see Figure 4.6) despite the elevated levels of EVs during the infection (see Figure 4.3). These results suggest that there is an increased release of specific EV populations during acute vivax infection. Interestingly, GO-term enrichment analysis over the human proteins exclusively detected in *Pv*EVs revealed several terms related to the upregulation of the immune response (see Figure 4.7). The identification of HLA molecules, Intercellular adhesion molecule 1 (ICAM-1) and Integrin alpha-L (ITGAL) (see Supplementary Dataset B.1) suggest that at least some subtypes of *Pv*EVs are released by immune cells and/or can target immune cells (Segura, Nicco, et al., 2005). However, we cannot know whether the components present on EVs would promote or inhibit host immunity without further functional analysis.

Overall, our study represents the first MS-based proteomics analysis of EVs from natural human vivax infections. We have demonstrated the association of parasite proteins to *Pv*EVs. These proteins, that could serve as potential antigens, and the presence of other molecules related to immune responses, indicate that *Pv*EVs might contribute to the immune regulation during malaria infection and might be of interest for vaccine development. Moreover, the altered quantity and composition of *Pv*EVs compared to *h*EVs suggests that EVs might be involved in several processes of inter-cellular communication that await further investigation.

8.1.3 *Future Work*

Reassessment of *Pv*EVs composition

A reassessment of the composition of circulating EVs in vivax infections using another EV isolation should be performed to validate the described cargo of *Pv*EVs. Currently, differential centrifugation with gradient is one the techniques that yields the purest EV preparations (Théry et al., 2018). However, to what extent it can be applied to reduced starting material, such as the case of plasma from *P. vivax* patients, remains to be assessed.

Enrichment of CD71+EVs

To maximize the chances of detection of potential parasite antigens associated to *Pv*EVs, we could try to select *Rex* coming from parasitized reticulocytes. We found that circulating EVs present CD71 (see Figure 4.2), the major component of *Rex* (Harding et al., 1983; Pan & Johnstone, 1983) and a reticulocyte-specific receptor for *P. vivax* (Gruszczyk et al., 2018). Importantly, *P. vivax*-infected reticulocytes are experiencing a rapid remodeling and accelerated "maturation" that includes the mobilization of vesicular components (Malleret et al., 2015). EVs derived from infected reticulocytes could be contributing to the increased levels of circulating EVs observed during acute *P. vivax* infections (see Figure 4.3). Therefore, one possible approach to capture *Rex* could be based on the selection of CD71+EVs. This could be performed with an immune-isolation procedure similar to the one established by Kowal *et al* to isolate different subsets of tetraspanin-bearing EVs (Kowal et al., 2016).

We refrained to use this approach in this initial proteomic characterization of *Pv*EVs for the several reasons. First, CD71 is not exclusively associated to *Rex* in plasma. There is a truncated soluble form of CD71 in circulation (Ahn & Johnstone, 1993) and CD71 can be expressed in other cells, such as activated T-cells (Motamedi, Xu, & Elahi, 2016), that could be releasing CD71+ EVs as well. Secondly, other EVs apart from *Rex* might be carrying parasite components. As evidenced in a liver-humanized mouse model that sustains liver-stage *P. vivax* parasites, EVs derived from parasitized hepatocytes can be present in circulation (Gualdrón-López et al., 2018). Finally, there might be other cells apart from those that are infected, whose release of EVs might affect multiple processes over the course of the infection. Despite the mentioned considerations, if our goal is to enrich *Rex* subpopulation and facilitate the detection of proteins associated to them as much as possible, the CD71 immune-isolation could still be a valid approach.

8.2 Exploring *Pv*EVs Involvement in the Promotion of Immune Responses

The association of MHC molecules to exosomes called the attention of many immunologists and numerous studies on the contribution of EVs derived from leukocytes to mediate immune responses have been carried out (Théry et al., 2009). However, to the best of our knowledge, Martin-Jaular *et al* reported for the first time an immune modulatory effect elicited by *Rex* (Martin-Jaular et al., 2011). This work showed that *Rex* derived from the *P.yoelii* infection contain parasite proteins and confer a long-lasting protective immune response when used in CpG-adjuvanted immunizations (Martin-Jaular et al., 2011). Moreover, these immunizations are associated with a spleen-dependent, effector

and non-exhausted memory T-cell response (Martín-Jaular et al., 2016). This thesis aimed at extending these observations to human *P. vivax* infections. Following our study on circulating EVs from natural human vivax infections, we aimed to shed light on their contribution to the immune response.

8.2.1 *Study of the Human Spleen*

The spleen has multiple relevant roles in the control of malaria infections (Del Portillo et al., 2012). Yet, knowledge on the human spleen is limited by technical and ethical limitations. Still it would be very relevant to gain insight on the human spleen-resident cells in order to discover novel processes mediated in this organ that could be of particular importance for human malaria parasites. Trying to gather understanding on the human spleen, we noticed that most of the reports were focused on the immune cells populations (Carpenter et al., 2018; Colovai et al., 2004; Langeveld & Gamadia, 2006; Mcilroy et al., 2001). Some of these works are indeed very complete, yet the red blood cells lineage, was not explored in any of them to our extent of knowledge. Therefore, we decided to conduct a holistic phenotyping approach of the cell populations of the human spleen.

After mechanical disaggregation and extensive filtration of the splenic tissue, we showed that mature RBCs are the major cell population in the spleen. Interestingly, reticulocytes correspond to approximately 0.3% of the total cells (see Figure 5.2). The presence of erythroid precursors in the spleen has been documented under stress erythropoiesis, where the spleen of both mouse and human increases its erythropoietic capacity (Socolovsky, 2007). We have also shown the presence of a minor population of hematopoietic stem cells, previously detected in the spleen from cadaveric donors (Söderdahl, Tammik, Remberger, & Ringdén, 1998). Note that human splenic hematopoiesis had previously been observed in patients with osteopetrosis (Freedman & Saunders, 1981).

Regarding the leukocyte populations, we have found high frequencies of neutrophils (around 30%) and monocytes (5%). These innate immune cells were found in a similar proportion to other studies on the human spleen (Carpenter et al., 2018; Meinderts et al., 2017). Unexpectedly, we could not detect macrophages in the total single-cell spleen suspension. Macrophages had been previously characterized in the human spleen by the expression of CD163 (Meinderts et al., 2017), as in our strategy. We suspect that the low detection of macrophages in the splenocytes suspension we obtained might be due to the absence of a prior enzymatic digestion of the splenic tissue. We observed that B-cells are more abundant than T-cells in the spleen, contrary to what happens in peripheral blood (see Figure 5.2). We observed around 1.6 ratio of CD4+/CD8+ T-cells in the total single-cell spleen suspension (see Figure 5.2) as well as in isolated T-cells (see Figure 5.6A). Similar ratio of B/T-

cells was observed in a prior study (Langeveld & Gamadia, 2006). However, Langeveld and Gamadia reported a greater percentage of CD8+ T-cells. We think that this inverse CD4+/CD8+ ratio in the spleen might be associated to a viral infection (Langeveld and Gamadia 2006). The CD4+/CD8+ ratio of T-cells that we have reported is in agreement with the work by Carpenter *et al* (Carpenter et al., 2018).

8.2.2 *Investigating PvEVs Interactions with the Human Spleen*

The role of the spleen is essential in the induction of immune responses to blood-borne pathogens (Bronte & Pittet, 2013) as well as in the protection elicited by *Rex* immunization in *P. yoelii* infections (Martín-Jaular et al., 2016). We explored the interaction of *Pv*EVs and *h*EVs with the human spleen immune cells, and we observed that both EV populations could interact with DCs. Interestingly, there was higher interaction of monocytes, B-cells and T-cells with *Pv*EVs compared to *h*EVs despite incubating the cells with equal amount of protein from both types of EVs (see Figures 5.5 and 5.6).

Currently, we are very interested in the potential phenotypic changes that *Pv*EVs can cause to spleen immune cells. We wonder whether components carried in *Pv*EVs could lead to the initiation of immune responses in the human spleen. EVs derived from cultures of *Pf*iRBCs promoted proinflammatory responses in peripheral monocytes *in vitro* (Mantel et al., 2013; Sisquella et al., 2017). It is possible that the enhanced interaction of *Pv*EVs could activate spleen monocytes. However, it is unclear whether monocyte activation would lead to clearance of malaria infection or immunopathology (Chua et al., 2013). An increased interaction of *Pv*EVs with spleen B- and T-cells could stimulate adaptive immune responses. Previous studies have reported that antigen-loaded DC-derived EVs facilitate the interaction between B- and T-cells and efficiently induce Th1-type memory (Qazi, Gehrmann, Domange Jordo, Karlsson, & Gabrielsson, 2009). Mice immunization with EVs from *P. yoelii*-infected reticulocytes promoted humoral responses (Martin-Jaular et al., 2011) and induction of spleen memory T-cells (Martín-Jaular et al., 2016). In addition, prior studies have shown that stimulation of human splenocytes with *Pv*EVs alters T-cell subsets (Martín-Jaular et al., 2016).

8.2.3 *Challenging Functional Assays with the Human Spleen*

As hypothesized in this thesis, potential antigens carried in EVs, presumably in *Rex*, could be presented to T-cells in the spleen. We wanted to test this hypothesis with human splenocytes, but functional assays with these cells are challenging.

First, there is scarcity of human spleen samples to which we have access. Secondly, although we work with fresh samples, there is still a large presence of neutrophils in the leukocyte-enriched samples that compromises the viability

of cell cultures (data not shown). Thirdly, we have obtained highly pure T-cells by either positive or negative isolation (see Figure 5.6A) and it would be very interesting to prime these cells with *Pv*EVs. However, for priming malaria-naive T-cells, the presence of antigen-presenting cells is fundamental (Guermonprez, Valladeau, Zitvogel, Théry, & Amigorena, 2002). Our attempts to enrich spleen DCs were not successful enough (see Figure 5.5A). Alternatively, we have tried to isolate spleen monocytes by positive selection with MACS CD14 microbeads to differentiate them *ex vivo* in the presence of GM-CSF and IL-4 to DCs, but so far, our attempts have failed (data not shown). This methodology is broadly used to obtain monocyte-derived DCs (mo-DCs) from circulating monocytes (Sallusto & Lanzavecchi, 1994) but it might not be applicable to obtain splenic mo-DCs. While there is evidence in mice that spleen monocytes can differentiate to DCs under microbial challenge, such as malaria (Hirako et al., 2016), other studies have shown that splenic monocytes cannot convert to DCs under inflammatory conditions (Drutman, Kendall, & Trombetta, 2012; Varol et al., 2007). To the extent of our knowledge, no one has assessed this in the human spleen. Therefore, we cannot discriminate if our inability to obtain *ex vivo* human splenic mo-DCs is based on technical or biological restrictions.

Given all the described limitations, we have decided to perform studies regarding the immunogenicity of *Pv*EVs with peripheral leukocytes, but we still believe that functional immune assays with spleen cells and EVs are worth exploring.

8.2.4 *Stimulation of T-cell Responses by* Pv*EVs*

Several reports on murine malaria models have demonstrated that CD8+ T-cell responses can be essential for protective immunity against blood-stage infection (Horne-Debets et al., 2013; Imai et al., 2010; Podoba & Stevenson, 1991). However, it has always been assumed that CD8+ T-cell responses would not contribute to the control of blood-stage *Plasmodium* infections given the lack of HLA class I molecules on red blood cells. Recent studies showing the importance of cytotoxic T-cell responses on *P. vivax*-infected reticulocytes via the HLA class I molecules present in the reticulocytes are urging to re-evaluate this paradigm (Burel et al., 2016; Junqueira et al., 2018). Importantly, not only the infected reticulocytes but the EVs derived from them might be able to carry antigen-HLA class I complexes that contribute to the cellular immune response against vivax. It is also possible that EVs provide antigens and/or antigen-HLA complexes that will be presented to T-cells via cross-presentation or cross-dressing. Both our MS data (see Supplementary Dataset B.1) and bead-based flow cytometry assays (see Figure 6.1) have corroborated the presence of HLA class I molecules in circulating EVs.

To understand the importance of the contribution of *Pv*EVs to the T-cell immune responses during infection, we set up an approach to evaluate whether

*Pv*EVs have the ability to stimulate T-cell responses from autologous malaria-exposed individuals (see Figure 6.2). Unfortunately, those experiments could not be performed yet due to low viability observed in the PBMCs from *P. vivax* patients after their shipment from malaria endemic regions.

Alternatively, we explored the stimulation capacity of *Pv*EVs over T-cells from individuals non-exposed to malaria. Given the ethical and technical limitations to work with tissue-resident cells, the use of PBMCs is the most convenient approach to understand human immune responses (Wagar, Difazio, & Davis, 2018). Using the monocyte-dependent approach that we had establish to evaluate T-cell responses by*Pv*EVs, we could not observe any activation of malaria-naive T-cells by means of CD69 upregulation and production of IFN-γ after 18h of stimulation (see Figure 6.5). While this system might be useful to detect rapid responses in antigen-experienced T-cells, it is not optimized enough to detect activation on naive T-cells. Moreover, we do not expect *Pv*EVs to be highly immunogenic since the parasite components that can act as potential antigens seem to be low abundant in the EVs as revealed by the reduced detection of parasite peptides in *Pv*EVs (see Supplementary Dataset B.2). Longer time of stimulation might be required to facilitate the encounter with the antigens. The reduced antigen-presenting ability of monocytes might be another drawback in this approach. Monocytes are likely to be the few APCs that we can get after thawing PBMCs, but in the case of using fresh PBMCs from non-exposed donors, other APCs, such as DCs, might enhance the chances of antigen presentation. Moreover, we ignore if other cells apart from APCs might be involved in the stimulation of T-cells by *Pv*EVs. Previous work has revealed that B-cells can contribute to the activation of naive T-cells by EVs (Segura, Amigorena, & Théry, 2005).

Preliminary results on the T-cell activation after 48h-stimulation by *Pv*EVs indicate that CD8+ T-cell responses might be activated by EVs during vivax infections. We observed a tendency of CD69 upregulation on CD8+ T-cells when PBMCs were stimulated by *Pv*EVs compared to *h*EVs. However, we could not detect a significant increase of IFN-γ production on*Pv*EVs-stimulated CD8+ T-cells compared to the cells stimulated with other EVs (see Figures 6.7 and 6.8). Future work will help us determine the relevance of these preliminary results.

It would be interesting to know whether *Pv*EVs promote the initiation of CD8+ T-cell responses against vivax, given the discussed potential of this mechanism for the control of blood-stage vivax malaria. Indeed, increased levels of circulating memory CD4+ T-cells and CD8+ T-cells have been reported during acute infections, and memory CD8+ T-cells were particularly augmented in immune individuals naturally exposed to *P. vivax*. Yet, it is unknown whether these cells were induced in response to blood- and/or liver-stage parasites (Jangpatarapongsa et al., 2006). Importantly, greater expansion of cytotoxic CD8+ T-cells was elicited in naive volunteers exposed to blood-

stage *P. vivax* parasites compared to individuals exposed to *P. falciparum* infection (Burel et al., 2016) and it has been recently demonstrated that their cytotoxic capacity can kill *P. vivax*-infected reticulocytes (Junqueira et al., 2018). Moreover, IFN-γ and perforin-producing CD8+ T-cells were associated with protection against murine blood-stage infection with *P. yoelii*, another retiulocyte-prone parasite (Imai et al., 2010). Of note, the protection elicicted by vaccination with EVs derived from *P. yoelii* infected reticulocytes was associated with an increase of effector memory CD8+ and CD4+ T-cells in the spleen of the immunized mice (Martín-Jaular et al., 2016). Unexpectedly, it has been reported that EVs derived from *P. berghei*-infected RBCs directly inhibit CD4+ T-cells. Histamine releasing factor and the elongation factor 1α, present on EVs from *Pb*-iRBCs, interfere with the phosphorylation pathways associated with TCR signaling. Despite the immunosuppressive effect on CD4+ T-cells, vaccination with EVs protected mice against infection (Demarta-Gatsi et al., 2019). These results might seem contradictory at the beginning, but distinct T-cell subsets might be activated or inhibited in the different studies. Furthermore, EVs are released from different malaria parasites and from distinct cellular origins and therefore are likely to trigger different effects. Given the plethora of factors that can alter EV composition and function, we are still at the beginning of the understanding of the multiple roles that EVs might have during malarial infections. However, the protective effects observed in EV immunization strategies in malaria (Demarta-Gatsi et al., 2019; Martin-Jaular et al., 2011) encourage us to keep pursuing EV-based vaccination approaches, particularly, those intended to elicit cytotoxic T-cell mediated immunity to vivax malaria.

8.2.5 *Future Work*

Analysis of human splenic immune responses to *Pv*EVs

Despite the mentioned limitations using human splenocytes, we would like to better understand the potential effects on the immune cells that differentially uptake *Pv*EVs compared to *h*EVs. The relevance of the spleen on the generation of immune responses against malaria encourages us to perform further experiments to gain insight on the processes that the EVs might be mediating in this organ.

Importantly, EVs might interact with acceptor cells in multiple ways. EVs can trigger further signaling in the recipient cell by acting at the surface level. They can also be internalized following different routes or fused to the plasma membrane having their cargo delivered to the acceptor cell (Mathieu, Martin-Jaular, Lavieu, & Théry, 2019). Defining the mechanism of uptake of EVs by human splenocytes will help us better understand their interaction and design future experiments to assess the changes on the recipient cells.

Examining the effect of the infections on small animal models containing human cells or tissues (*humanized* mouse models) is a promising model to understand human responses *in vivo* (Minkah & Kappe, 2019). A human spleen-engrafted model has already been used as a valuable preclinical model to down-select malaria vaccine candidates (Ghosn et al., 2018). Perhaps a similar mouse model engrafted with human splenocytes that we have isolated and characterized could help us better evaluate the potential interactions of malarial parasites and/or parasite-derived components such as *Pv*EVs with the spleen.

Optimization of assays to measure T-cell activation

The stimulation of malaria naive PBMCs by *Pv*EVs has given some initial promising results on terms of CD69 expression on CD8+ T-cells, but not in terms of IFN-γ production. While the expression CD69 is an accurate predictor of the proliferative capacity of lymphocytes in general (Mardiney, Brown, & Fleisher, 1996), the release of IFN-γ in naive CD8+ T-cells might be rapid and transient to be detectable, particularly in low-level responses. Even though flow cytometry allows the identification of responding cells and simultaneous collection of several readouts, in future experiments, we might enumerate IFN-γ producing cells by a more sensitive technique such as Enzyme-Linked ImmunoSpot (ELISPOT) (Karlsson et al., 2003). This technology might also be useful to determine positive responses on T-cells from vivax exposed individuals, where the frequency of activated cells is expected to be low as well.

In future studies, we will try to determine the cells involved in the potential presentation of antigens to T-cells. For instance, it would be interesting to assess if T-cells from malaria exposed individuals can be directly activated by *Pv*EVs without further involvement of APCs.

Towards the immunopeptidome of *Pv*EVs and its applications

If future experiments confirm that *Pv*EVs promote the stimulation of CD8+ T-cell responses in malaria exposed or naive individuals, it would be essential to unveil the antigenic proteins, or even better, the peptides recognized. Combining experimental profiling of HLA-bound peptide approaches and *in silico* predictions would accelerate the description of the immunopeptidome of *Pv*EVs and the identification of potential vivax T-cell epitopes. This strategy is rapidly evolving since it is critical to identify the most efficient portions of antigens to elicit targeted and potent immune responses. This profiling approach has already been used to elucidate functions of CD4+ T-cells in malaria infection (Draheim et al., 2017) and has also been applied to EVs (Synowsky et al., 2017). In our case there could be two novel sources of T-cell epitopes associated to *Pv*EVs: 1) peptides bound to the HLA molecules present

on the *Pv*EVs and 2) Peptides presented on the surface of APCs, preferentially DCs, that have processed *Pv*EVs. It is likely that the last one would have a greater contribution to the presentation of antigens, but it would be interesting to explore and compare both of them. Briefly, immune-isolation against HLA molecules of lysed *Pv*EVs or *Pv*EV-primed DCs followed by the extraction of bound peptides and identification of those by MS would yield a potential list of epitopes that could be down-selected according to vivax specificity and *in silico* predictions.

These investigations could have several applications. Peptides with the sequence of these epitopes could be synthetized to stimulate PBMCs from vivax exposed individuals and evaluate their antigenicity as well as determine if they are targets of natural immunity. Potential vivax epitopes would be more confidently selected with this strategy than just based on *in silico* predictions as presented in Chapter 4 (see Section 4.3).

Indeed, the potential identification of prominent vivax epitopes would set the basis for the development of reporter CD8+ T-cell hybridomas, which to the best of our knowledge have never been developed against vivax antigens. These parasite-specific reporter T-cell lines would help us dissect in a more efficient way the presentation mechanisms of the antigens contained in *Pv*EVs, as it has been elegantly studied in melanoma-derived EVs carrying tumor antigens with melanoma-specific cytotoxic T-cell clones (Wolfers et al., 2001). Moreover, those lines could be a helpful model to further elucidate the role of cytotoxic T-cell activity in the killing of *P. vivax*-infected cells.

*Pv*EVs immunogenic potential: only a T-cell matter?

We still need a better understanding of immunity against *P. vivax* to elucidate which are the protective mechanisms that the vaccine strategies should promote. In this thesis we have focused on assessing the potential of *Pv*EVs to promote T-cell activation given the relevance that this mechanism might have for vivax control (Burel et al., 2016; Hojo-Souza et al., 2015; Junqueira et al., 2018) and because most of the activating immune responses by EVs have focused as well on their ability to stimulate T-cell responses (Robbins & Morelli, 2014).

However, it is likely that other components of immunity might be critical for protection as described in Chapter 1 (see Section 1.1.4). Importantly, naturally acquired humoral responses have been correlated with protection in vivax infected patients (França et al., 2017). Thus, vaccines that promote both humoral as well as T-cell responses are likely to be the most effective ones. Note that immunization with *Rex* from *P. yoelii* infections could both promote humoral and cellular responses (Martín-Jaular et al., 2016; Martin-Jaular et al., 2011). Therefore, it could be interesting to extend our studies on *Pv*EVs

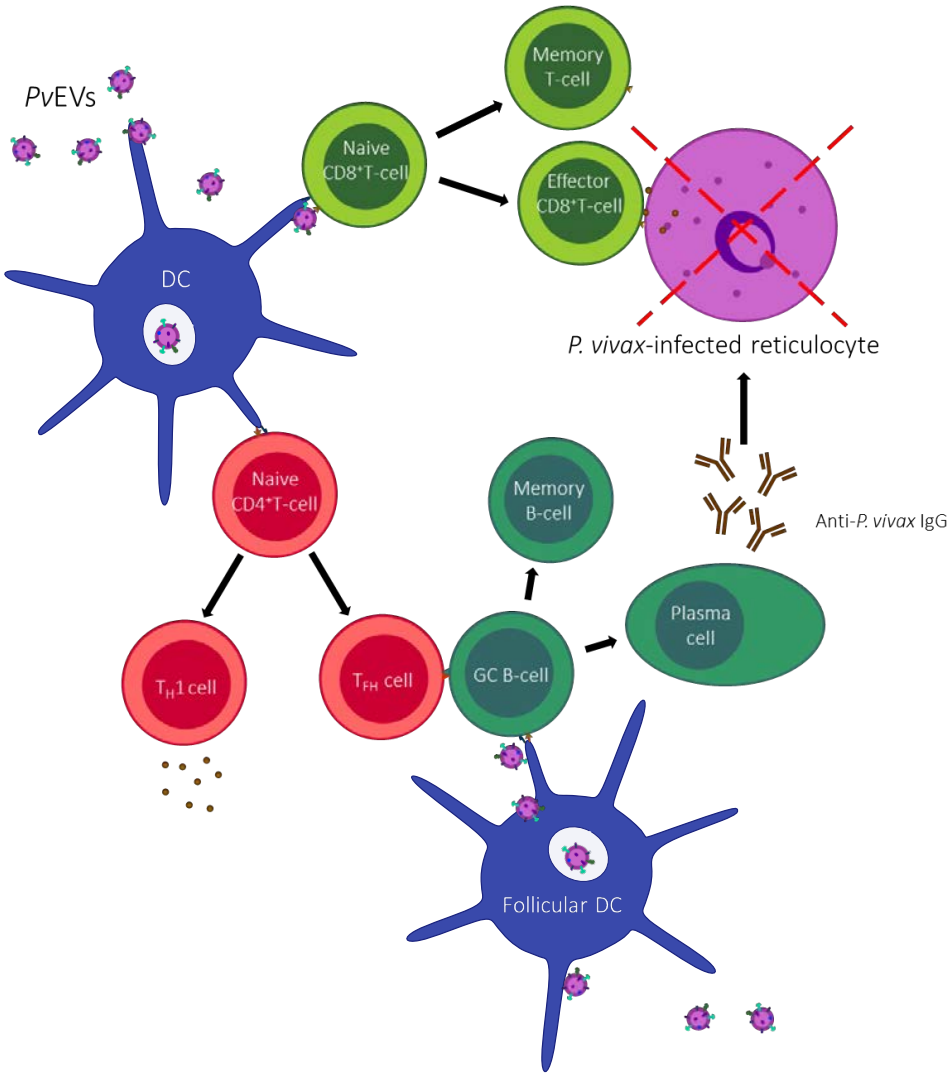

Figure 8.1 **Potential adaptative immune responses mediated by *Pv*EVs.** Circulating EVs in vivax infections, presumably from infected reticulocytes could carry vivax antigens that can be presented to T and/or B cells and activate cellular or humoral anti-malarial immune responses. T_H1 cell, T helper 1 cell; T_{FH} cell, T follicular helper cell; GC B-cell, Germinal center B-cell.

immunogenicity to other potential target immune cells, such as B-cells (see Figure 8.1).

8.3 *Rex* as Vaccine Platform against *P. vivax* Malaria

Rex were originally discovered as a cargo-disposal mechanism for destruction of obsolete proteins during the reticulocyte maturation (Harding et al., 1983; Johnstone et al., 1987; Pan & Johnstone, 1983). Yet, *Rex* from the reticulocyte-prone malaria infection with *P. yoelii* are able to induce protective responses (Martin-Jaular et al., 2011). These studies represent a proof-of-concept for the use of *Rex* as a new vaccine delivery platform against reticulocyte-prone malaria infections. In this thesis we have demonstrated that circulating EVs from vivax infections carry parasite proteins and express CD71, thus suggesting that *Rex* might represent at least part of the EVs carrying potential antigens. Moreover, preliminary results on *Pv*EVs immunogenicity suggest that they might have a discrete ability to stimulate CD8+ T-cells. Taken all these findings together, we believe human *Rex* could facilitate the development of a novel platform for vaccination against blood-stage *P. vivax* malaria.

8.3.1 *Rex: Vaccine Platform and More*

Most of the works that had been conducted over *Rex* were limited to animal models. To the extent of our knowledge, only seven proteins (TfR, Stomatin, Flotillin-1 and 2, CD55, CD58 and CD59) had been previously reported in *HuRex* using non-high-throughput technologies (Blanc & Vidal, 2010; Gassart et al., 2003; Rabesandratana, Toutant, Reggio, & Vidal, 1998). We conducted the first MS-based proteomic analysis of human *Rex* and identified 367 proteins. Comparing this novel proteome with MS proteomes from human reticulocytes (Chu et al., 2017; Gautier et al., 2016; Wilson et al., 2016) and mature RBCs (Bryk & Wiśniewski, 2017; D'Alessandro et al., 2017; Pasini et al., 2006; Wilson et al., 2016), we found many common proteins, especially from plasma membrane and cytosol (see Figure 7.6 and Supplementary Dataset B.6). These data are largely in agreement with the idea that exosomes reflect a "cell biopsy" but at the same time are enriched in specific cargo (Raposo & Stoorvogel, 2013; Yáñez-Mó et al., 2015). In addition, the complete MS proteome of *Rex* from rats is available (Carayon et al., 2011) and its comparison with the *HuRex* proteome revealed more than 200 conserved proteins (see Supplementary Dataset B.8). This dataset represents the first comprehensive list of proteins from *HuRex* and a valuable resource to pursue different studies (see Figure 8.2). Remarkably, several receptors and proteins involved in entrance of the parasite into the host cell, such as CD98 (Malleret et al., 2017), CD71 (Gruszczyk et al., 2018), band 3 (Alam, Zeeshan, Rathore, & Sharma, n.d.) and erythrocyte band 7 (Hiller, Akompong, Morrow, Holder, & Haldar, 2003) are selectively removed

in *HuRex*. Our study expands the knowledge of the biology of the cell that *P. vivax* preferentially invades, and suggests a possible partial explanation of the failure of *in vitro* culture systems. Besides, this proteome might help identify EV-mediated mechanisms of pathology and non-invasive biomarkers for other diseases affecting RBCs apart from malaria (Hebbel & Key, 2016).

Importantly, we have identified HLA class I molecules in *HuRex*, antigen-presenting molecules that have been associated to EVs coming from reticulocyte-prone infections, where parasite proteins have been detected as well. MHC class I molecules were identified in *Rex* derived from murine infections with *P. yoelii* (Martín-Jaular et al., 2016) and HLA class I molecules has been associated to the circulating EVs from vivax infections. It is unlikely that HLA class I-peptides complexes on the EVs would directly stimulate T-cell responses, but prior studies have demonstrated that peptide-bearing EVs can induce antigen-specific T-cell activation *in vivo* and *in vitro* in the presence of DCs (Théry et al., 2002). Therefore, we conducted a functional assay (Izquierdo-Useros et al., 2009) to demonstrate the potential capacity of *HuRex* to interact with DCs. This assay previously demonstrated that ganglioside-containing vesicles such as viral-like particles or exosomes could be captured by mDCs expressing Siglec-1 (CD169) (Izquierdo-Useros et al., 2012).

Noticeably, our results demonstrated Siglec-1-dependent capture of *HuRex* by mDCs as well as co-localization of *HuRex* with VLPs (see Figure 7.8), indicating they follow the same trafficking route (Izquierdo-Useros et al., 2009). The interaction of *HuRex* with CD169-expressing DCs suggests that CD169+APCs could facilitate the presentation of potential antigens carried in *HuRex*. Interestingly, it has been shown that CD169 mediates the capture of EVs in the spleen and the lymph node (Saunderson, Dunn, Crocker, & McLellan, 2014), and tumor antigen targeting to CD169+APCs induces activation of CD8+ T-cell responses (van Dinther et al., 2019). We can speculate that *HuRex* could be able to eventually reach those lymphoid organs and activate CD8+ T-cell responses.

8.3.2 *Future Work*

Comparison of different vaccine platforms

EVs present several advantages that make them an attractive delivery platform for vaccination: 1) protein conformation is more stable in EVs than in soluble form (Y. Yang, Hong, Cho, Kim, & Kim, 2018). 2) EVs have the ability to circulate through body fluids, which might improve antigen accessibility to the lymphoid organs and 3) EVs' natural capacity to transport and spread potential antigens between immune cells (Jeffrey Sean Schorey et al., 2015), facilitating for instance, the antigen uptake by APCs. Note that there are

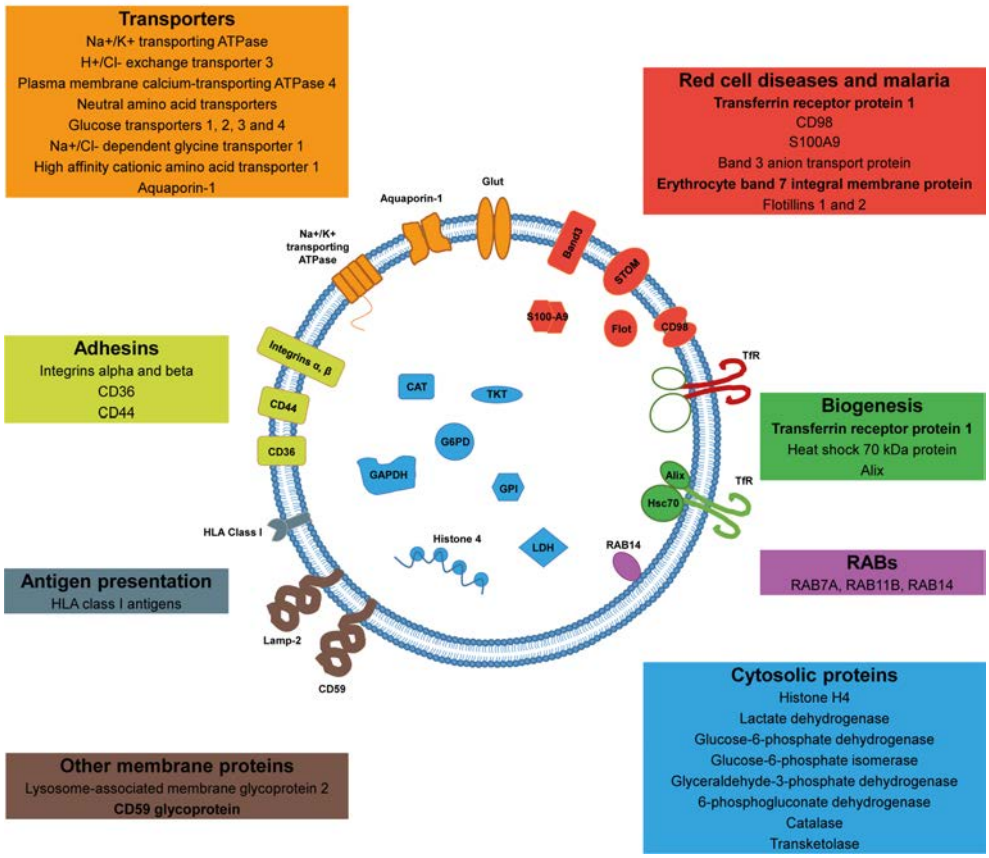

Figure 8.2 **Schematic representation of *HuRex* proteome.** Transporters (in orange); Adhesins (in lime); Antigen presentation (in grey); Other membrane proteins (in brown); Proteins related to red cell diseases and malaria (in red); Proteins implicated in biogenesis (in green); RABs (in pink); Cytosolic proteins (in blue). Proteins in bold have been previously associated to *HuRex* (TfR, STOM, CD59) (Blanc & Vidal, 2010; Gassart, Ge, & Fe, 2003; Rabesandratana, Toutant, Reggio, & Vidal, 1998).

other promising particle-based platforms that share multiple characteristic with EVs, such as their nanometric size, which largely affects the antigen distribution (Bachmann & Jennings, 2010). VLPs are one important example. Remarkably, VLPs carrying malaria vivax antigens delivered in combination with viral vectored antigens or adjuvants are yielding promising results for vaccine development (Atcheson et al., 2018; Cabral-Miranda et al., 2017). Comparison of exosomes and VLPs has shown that both platforms have similar efficacy inducing cross-presentation of viral antigens (Lattanzi & Federico, 2012). Perhaps, it would be of interest to compare the immunogenic capacity of *Rex versus* VLPs containing parasite proteins identified in *Pv*EVs or other potential antigens.

Perspectives on *Rex*-based vaccination

If we were able to determine that *Pv*EVs activate vivax specific CD8+ T-cell responses and discover the epitopes recognized by T-cells, synthetic peptides of those epitopes could be loaded on *HuRex* to evaluate their ability to prime CD8+ T-cell responses able to kill vivax-infected reticulocytes.

To translate the use of *HuRex* into an affordable clinical application, we should be able to perform immunizations with heterologous EVs. However, that poses the risk of introducing "non-self" human antigens. While the use of autologous EVs could represent a safer approach, it is unlikely that a laborious large-scale production of autologous EVs would be applicable for the numerous populations at risk for vivax malaria. Artificial EVs could be generated to make an EV-based clinical application logistically viable. One potential approach has already been designed using EV-mimetic nanovesicles such as liposomes coated with HLA class I-peptide complexes and a range of adhesion, early activation, late activation, and survival T-cell receptors (De La Peña et al., 2009). Still, these synthetic vesicles might not be as stable as the naturally produced EVs and likely will not have their intrinsic targeting capacities.

Future studies will help us uncover the key components responsible for EV-mediated immune responses. Apart from HLA class molecules and vivax antigens, other molecules are likely to be required for an efficient targeting and stimulation. This could guide the design of affordable and effective EV-based strategies against vivax malaria.

8.4 Common Discussion

This thesis aimed at providing insights into how EVs represent a novel approach for antigen discovery and contribute to cell-mediated immunity that might be critical for vivax control. It also explored the particular use of *Rex* as a vaccine platform. Even if we are far from a clinical application, we believe this work provides valuable insights for vaccine development against *P. vivax* malaria.

Given the lack of a continuous *in vitro* culture of *P. vivax* (Noulin et al., 2013), studying *Rex* derived from*P. vivax*-infected reticulocytes would have been challenging. We circumvented this limitation with an analysis of plasma-derived EVs from vivax patients and of *Rex* derived from human reticulocytes. These analyses have given us an idea about the potential composition of *Rex* from infected reticulocytes. Concretely for circulating EVs from vivax patients, we have shed light on EVs composition and potential functions in natural vivax infections, which had been largely unexplored until now. Importantly, we demonstrated the presence of HLA class molecules in these vesicles, thus suggesting that *Rex* have the capacity to mediate immune responses during vivax infections. Furthermore, multiple parasite proteins were found associated to EVs from vivax patients, thus reassuring the potential of circulating EVs in the search of novel antigens. To the best of our knowledge, this represents the first identification of parasite proteins in EVs derived from natural vivax infections.

*Pv*EVs could mediate multiple host-pathogen interactions in the course of an infection. We assessed specifically their interaction with the human spleen and the capacity to stimulate T-cell responses since the proof-of-concept of *Rex* vaccination in the reticulocyte-prone *P. yoelii* model demonstrated that protection was associated with a spleen-dependent memory T-cell response (Martín-Jaular et al., 2016). Our work shows that many leukocytic populations from the human spleen have a higher interaction with circulating EVs derived from infected patients compared to EVs from healthy donors. Despite the discussed challenges of working with human spleen samples, future studies will be performed to decipher the mechanisms and effects of this preferential uptake. The recent evidence that cytotoxic T-cell might be critical to control blood-stage vivax infection (Burel et al., 2016; Junqueira et al., 2018) encourages us to keep focused on the evaluation of T-cell activation by EVs in malaria exposed and naive individuals. After the implementation of an approach to study these responses, preliminary results indicate that EVs might contribute to the stimulation of CD8+ T-cell responses. Further experimentation is guaranteed to re-evaluate those findings and identify in EVs potential targets of cell-mediated immunity.

Having been demonstrated that *Rex* protect against the reticulocyte-prone parasite *P. yoelii* (Martín-Jaular et al., 2016; Martin-Jaular et al., 2011) and bearing in mind that reticulocytes are the preferential target of*P. vivax* blood-stages (Kitchen, 1937), human *Rex* carrying vivax antigens represent a promising vaccine platform against blood-stage vivax malaria. Our MS-proteomics over *HuRex* extended the existing limited knowledge on the composition of these vesicles (Blanc & Vidal, 2010) and the identification of HLA class I molecules reinforced the potential of *Rex* to facilitate the induction of cytotoxic T-cell responses. The interaction of *HuRex* with Siglec-1 expressing DCs further emphasizes the contribution of *HuRex* to antigen presentation by the transfer of antigens or antigen/HLA complexes carried in *HuRex*.

Altogether, the findings of this thesis indicate that EVs open a promising avenue for controlling vivax malaria. Our work supports further studies on the use of EVs, particularly *Rex*, as source of novel antigens and as a new vaccine platform against *Plasmodium vivax* malaria.

Chapter

9 Conclusions

The main conclusions drawn from the studies performed during this thesis are summarized as follows:

1. Circulating EVs from natural *P. vivax* infections (*Pv*EVs) present an altered cargo as revealed by a comparative MS-proteomic analysis with EVs from healthy donors (*h*EVs).

2. *Pv*EVs contain parasite proteins that could serve as antigens. Indeed, two of the identified vivax proteins present promising cytotoxic T-cell epitopes as evidenced by *in silico* analysis. These results encourage the use of *Pv*EVs in antigen discovery.

3. *Pv*EVs are composed by a less varied repertoire of human proteins than *h*EVs. Yet, there are proteins exclusively associated to *Pv*EVs that are related to immune functions as revealed by GO enrichment analysis.

4. The presence of common proteins in circulating EVs and *Rex*, such as CD71, and the identification of parasite proteins in *Pv*EVs suggest that at least part of the EVs in circulation derive from *P. vivax*-infected reticulocytes.

5. The detection of HLA class I molecules and parasite proteins on *Pv*EVs highlights the possibility that *Pv*EVs represent potential antigen-carrying vesicles that might promote immune responses. Particularly, the presence of HLA class I molecules indicates they might be involved in the stimulation of CD8+ T-cell responses.

6. An increased interaction of human spleen monocytes, B-cells and T-cells is observed with *Pv*EVs compared to *h*EVs.

7. Preliminary results indicate that CD8+ T-cells responses might be activated by *Pv*EVs.

8. MS-proteomic analysis of *HuRex* revealed over 300 proteins associated to these vesicles, including HLA class I molecules. These data represent a valuable resource to improve our understanding on human erythropoiesis and an opportunity to develop further studies on red cell diseases.

9. Siglec-1-expressing DCs actively take up *HuRex*. These results further support the role of *Rex* as conveyors of immune responses and as potential vaccine platform for human reticulocyte-prone malaria infections such as vivax malaria.

Chapter

10 Contributions

This chapter presents the main publications and manuscripts that have been produced in the context of this thesis. Note that the contributions are ordered in the same way the findings related to them were presented in the thesis:

1. **Plasma extracellular vesicles signal spleen fibroblasts facilitating *Plasmodium vivax* adherence.** Toda H, Díaz-Varela M, Segui-Barber S, Roobsoong W, Baro B, Garcia-Silva S, Galiano A, Gualdrón-López M, Almeida ACG, Brito MAM, Cardoso de Melo G, Marcelo Monteiro W, Borras Eva, Sabido E, Almeida IC, Chojnacki J, Martinez-Picado J, Calvo M, Armengol P, Lauzurica R, Marcilla A, Peinado H, Galinski MR, Lacerda MVG, Sattabongkot J, Fernandez-Becerra C* and del Portillo HA* (*co-corresponding authors) (Manuscript under review)

   This manuscript has been submitted for publication and is under review process. Among other findings, it includes the studies on the isolation and proteomic characterization of *Pv*EVs that were presented in the Chapter 4.

2. **Interaction studies of plasma-derived extracellular vesicles from *Plasmodium vivax* infected patients with human spleen.** Gualdrón-López M*, Díaz-Varela M*, Toda H, Aparici-Herraiz I, Cos-Pedró L, Fernandez M, Lauzurica R, Fernandez-Becerra C and del Portillo HA. (*co-first authors) (Manuscript under preparation)

   This manuscript is under preparation and is close to be submitted for publication. It contains the findings on the isolation and phenotyping of cell populations from the human spleen as well as on the evaluation

of the interaction of these human splenocytes with *Pv*EVs that were presented in the Chapter 5.

3. **Proteomics study of human cord blood reticulocyte-derived exosomes.** Díaz-Varela M, de Menezes-Neto A, Perez-Zsolt D, Gámez-Valero A, Seguí-Barber J, Izquierdo-Useros N, Martinez-Picado J, Fernández-Becerra C and del Portillo HA. 2018. Scientific Reports, 8(1), p.14046. (Published article)

   Proof: Check Appendix D for the proof of this paper (e.g., link, screenshot).

   This article contains the proteomic study of *HuRex* and the assessment of their interaction with antigen-presenting cells that were presented in Chapter 7.

Other relevant contributions outside the main objectives of this thesis:

4. **Spleen-Dependent Immune Protection Elicited by CpG Adjuvanted Reticulocyte-Derived Exosomes from Malaria Infection Is Associated with Changes in T-cell Subsets' Distribution.** Martín-Jaular L, de Menezes-Neto A, Monguió-Tortajada M, Elizalde-Torrent A, Díaz-Varela M, Fernández-Becerra C, Borras FE, Montoya M, and del Portillo HA. 2016. Frontiers in Cell and Developmental Biology, 4 (November): 1–11. (Published article)

   Proof: Check Appendix D for the proof of this paper (e.g., link, screenshot).

   The PhD candidate contributed to this published study, that was carried out using experimental infections with *P. yoelii*. Since it was a little off topic from the main objectives of this thesis, it was not presented as a main finding of this work. However, it has been extensively discussed alongside the main findings of this thesis.

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Appendix

A Supplementary Figures

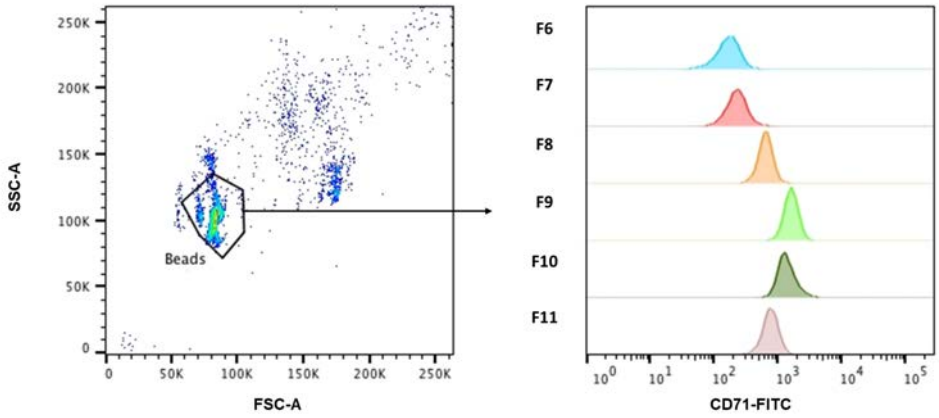

Figure A.1 **Gating strategy followed in bead-based flow cytometry analysis of EV-enriched fractions.** EV-coupled beads were first gated according to FSC-A/SSC-A. Expression of markers (e.g., CD71) was compared among different fractions using the median of FITC or AF488 fluorescence of the beads population.

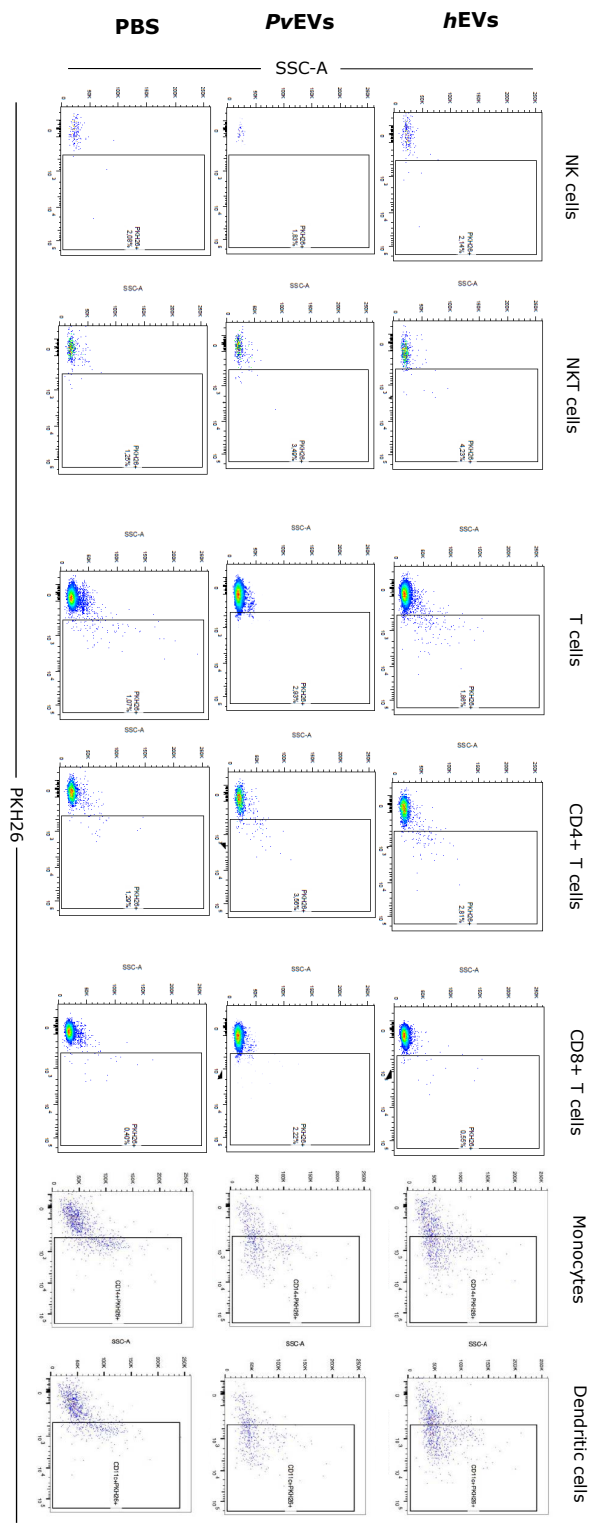

Figure A.2 **Gating strategy applied to determine PKH26+ in total splenocytes.** Gating strategy for populations of NK cells, NKT cells, total T-cells, CD4+ T-cells, C8+ T-cells, monocytes and DCs (from left to right). Data shows a representative gating from one spleen donor.

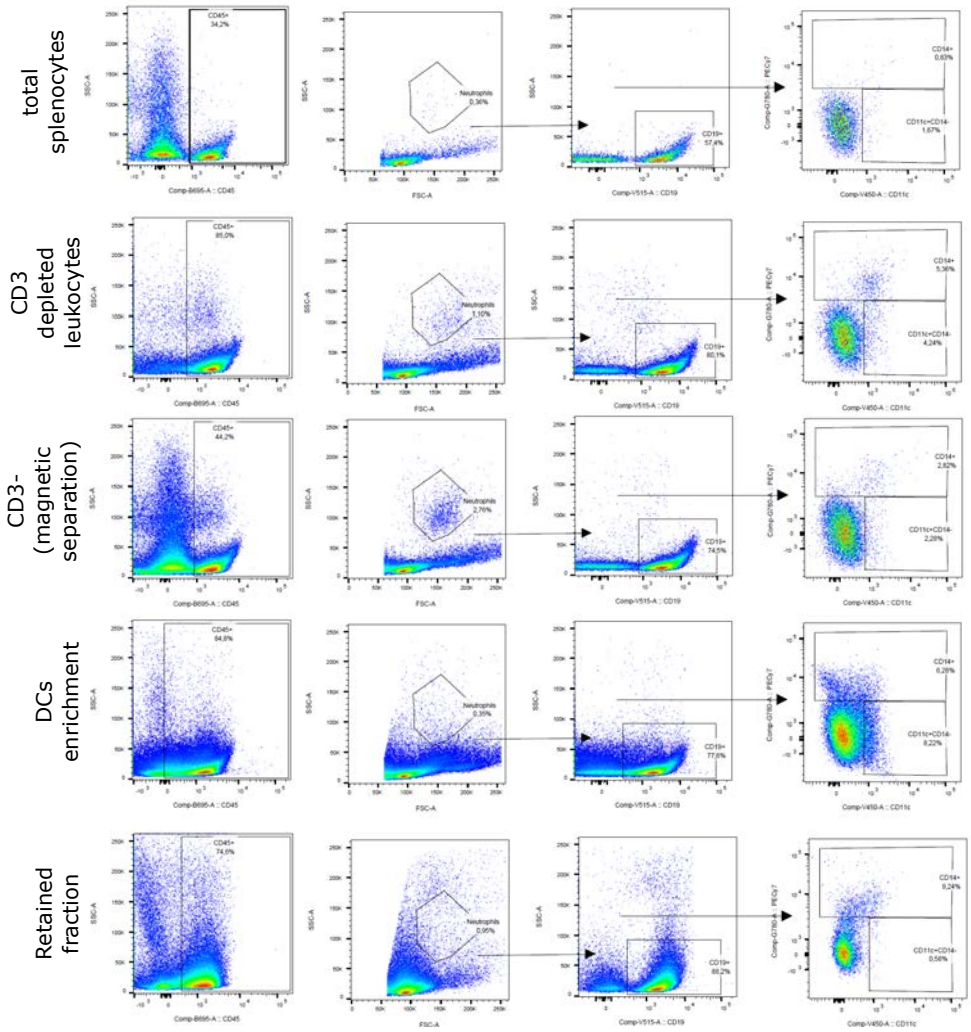

Figure A.3 **Gating strategy applied to determine sorted phagocytic and B-cells.** Gate strategy to identify DCs, monocytes and B-cells during DC enrichment procedure. Plots shown corresponds to one purification from one spleen donor.

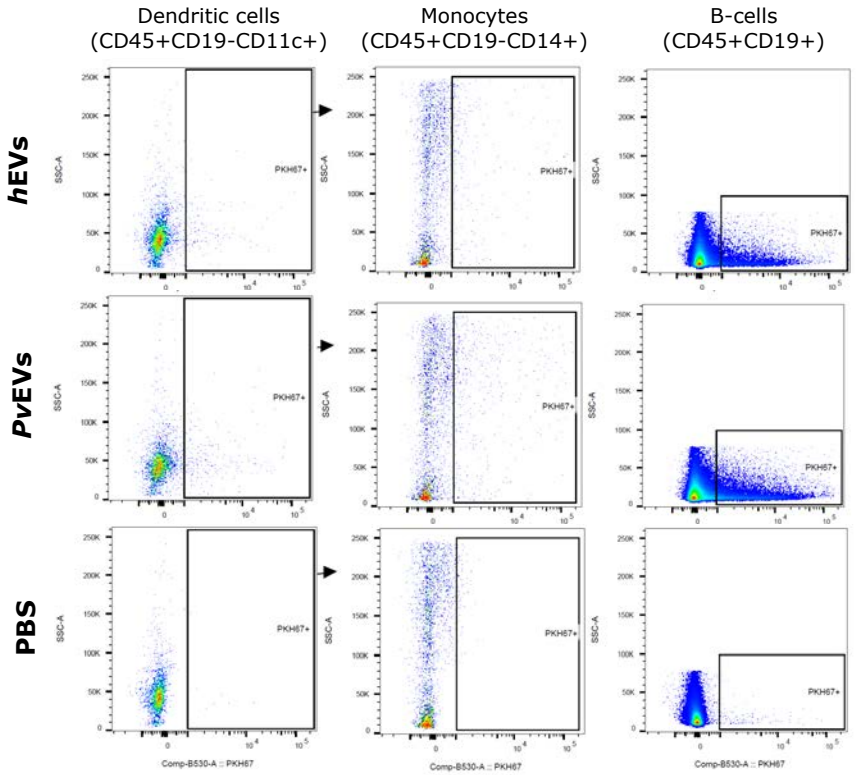

Figure A.4 **Gating strategy applied to determine PKH67+ sorted phagocytic and B-cells.** Gate strategy to determine the frequency of PKH67+ cells after uptake. Data shows a representative gating from one spleen donor.

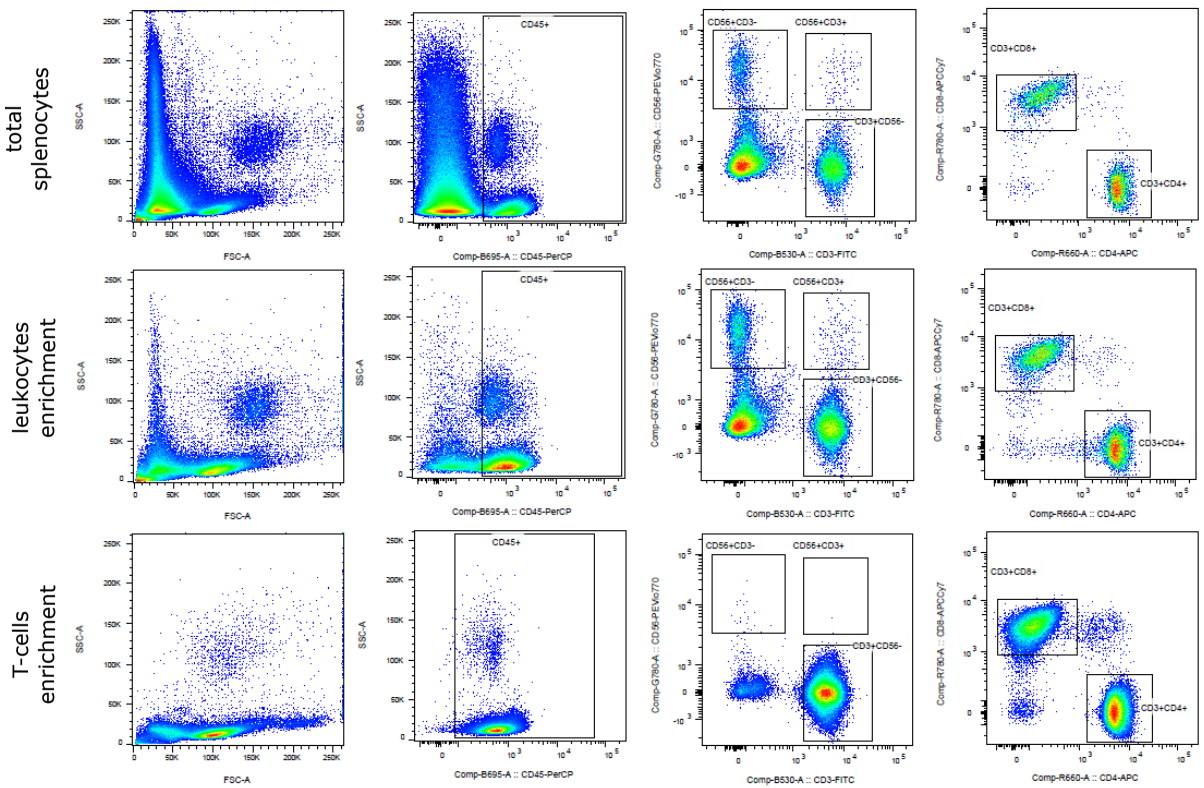

Figure A.5 **Gating strategy applied to determine sorted T-cells.** Gate strategy to identify T-cells during T-cell enrichment procedure. Plots shown corresponds to one purification from one spleen donor.

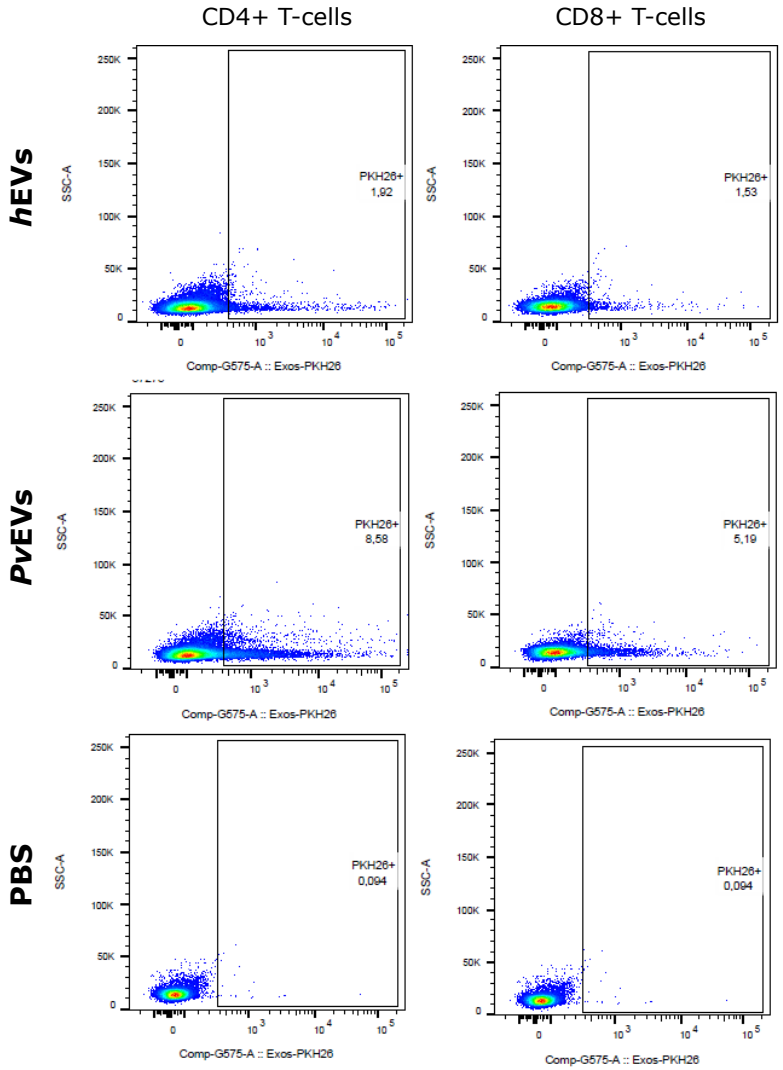

Figure A.6 **Gating strategy applied to determine PKH+ sorted T-cells.** Gate strategy to determine the frequency of PKH+ T-cells after uptake. Data shows a representative gating from one spleen donor.

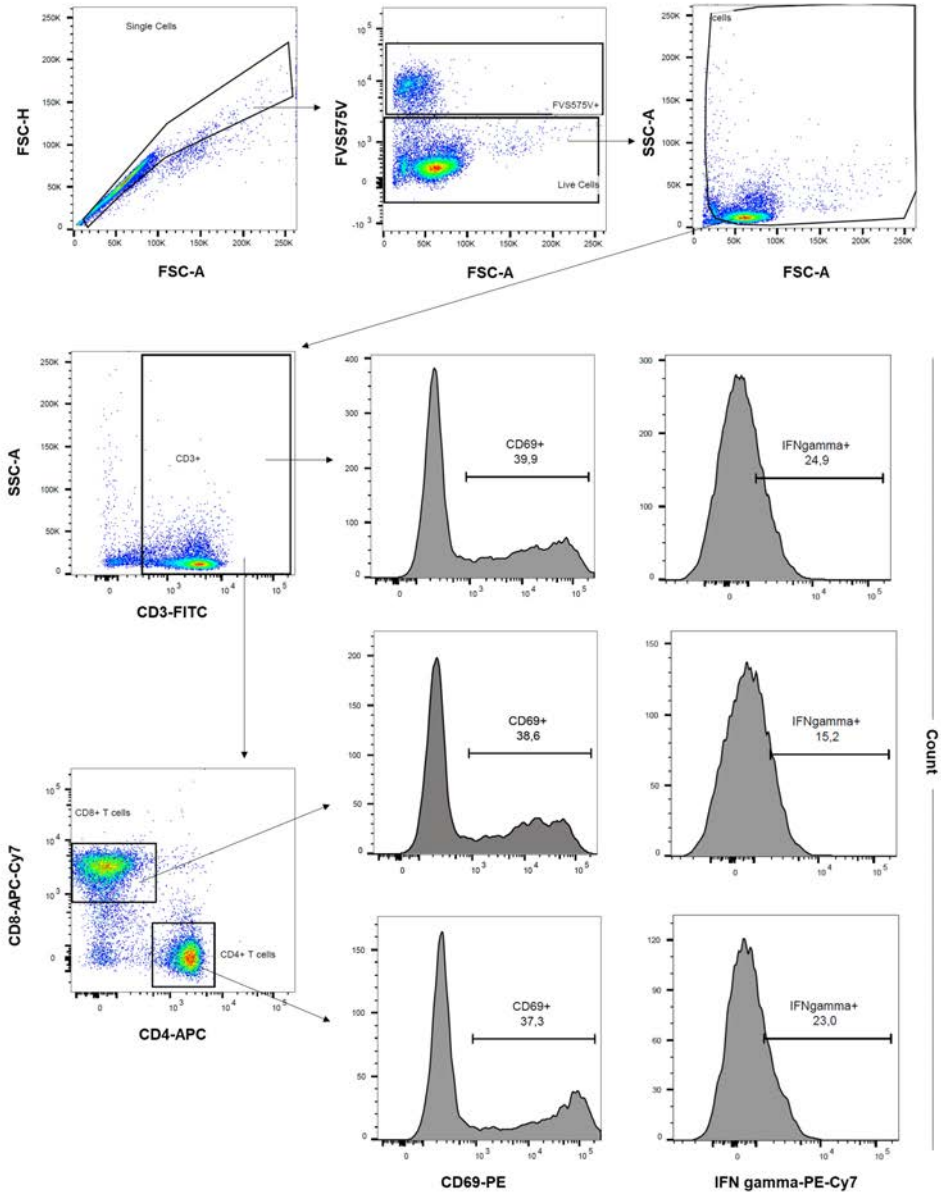

Figure A.7 **Gating strategy followed to quantify expression of CD69 and IFN-γ production on T-cells.** Plots show a representative example of T-cells from one donor stimulated with anti-CD2/CD3/CD28 microbeads.

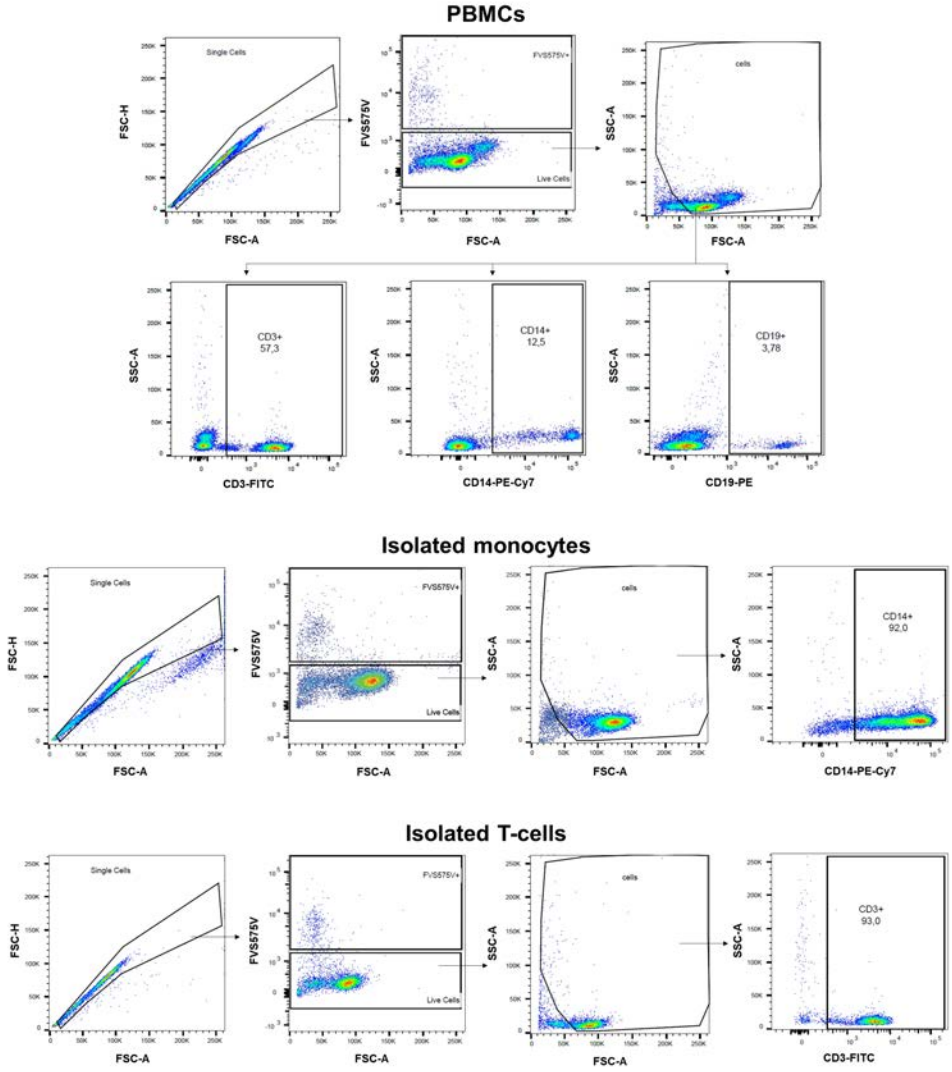

Figure A.8 **Gating strategy to analyze the frequency of T-cells (CD3+), monocytes (CD14+) and B-cells (CD19+) on total PBMCs and after monocyte or T-cell isolation procedures.** Plots show a representative gating from one donor.

Appendix

B Supplementary Datasets

B.1 Dataset B1: Human proteins identified in circulating EVs by LC-MS/MS

Dataset can be found at https://tinyurl.com/y4opooxx.

Table 1. Humans proteins identified in the healthy donors and *P. vivax* patients. Keratins, immunoglobulins and abundant plasma proteins as published in (de Menezes-Neto et al., 2015) have been removed.

Table 2. Human proteins exclusively present in *P. vivax* patients. Proteins identified in 5 or more patients are highlighted in bold.

Table 3. Human proteins exclusively present in healthy donors. Proteins identified in 5 or more patients are highlighted in bold.

Table 4. Human proteins with differences in abundance between *P. vivax* patients and healthy donors. Only proteins with a statistically significant abundance change are shown.

Table 5. Proteins removed from the total list considered as contaminants. Categories include *Bos taurus* contaminants, immunoglobulins, keratins and abundant plasma proteins as reported in (de Menezes-Neto et al., 2015).

B.2 Dataset B2: *P. vivax* proteins identified in *Pv*EVs by LC-MS/MS

Dataset can be found at https://tinyurl.com/y3yvp9s4.

Table 1. *P. vivax* proteins identified in EVs from *P. vivax* infected patients. Total proteins identified: 20. Descriptions of Uniprot were used to retrieve protein sequences of each entry. Blast analysis was performed in the PlasmoDB

database to retrieve the corresponding gene name and description of orthologues in *P. vivax* SalI and PVP01 strains.

Table 2. *P. vivax* proteins identified as false positives. Exclusion criteria was based on the detection of peptides in EVs from healthy donors.

B.3 Dataset B3: Gene ontology enrichment analysis of human proteins exclusively identified in *Pv*EVs

Dataset can be found at https://tinyurl.com/y3uha7ke.

Table 1. GO analysis for the category "Biological Process".

Table 2. GO analysis for the category "Cellular component".

Table 3. GO analysis for the category "Molecular function".

In all tables GO terms with $p<0.05$ are highlighted in color.

B.4 Dataset B4: List of proteins identified in human reticulocyte-derived exosomes from different preparations by LC-MS/MS

Dataset can be found at https://tinyurl.com/yy52j46x.

List of grouped proteins that have been identified at 1 %FDR and by at least 2 unique peptides, or with 1 unique peptide if they are present in two or more preparations. After filtering out contaminant proteins, a list of 367 proteins has being generated (presented in Sheet 1 "*HuRex* proteome"). Proteins are listed based on their number of unique peptides. Sheets from 2 to 13, contain the lists of proteins identified for different subcellular locations according to GO annotation: "2.ER or secreted", "3.Cytosol", "4.Plasma membrane", "5.Nucleus", "6.Cytoskeleton", "7.Lysosome", "8.Endosome", "9. Golgi apparatus", "10. Endoplasmic reticulum", "11.Mitochondrion", "12.Peroxisome", "13.Not retrieved". Sheet 14 shows the list of potential contaminants removed from the final list. Table abbreviations: ER: extracellular region.

B.5 Dataset B5: Gene Ontology enrichment analysis of human reticulocyte-derived exosomes

Dataset can be found at https://tinyurl.com/yyfmnmzx.

GO term-enrichment analysis of *HuRex* proteome at biological process (Sheet 1), cellular component (Sheet 2) and molecular function (Sheet 3) level performed with Database for Annotation, Visualization and Integrated Discovery (David 6.8). All GO terms with their statistical analysis are shown.

B.6 Dataset B6: Lists of proteins from the comparative analysis with human red cell MS proteomes

Dataset can be found at https://tinyurl.com/yxc6fxco.

In sheet 1: list of proteins common to both reticulocyte and mature RBC core proteomes (white), to reticulocytes (cyan), to mature RBCs (yellow) and those exclusively related to *HuRex* (green) that were obtained according to the analysis shown in Figure 7.6.

In sheet 2: plasma membrane proteins and in sheet 3: cytosol proteins.

B.7 Dataset B7: Relative protein abundance in *HuRex* by NSAF

Dataset can be found at https://tinyurl.com/yx9v6yg6.

Relative protein abundance was estimated with the calculation of the protein quantification index NSAF for every protein, denoted by each gene ID, identified in every *HuRex* preparation. Mean, median and standard deviation of the NSAF values from the all preparations are included in the last three columns.

B.8 Dataset B8: *Rex* proteins conserved among different species

Dataset can be found at https://tinyurl.com/yxtg6h7q.

Proteins common between *HuRex* and previously reported proteome from rat exosomes at day 2 of *in vitro* differentiation (Carayon et al., 2011).

Appendix

C Summarized Spanish Version

C.1 Introducción

C.1.1 *Malaria*

Casi la mitad de la población mundial vive en riesgo de infección por malaria. Según las estimaciones mundiales más recientes, en el año 2017 ocurrieron 219 millones de casos de malaria y 435.000 muertes (WHO, 2018). De las cinco especies que pueden causar malaria humana, *Plasmodium falciparum* y *Plasmodium vivax* son las principales contribuyentes a la morbilidad por malaria (WHO, 2018). Es de destacar que *P. vivax* es el parásito de la malaria humana más extendido geográficamente, lo que pone a casi 2.500 millones de personas en riesgo de infección (Howes et al., 2016).

P. falciparum causó la mayoría de los casos de malaria en la mayoría de las regiones de la OMS. Aun así, *P. vivax* fue responsable de al menos 7,5 millones de casos de malaria clínica en el último año. *P. vivax* es el parásito más frecuente en la Región de las Américas de la OMS, representando más del 70% de los casos de malaria, y también provoca alrededor de un 30% de los casos de malaria en las regiones de la OMS del Sudeste Asiático, Mediterráneo Oriental y el Pacífico occidental (WHO, 2018). Aunque éstas son las mejores estimaciones que podemos obtener con las metodologías actuales, no debemos olvidar que es probable que se estén subestimando las infecciones por *P. vivax* debido al bajo número de parásitos que suele haber en circulación. Un gran número de las infecciones son submicroscópicas (Moreira et al., 2015) y muchas de las coinfecciones con *P. falciparum* tienden a clasificarse como monoinfecciones por *P. falciparum* ya que la mayor densidad de este último

parásito puede enmascarar a los parásitos de *P. vivax* (Mayxay et al., 2004). Desafortunadamente, sin una estimación precisa de las infecciones por *P. vivax*, es difícil que se desarrollen e implementen estrategias de control eficientes.

Las personas que contraen malaria a menudo experimentan fiebre, dolor de cabeza y escalofríos cuando no son inmunes. No todas las personas son igualmente vulnerables a la malaria. Los niños son el grupo más afectado, representando alrededor del 60% de las muertes por malaria en 2017. Las mujeres embarazadas, los pacientes con SIDA y los migrantes no inmunes también se encuentran dentro de los grupos de mayor riesgo (WHO, 2018). Los episodios severos de malaria pueden causar convulsiones, coma e insuficiencia en múltiples órganos. Las principales complicaciones incluyen malaria cerebral, anemia severa y acidosis respiratoria. Siempre se ha creído que solo *P. falciparum* puede causar malaria grave, pero varios informes en la última década indican que *P. vivax*, una especie de malaria calificada erróneamente como benigna, puede provocar también malaria grave (Baird, 2013).

P. vivax plantea desafíos adicionales para su control y prevención en comparación con otras especies causantes de malaria. Por ejemplo, los parásitos de *P. vivax* pueden desarrollar etapas latentes en el hígado, denominados hipnozoitos, que, si no se tratan adecuadamente con primaquina, podrían volver a despertarse y provocar una recaída. Sin embargo, la administración de primaquina probablemente causará hemólisis a pacientes con una deficiencia hereditaria de la enzima glucosa-6-fosfato deshidrogenasa (en inglés *Glucose-6-phosphate dehydrogenase*, y abreviado como G6PD) (Baird et al., 2016). Por lo tanto, es crucial para el control de vivax, que se desarrollen herramientas de diagnóstico asequibles y precisas para detectar las etapas hepáticas y la deficiencia de G6PD. También se deberían desarrollar herramientas para detectar la baja parasitemia de *P. vivax* (The malERA Consultative Group on Diagnoses, 2011).

C.1.1.1 Biología de *Plasmodium vivax*

Plasmodium, incluido en el filo Apicomplexa (N. Levine, 1970). Cualquier especie de *Plasmodium* que infecta a humanos también parasita a mosquitos pertenecientes al género *Anopheles* para completar su complejo ciclo de vida. Estos parásitos experimentan varias transformaciones para poder infectar varios tipos de células en sus diferentes huéspedes (Mueller et al., 2009). En esta sección nos centraremos específicamente en el ciclo de vida de *P. vivax* dentro del huésped humano.

La primera forma de *Plasmodium* que entra naturalmente en un huésped humano es el esporozoíto. Los esporozoítos son inoculados en la piel por mosquitos hembra. Una vez alcanzan el torrente sanguíneo, llegan al hígado donde se diferencian a esquizontes tisulares dentro de los hepatocitos. Es-

tos esquizontes pueden originar después de miles de replicaciones mitóticas merozoitos que lleguen al torrente sanguíneo o pueden derivar a una forma latente, el hipnozoito (Krotoski, 1985). En su fase eritrocitaria, los merozoitos de *P. vivax* invaden predominantemente, si no exclusivamente, los eritrocitos inmaduros, también conocidos como reticulocitos (Kitchen, 1937). Este tropismo por reticulocitos es uno de los principales factores que contribuyen a la falta de un cultivo *in vitro* continuo para *P. vivax* (Noulin et al., 2013), lo cual ha dificultado enormemente la investigación en este parásito. Durante esta fase, los reticulocitos infectados se agradan y se vuelven más deformables. También se producen complejos de caveolas y vesículas que se reconocen en frotis de sangre teñidos con Giemsa como puntos de Schüfnner (Aikawa et al., 1975). En lugar de invadir nuevos reticulocitos, algunos de los parásitos pueden diferenciarse gametocitos masculinos y femeninos que, si llegan al mosquito *Anopheles*, desarrollarán el ciclo sexual donde tras varias transformaciones, se producirán nuevos esporozoitos.

C.1.1.2 Respuestas inmunes contra *P. vivax*

La inmunidad natural producida frente a malaria es muy compleja y aún no se conocen bien los mecanismos que la producen. Ésta varía de acuerdo a muchos factores, tales como la edad del huésped, el nivel de transmisión y la especie y estadio del parásito, entre muchos otros (Longley et al., 2016). Las observaciones en pacientes infectados naturalmente como en infecciones experimentales sugieren que tanto el mantenimiento de anticuerpos citofílicos como la inducción de las células B y T de memoria son esenciales para la generación de protección duradera frente a malaria (Long & Zavala, 2017). Los estudios epidemiológicos han demostrado que la inmunidad natural frente *P. vivax* se adquiere más rápido que la inmunidad frente a *P. falciparum*. En lugares donde ambos parásitos son altamente endémicos, la mayoría de los niños son inmunes a vivax a la edad de 5 años, mientras que continúan en riesgo de enfermedad clínica frente a falciparum (Mueller et al., 2013). Estas observaciones junto con las características biológicas únicas de cada especie de Plasmodium sugieren que los mecanismos de protección contra *P. vivax* y *P. falciparum* pueden diferir sustancialmente.

Los estudios seminales de Cohen *et al* destacaron la importancia de los anticuerpos en la inmunidad natural contra la malaria, al demostrar que la transferencia de sueros de adultos inmunes a niños protegía a los niños frente a manifestaciones clínicas (S. Cohen et al., 1961). Sin embargo, hay más de 5.000 proteínas codificadas en el genoma de *P. vivax* (Auburn et al., 2016), por lo que no es sencillo determinar cuáles de ellas representan el mejor objetivo para una vacuna altamente eficaz. La proteína de unión a Duffy de *P. vivax* (en inglés *P. vivax Duffy binding protein*, cuya sigla es PvDBP) es una proteína

de gran importancia en la invasión de merozoitos y, por lo tanto, un relevante objetivo para vacunación. A lo largo de los años, se ha acumulado evidencia de que los anticuerpos dirigidos contra esta proteína bloquean la interacción con su receptor e inhiben la invasión de eritrocitos por *P. vivax* (Grimberg et al., 2007; Michon et al., 2000). A pesar de la naturaleza polimórfica de la PvDBP, recientes estudios demuestran que anticuerpos derivados de humanos de individuos con inmunidad adquirida de forma natural se dirigen a epítopos conservados en el dominio de unión a su receptor (Urusova et al., 2019). Muchos trabajos han estudiado la generación de respuestas humorales contra otras proteínas de superficie de merozoito (en inglés *merozoite surface protein*, MSP), siendo MSP1 la más estudiada. Sin embargo, pocos trabajos han podido asociar los anticuerpos específicos contra MSP1 con protección (Nogueira et al., 2006; Versiani et al., 2013). Recientes análisis de respuestas humorales adquiridas naturalmente frente a EBP y RBP [del inglés *erythrocyte binding protein* (EBP) y reticulocyte binding protein (RBP) respectivamente)] entre otras proteínas, han sido asociadas con protección (França et al., 2017). Además, este estudio investigó la posible eficacia protectora de los anticuerpos contra múltiples combinaciones de antígenos para guiar el desarrollo de vacunas contra *P. vivax* basadas en múltiples componentes.

Las respuestas celulares parecen ser críticas para limitar tanto la infección asintomática de la etapa hepática como la etapa sintomática, pero la mayor parte de la evidencia de inmunidad mediada por células T proviene de modelos de roedores que estudian la protección contra las etapas pre-eritrocíticas (Kurup, Butler, & Harty, 2019). Es de destacar que estudios *in vitro* también han mostrado respuestas específicas de células T CD8+ contra formas hepáticas de *P. vivax* (Vichchathorn et al., 2006). En malaria, las respuestas de las células T a la infección sanguínea siempre se han pasado por alto debido a que los eritrocitos maduros no expresan antígenos leucocitarios humanos (del inglés *Human Leukocyte Antigen*, cuya sigla es HLA). Sin embargo, *P. vivax* invade reticulocitos, células que sí presentan moléculas HLA de la clase I (Silvestre et al., 1970). Burel *et al* exploraron por primera vez las posibles respuestas celulares diferenciales en voluntarios naive infectados con formas sanguíneas de *P. vivax* o *P. falciparum*, y demostraron que solo los voluntarios infectados con *P. vivax* tenían un subconjunto expandido de células T CD8+ citotóxicas (Burel et al., 2016). Además, un estudio reciente ha demostrado que los reticulocitos infectados con *P. vivax* pueden ser reconocidos y destruidos por las células T CD8+ a través de sinapsis inmunológicas dependientes de las moléculas HLA presentes en los reticulocitos (Junqueira et al., 2018). Este podría ser un mecanismo de defensa diferencial entre la entre la infección por *P. vivax* y por *P. falciparum*. Se deben realizar más estudios para determinar el papel de las estas respuestas en la infección sanguínea de *P. vivax* y su potencial para vacunación.

El bazo es un órgano esencial para el control de las infecciones por malaria ya

que es el lugar donde se filtran los glóbulos rojos infectados y dónde se generan las respuestas inmunes adaptativas (Engwerda et al., 2005). El bazo tiene una arquitectura muy compleja que facilita la captura de antígenos en circulación y su presentación a linfocitos T y B para que se generen respuestas antígeno-específicas (Lewis et al., 2019). Sin embargo, la mayoría de los trabajos sobre el desarrollo de respuestas esplénicas frente a malaria provienen de modelos de roedores y no está claro en qué medida estos resultados se pueden extrapolar a infecciones humanas (Del Portillo et al., 2012). A pesar de que los estudios sobre el bazo de pacientes estén limitados por razones éticas y técnicas, sería relevante tener una comprensión más profunda de la interacción del bazo humano con parásitos causantes malaria humana.

C.1.1.3 Vacunas contra *P. vivax*

Para ayudar a reducir la enfermedad y la transmisión de *P. vivax*, la comunidad científica debería hacer un mayor esfuerzo en el desarrollo de vacunas altamente efectivas (Mueller et al., 2015). Eso también se aplica a *P. falciparum*. Ahora mismo la vacuna más avanzada, la RTS,S, dirigida contra falciparum, confiere niveles parciales de protección (RTS,S Clinical Trials Partnership, 2015). El desarrollo de vacunas contra *P. vivax* ha sido muy limitado, con menos de 10 candidatos a vacuna que se hayan sometido o estén ensayos clínicos [1]. Esta situación deriva, al menos en parte, de la falta de comprensión de la biología del parásito *P. vivax* y la inmunidad contra el mismo. Es de vital importancia que se lleven a cabo mayores esfuerzos de financiación e investigación para poder subsanar esta falta de conocimiento y desarrollar nuevas estrategias de vacunación contra vivax.

A pesar de las limitaciones varios ensayos clínicos con estrategias de vacunación dirigidas a la PvDBP han dado resultados prometedores. En uno de ellos fue demostrado que partículas virales (en inglés *viral-like particles*, VLP) codificando esta proteína son seguras y producen respuestas humorales capaces de inhibir in vitro la unión de esta proteína con su receptor (Payne et al., 2017). En otro ensayo, la misma región de esa proteína también provocó una respuesta humoral al ser administrada con una emulsión estable de adyuvante lípido de glucopiranosilo (en inglés, *glucopyranosyl lipid adjuvant-stable emulsion*, GLA-SE) (K. Singh et al., 2018). De relevancia, otros modelos pre-clínicos que utilizan VLPs codificando proteínas del esporozoito destacan el potencial de esta plataforma para aumentar la eficacia de los candidatos a vacunas (Atcheson et al., 2018; Salman et al., 2017).

A pesar de que diseñar vacunas contra malaria es un gran desafío, aún hay muchas estrategias por desarrollar y mejorar. Descubrir cuales son los antígenos y epítopos clave, maximizar la actividad de los anticuerpos y de las

[1] WHO Malaria Vaccine Rainbow Table and clinicaltrials.gov

células T así como optimizar el uso de adyuvantes y plataformas de vacunas son algunas de las muchas alternativas que se pueden seguir investigando en el futuro (Beeson et al., 2019).

C.1.2 *Vesículas Extracelulares*

Las vesículas extracelulares (VEs) son pequeñas vesículas membranosas que pueden originarse a partir de cualquier tipo de célula y detectarse en múltiples fluidos biológicos (Yáñez-Mó et al., 2015). Su potencial como comunicadores intercelulares se ha demostrado en múltiples procesos fisiológicos y patológicos (Valadi et al., 2007), incluidas las infecciones por malaria (Regev-Rudzki et al., 2013).

Las VEs son muy heterogéneas, pero se han definido dos subconjuntos principales: exosomas, que tienen un origen endocítico y un tamaño más reducido (50-150 nm) y microvesículas (MVs), que se liberan directamente desde la membrana plasmática y tienen un tamaño más grande (generalmente 50-500 nm). La composición de estas vesículas es variable y en gran medida, depende del tipo celular del que derivan. Han sido reportadas en varios tipos de VEs las tetraspaninas, varios componentes de la maquinaria ESCRT (del inglés *endosomal sorting complexes required for transport*), proteínas involucradas en la adhesión celular y el tráfico intracelular así como chaperonas y enzimas metabólicas y señalizadoras (Van Niel, D'Angelo, & Raposo, 2018).

C.1.2.1 Aislamiento y caracterización de VEs

La mayoría de los estudios sobre VEs se han especializado más en sus potenciales funciones que en estudiar su origen. Por lo tanto, normalmente tratamos con poblaciones de VEs heterogéneas donde no sabemos qué subpoblación(es) específica(s) es(son) responsable(s) de un efecto dado (Tkach et al., 2018).

En este momento, el campo de VEs está muy confundido por la falta de métodos estandarizados para identificar y aislar los diferentes subtipos de vesículas. De todas formas, existen metologías ampliamente utilizadas para su aislamiento, como la centrifugación diferencial, que fue la primera técnica en utilizarse (Théry et al., 2006). De forma general, en esta técnica se centrifugan los fluidos biológicos o los sobrenadantes de cultivos celulares a velocidades que permitan eliminar debris celular primero y a mayores velocidades después para aislar MVs y exosomas, siendo preciso para estos últimos una ultracentrifugación a alta velocidad (Théry et al., 2006). Cuando la ultracentrifugación se acopla a gradientes de densidad, se pueden resolver mejor las diferentes poblaciones de VEs (Jeppesen et al., 2019; Kowal et al., 2016). Sin embargo, ésta es una técnica que produce material muy puro, pero muy escaso, no es fácil determinar hasta qué punto se podría aplicar sobre muestras de limitado volumen inicial. Por ello se están popularizando otros métodos alternativos. Algunos de ellos

se basan en separaciones por inmuno-afinidad utilizando anticuerpos contra tetraspaninas (F. Yang et al., 2017). Sin embargo, dado que la distribución de estas moléculas no es homogénea en las VEs, es posible que se comentan sesgos. Otra limitación importante es la contaminación de la muestra EV con moléculas aislantes. La cromatografía de exclusión por tamaño (en inglés *size-exclusion chromatography*, SEC) también se utilizando cada vez más en el enriquecimiento de VEs ya que es una técnica escalable y rentable que proporciona una alta pureza y rendimiento de VEs (Monguió-Tortajada et al., 2019). SEC se ha aplicado ya en gran variedad de fluidos biológicos como orina (Lozano-Ramos et al., 2015) y plasma (de Menezes-Neto et al., 2015) así como en sobrenadante de cultivo celular (Monguió-Tortajada et al., 2017). Una de sus desventajas es que diluye bastante las muestras de VEs, por lo que para muchas aplicaciones es necesaria una concentración previa. Dado que cada una de las técnicas de aislamiento tiene sus ventajas e inconvenientes, es primordial tener claro el objetivo del estudio de VEs para elegir la técnica que mejor se adecue.

La caracterización de VEs es crucial para determinar la naturaleza de las mismas, desentrañar sus potenciales funciones biológicas y/o aplicarse terapéuticamente. Entre otras, se suelen utilizar varias técnicas para determinar su tamaño y morfología tales como la microscopía electrónica, el análisis de seguimiento de nanopartículas (del inglés *nanoparticle tracking analysis*, NTA) y la dispersión de luz dinámica (del inglés *dynamic light scattering*, DLS). Son esenciales también las técnicas que caracterizan su contenido molecular. Está especialmente extendido el análisis de su composición proteica por *western blot*, citometría de flujo y la espectrometría de masas de proteínas. Esta última técnica es altamente informativa y contribuye enormemente a la comprensión de las funciones de VEs así como a la búsqueda de posibles dianas de diagnóstico o terapéuticas (Kreimer et al., 2015).

C.1.2.2 VEs en las respuestas inmunes

Las VEs liberados tanto por las células inmunes como por las no inmunes tienen funciones relevantes en la regulación de la respuesta inmunitaria. Los mecanismos por los cuales las VEs podrían mediar la respuesta inmune son muy variados. Múltiples informes han descrito la acción de las VEs sobre el sistema inmune innato (Groot Kormelink et al., 2018) y aún más se han centrado en su contribución a las respuestas inmunes adquiridas (Robbins & Morelli, 2014). Curiosamente, se ha demostrado que los EV ejercen efectos inmunoestimuladores o inmunosupresores, con un enorme potencial para las estrategias de vacunación positivas o negativas.

VEs derivadas de células inmunes, especialmente de células presentadoras de antígenos (abreviado como CPAs) contienen moléculas HLA de la clase I y II y por lo tanto, pueden facilitar la presentación de antígenos a células T

CD8+ y CD4+, respectivamente. Se ha demostrado que la capacidad de las VEs para estimular respuestas T depende de la intervención de CPAs, como por ejemplo de las células dendríticas (abreviado como CDs) (Théry et al., 2002). Mientras que algunos de las VEs pueden permanecer unidos a la superficie de las CPAs, y presentar los antígenos unidos a las moléculas de HLA vesiculares directamente a las células T, otras vesículas pueden ser internalizadas por las CPAs y los antígenos que portan podrían ser cargados en las moléculas HLA de las células para su presentación (Robbins & Morelli, 2014).

También se ha observado que VEs derivadas de DCs cargadas con péptidos de virus de Epstein-Barr, citomegalovirus e influenza estimulan directamente células T CD8+ (Admyre et al., 2006). Sin embargo, debe tenerse en cuenta que probablemente éstas eran células T de memoria. Los EV libres podrían funcionar como CPAs para células T específicas de antígeno o anteriormente expuestas al mismo, pero dada su débil capacidad de estimulación, es improbable que puedan presentar antígenos directamente a células T naive sin la mediación de CPAs (Robbins & Morelli, 2014).

Independientemente del mecanismo de presentación de antígenos que puedan facilitar, las VEs son además una fuente potencial de antígenos. El campo del cáncer fue pionero en la búsqueda de nuevos antígenos en VEs derivadas de células tumorales (Wolfers et al., 2001). Años más tarde, se identificaron componentes microbianos en VEs derivadas de células infectadas en infecciones virales, parasitarias, fúngicas y bacterianas, y se han descrito tanto respuestas inmunes inhibitorias como estimulatorias mediadas por estas VEs (Jeffrey Sean Schorey et al., 2015).

Dado el potencial de las VEs para modular las respuestas inmunes, varios trabajos han investigado su potencial para el desarrollo de vacunas. Los estudios más avanzados se han llevado con VEs derivadas de CDs para facilitar el reconocimiento y control de células tumorales en ensayos clínicos (Besse et al., 2016). También se está explorando esta aplicación en enfermedades infecciosas. Se ha demostrado que la liberación de VEs durante la infección por *Mycobacterium tuberculosis* contribuye significativamente a las respuestas de células T (Smith et al., 2017) y que las VEs de CDs cargadas con antígenos microbianos pueden mediar inmunidad protectora contra la toxoplasmosis (Aline et al., 2004; Beauvillain et al., 2007) y la leishmaniasis cutánea (Schnitzer et al., 2010).

C.1.2.3 VEs en malaria

La producción de VEs ha sido documentada en múltiples infecciones. En organismos infectados, se ha demostrado que las VEs transportan señales tanto de los patógenos como de los huéspedes, lo que permite rutas de comunicación extracelulares entre los patógenos y entre los patógenos y sus huéspedes (Jeffrey Sean Schorey & Harding, 2016). Se ha planteado la hipótesis de que esta

transferencia de componentes es un contribuyente importante en la coevolución huésped-patógeno (Barteneva et al., 2013).

Las VEs en las infecciones pueden jugar papeles opuestos. En algunos contextos, se ha demostrado que las VEs promueven respuestas inmunitarias que protegerían al huésped (Aline et al., 2004; Bhatnagar et al., 2007; Martin-Jaular et al., 2011) mientras que, en otros parecen favorecer la supervivencia y la propagación de los patógenos (Abrami et al., 2013; Cestari et al., 2012). Ambos fenómenos pueden estar ocurriendo en el curso de una infección dependiendo del tipo y la fase de patógeno y/o las condiciones ambientales específicas.

La producción de VEs por parásitos y células parasitadas se ha descrito en infecciones como toxoplasmosis, tripanosomiasis, leishmaniasis y malaria, que afectan a millones de personas en todo el mundo. De hecho, el número de estudios relacionados con VEs está creciendo rápidamente para parásitos protozoarios como la malaria (Marcilla et al., 2014).

En los últimos años, múltiples trabajos han resaltado la relevancia de las VEs con respecto a la patogénesis de la malaria y la comunicación intercelular que estudian las infecciones naturales de la malaria y utilizan diferentes modelos experimentales (Sampaio et al., 2017). Los estudios iniciales se centraron principalmente en la asociación de MVs y la malaria severa, en concreto con malaria cerebral (Combes et al., 2004). Años más tarde, una elevada producción de VEs fue reportada en infecciones naturales en Tailandia, entre las cuales había infecciones causadas por *P. vivax* (Nantakomol et al., 2011) y en infecciones de *P. vivax* en Brasil (Campos et al., 2010).

En un modelo de malaria murino infectado con *P. berghei* se demostró que las MVs activaban macrófagos y contribuían una inflamación sistémica (Couper et al., 2010). Por otro lado, en un modelo murino infectado con *P. yoelii*, un parasito con predilección por invadir reticulocitos como vivax se aislaron VEs circulantes de menor tamaño que contenían proteínas del parásito. Cuando se aislaron exosomas de cultivos *in vitro* de reticulocitos infectados con *P. yoelii* y se utilizaron en combinación al adjuvante CpG para inmunizar ratones, éstos produjeron respuestas humorales y sobrevivieron a un desafío letal de malaria. Ésta ha sido la primera evidencia de que las VEs pueden estar involucradas en un efecto protector frente a malaria y, por lo tanto, futuros estudios deberían investigar su uso en vacunación (Martin-Jaular et al., 2011).

Varios estudios posteriores de VEs en malaria se han beneficiado del cultivo *in vitro* de falciparum para estudiar la composición y funciones de las VEs derivadas de eritocitos infectados por *P. falciparum*. Se ha descubierto que dichas VEs median la capacidad de comunicación entre parásitos para, por ejemplo, fomentar la gametocitogénesis o la resistencia a drogas, así como para regular la respuesta inmune del huésped entre otras funciones (Mantel et al., 2016; Mantel et al., 2013; Regev-Rudzki et al., 2013; Sisquella et al., 2017).

De importancia para vivax, un estudio sobre las VEs circulantes en un mod-

elo de ratón humanizado en hígado capaz de sostener etapas pre-eritrocíticas de *P. vivax*, ha identificado proteínas de vivax en dichas vesículas, abriendo nuevos caminos en la búsqueda de biomarcadores para las formas hepáticas del parásito, incluido el hipnozoito (Gualdrón-López et al., 2018).

Aunque el estudio de las VEs liberadas *in vitro* de las células infectadas por *P. vivax* es limitado debido a la falta de un cultivo *in vitro* continuo (Noulin et al., 2013), en el futuro sería factible y relevante investigar las VEs circulantes de las infecciones por *P. vivax* para comprender sus posibles funciones durante la infección.

C.1.2.4 Exosomas derivados de reticulocitos: ¿una posible plataforma de vacuna contra malaria?

Además de ser las células diana de muchas especies de *Plasmodium*, los reticulocitos son las células donde se describió por primera vez la liberación de exosomas. Este descubrimiento, casi fortuito, fue realizado por dos grupos independientes que estudiaban el destino del receptor de transferrina (Harding et al., 1983; Pan & Johnstone, 1983). Se descubrió que la vía exosomal ayudaba a los reticulocitos a eliminar selectivamente proteínas obsoletas durante su maduración a los eritrocitos.

Martin-Jaular *et al* fueron pioneros en el estudio de exosomas en infecciones de malaria propensas a invadir reticulocitos. Utilizaron como modelo de estudio la infección por *P. yoelii* para determinar si durante esta infección, los exosomas derivados de reticulocitos (en inglés *reticulocyte-derived exosomes*, cuyo acrónimo es *Rex*) podrían contener proteínas del parásito y modular la respuesta inmune (Martin-Jaular et al., 2011). Descubrieron que cuando los *Rex* derivados reticulocitos infectados se utilizaban en inmunizaciones en combinación con el adyuvante CpG-ODN, se podía conferir una protección letal y duradera en aproximadamente el 85% de los ratones testados (Martin-Jaular et al., 2011) Posteriormente, se demostró que el efecto protector de estas inmunizaciones está asociado con una respuesta de células T de memoria dependiente del bazo, efectora y no agotada (Martín-Jaular et al., 2016).

Estos resultados fomentan el estudio del papel de *Rex* derivados de infecciones de *P. vivax* en la presentación de antígenos y la exploración de *Rex* humanos (abreviado como *HuRex*) como una plataforma para la vacunación contra la malaria vivax.

C.2 Hipótesis y Objetivos

Nuestra hipótesis es que las VEs de infecciones de *P. vivax* (*Pv*VEs), particularmente los exosomas derivados de reticulocitos infectados por *P. vivax*, contienen proteínas del parásito que sirven como antígenos y están involucradas en respuestas inmunitarias antipalúdicas. Proponemos que estas vesículas portadoras

de antígenos pueden llegar al bazo humano y facilitar allí la presentación de antígenos a las células T. Por lo tanto, los exosomas derivados de reticulocitos humanos podrían utilizarse en el descubrimiento de antígenos y representar una novedosa plataforma de vacunación capaz de activar respuestas T citotóxicas contra la malaria vivax.

Los objetivos principales de esta tesis son: (i) evaluar el potencial de las VEs de infecciones de *P. vivax* como fuente de antígenos y como activadoras de respuestas inmunitarias, y (ii) explorar los exosomas derivados de reticulocitos humanos como plataforma de vacunación contra malaria vivax.

Los objetivos específicos de esta tesis son:

- ◇ Aislamiento y caracterización proteómica de las VEs de infecciones de *P. vivax* para descubrir posibles antígenos asociados a estas vesículas y explorar los posibles roles de los *Pv*VEs durante las infecciones naturales de *P. vivax*.

- ◇ Investigación de las interacciones entre las poblaciones de células inmunes del bazo humano y las *Pv*VEs para evaluar la participación de estas vesículas en el inicio de las respuestas inmunes esplénicas.

- ◇ Implementación de una estrategia para evaluar la activación de células T *in vitro* por VEs con el fin de medir la capacidad de los *Pv*VEs para estimular células T de individuos naive o expuestos a malaria vivax.

- ◇ Aislamiento y análisis proteómica de *HuRex* y estudio de su interacción con células presentadoras de antígenos para explorar su uso como plataforma de vacuna.

C.3 Resultados

C.3.1 *Caracterización de las VEs de infecciones de P. vivax: implicaciones en el descubrimiento de antígenos*

Con el objetivo de aislar las VEs circulantes de infecciones naturales de *P. vivax*, procesamos plasma de pacientes infectados con *P. vivax* mediante cromatografía de exclusión por tamaño (SEC). Las fracciones cromatográficas eluidas se analizaron para determinar la concentración de proteínas y se unieron a perlas de látex de 4 μm para identificar y caracterizar fracciones enriquecidas en VEs mediante citometría de flujo evaluando la presencia de marcadores clásicos de VEs (CD9, CD63, CD81), así como del CD5L y CD71. También se midió el tamaño y concentración de VEs utilizando NTA. Como control, se aislaron y caracterizaron VEs del plasma de donantes sanos (*h*VEs). Descubrimos que tanto las VEs de pacientes como controles aisladas por SEC presentaban los

marcadores evaluados y que la cantidad de VEs en las fracciones de los pacientes era mayor que en las de controles sanos, aunque hubiéramos procesado el mismo volumen de plasma.

Para identificar proteínas de *P. vivax* asociadas a los EV circulantes de las infecciones de vivax, se analizaron los EV derivados del plasma de diez pacientes diferentes de *P. vivax* y los EV del plasma de diez donantes sanos mediante un análisis proteómico basado en espectrometría de masas. En general, identificamos 20 proteínas del parásitos y 533 proteínas humanas entre las 20 preparaciones de VEs. Se detectaron tres proteínas de *P. vivax* con al menos dos péptidos únicos: MSP3.1 (del inglés *merozoite surface protein* 3.1) (PVP01_1031700); PHISTc (del inglés *Plasmodium helical interspersed subtelomeric* C) (PVP01_0119200) y GAPDH (Gliceraldehído-3-fosfato deshidrogenasa) (PVP01_1244000) y dos de ellas fueron validadas por *western blot*. De interés, pudimos predecir varias secuencias en MSP3.1 y PHISTc que podrían representar epítopos citotóxicos de células T. Tanto las proteínas de merozoito como las proteínas de parásito exportadas a los eritrocitos infectados se han asociado a VEs derivadas de glóbulos rojos infectados con malaria.

En cuanto a las proteínas humanas, encontramos un conjunto más restringido de proteínas en los *Pv*VEs en comparación con los *h*VEs, a pesar de detectar una mayor cantidad de VEs en el plasma de pacientes infectados. Para obtener información sobre los procesos que las *Pv*VEs podrían estar mediando durante las infecciones de vivax, realizamos un análisis de enriquecimiento en términos de Ontología Génica (en inglés *Gene Ontology*, GO) sobre el conjunto de proteínas exclusivamente detectadas en *Pv*VEs. Los términos GO enriquecidos más significativamente para la categoría de proceso biológico correspondieron a "respuesta inflamatoria", "migración de leucocitos" y "activación de células T", lo que sugiere que las VEs de las infecciones por *P. vivax* pueden estar contribuyendo a la regulación de la respuesta inmune.

C.3.2 *Interacción de PvVEs con esplenocitos humanos*

Dado que el bazo es un órgano crucial en el control de la malaria debido a su capacidad de eliminación de glóbulos rojos parasitados y su reactividad inmunológica, decidimos evaluar las posibles interacciones de los esplenocitos humanos, en particular las células inmunes, con las *Pv*VEs.

Sin embargo, los estudios sobre bazo humano aún son bastante limitados, por ello, llevamos a cabo un fenotipaje por citometría de flujo de varias poblaciones celulares de la línea roja y blanca, así como de células madre hematopoyéticas en bazos humanos provenientes de donantes de trasplante renal.

Posteriormente, evaluamos la interacción de las VEs derivadas del plasma de pacientes de vivax y de donantes sanos con los esplenocitos. Marcamos

las *Pv*VEs y *h*VEs con un tinte lipofílico fluorescente (PKH) y después las incubamos *in vitro* con esplenocitos totales o con células inmunes enriquecidas. Después de la incubación, comparamos la proporción de diferentes poblaciones celulares teñidas positivamente con el PKH por citometría de flujo como una medida de la interacción entre las VEs y las células. Como control negativo, teñimos PBS en las mismas condiciones que las EVs, y lo incubamos con los esplenocitos en paralelo a las VEs.

Observamos que existe una mayor interacción entre monocitos, linfocitos B y T aislados del bazo humano con *Pv*VEs respecto con *h*VEs. La relevancia biológica de estos hallazgos se determinará con futuros estudios.

C.3.3 *Inmunogenicidad de las PvVEs*

La capacidad que tienen algunas VEs de modular la presentación de antígenos a las células T, así como el transporte y la propagación de antígenos, ha fomentado el estudio de las VEs para el desarrollo de vacunas.

Idealmente nos hubiera gustado evaluar la capacidad de los *Pv*VEs en estimular respuestas de las células T del bazo humano, dado que el bazo es el lugar donde se inducen las respuestas T específicas a antígenos circulantes. Sin embargo, las limitaciones éticas, técnicas y biológicas de trabajar con esplenocitos humanos han hecho que redirijamos nuestras investigaciones de inmunogenicidad de *Pv*VEs a células mononucleares de sangre periférica (en inglés, *peripheral blood mononuclear cells*, abreviado como PBMCs).

Es importante destacar que el trabajo de Junqueira *et al* describió que los reticulocitos infectados con *P. vivax* presentan antígenos a las células T CD8+ a través de moléculas HLA de clase I. Por ello, evaluamos la presencia de moléculas HLA clase I en VEs circulantes, ya que podrían facilitar el reconocimiento de potenciales antígenos unidos a las mismas. Medimos su expresión con un anticuerpo contra moléculas HLA de clase I y citometría de flujo. Encontramos que las fracciones SEC enriquecidas en VEs presentaban moléculas de HLA clase I y eran coincidentes con las fracciones que tenían mayor expresión de CD71 y CD9. Estas fracciones fueron utilizadas en ensayos inmunológicos posteriores.

A continuación, establecimos un sistema para evaluar si las células T de individuos expuestos a la malaria se activan mediante VEs circulantes autólogos que podrían transportar proteínas de parásito y actúen como antígenos. Además, ya que se tratan de células expuestas, queremos saber si las VEs pueden facilitar la presentación de antígenos de un manera dependiente o independiente de CPAs. Desafortunadamente los PBMCs de individuos expuestos a malaria presentaban una baja viabilidad y los experimentos tendrán que ser llevados a cabo cuando se colecten, envíen y recuperen PBMCs con alta viabilidad. Estos estudios son muy relevantes porque nos ayudarán a determinar si los posibles antígenos transportados en las *Pv*VEs son dianas de la inmunidad

celular adquirida de forma natural.

En paralelo, exploramos la capacidad de *Pv*VEs de estimular células T naive. Resumidamente, incubamos PBMCs de individuos no expuestos a malaria con *Pv*VEs por 48h. Como controles, estimulamos PBMCs de los mismos donantes con *h*VEs heterólogos y VEs autólogos en paralelo. Después de la incubación, comparamos la proporción de células T que expresaban el marcador de activación CD69 y producían IFN-γ por citometría de flujo como medida de activación de las células T. Encontramos que las células T CD8+ expuestas a *Pv*VEs presentan una mayor expresión de CD69 en comparación con *h*VEs autólogos y heterólogos. Estos resultados preliminares sugieren que las VEs circulantes podrían activar células T CD8+ en infecciones de *P. vivax*, pero se requiere más experimentación para corroborarlo.

C.3.4 *Exosomas derivados de reticulocitos humanos: una posible plataforma de vacunación contra malaria vivax*

La protección conferida con la inmunización basada en *Rex* contra la infección de *P. yoelii* evidenció que los *Rex* podrías representar una potencial plataforma para vacunación contra infecciones de malaria propensas a invadir reticulocitos humanos como *P. vivax*. Para estudiar ese potencial, investigamos la composición de exosomas derivados de reticulocitos humanos y evaluamos la interacción de dichos exosomas con CPAs.

Se obtuvieron muestras enriquecidas en reticulocitos humanos a partir de sangre de cordón umbilical. Estas muestras fueron luego cultivadas *in vitro* durante 36h y los sobrenadantes de los cultivos fueron procesados para aislar *HuRex* mediante ultracentrifugación o SEC. Los *HuRex* fueron analizados por NTA y microscopía electrónica, revelando un tamaño y morfología típico de exosomas. Además, para confirmar su origen reticulocítico se determinó la presencia del receptor de transferrina en los mismos mediante *western blot* e inmuno-microscopía electrónica.

La caracterización proteómica por espectrometría de masas, se realizó sobre *HuRex* de seis donantes diferentes. Como la investigación sobre la carga molecular de las VEs se confunde, en gran medida, por la falta de una metodología de obtención de VEs, aplicamos varias estrategias para aislar *HuRex*. Las preparaciones de *HuRex* que se obtuvieron en ausencia de suero de tres donantes diferentes se aislaron mediante SEC y UC. Las preparaciones de *HuRex* en presencia de suero se obtuvieron de otros tres donantes y se purificaron con las mismas técnicas. En total, fueron identificadas 367 proteínas diferentes en el conjunto de datos de diez preparaciones, aunque los números de proteínas de cada donante fueron diferentes entre sí debido a la variabilidad intrínseca de las muestras. Análisis adicional por *western blot* confirmó de detección de la proteína estomatina, previamente asociadas a *HuRex*, y de las nuevamente detectadas

proteínas de shock térmico de 70 KDa y gliceraldehído-3-fosfato deshidrogenasa. De interés, identificamos en *HuRex* moléculas de HLA de la clase I.

Analizamos la similitud del proteoma de *HuRex* con proteomas previamente descritos de reticulocitos y eritrocitos humanos, y encontramos que muchas de las proteínas identificadas en *HuRex* ya habían sido descritas en estas células.

Por último, realizamos un ensayo funcional para determinar si los *HuRex* podrían ser captados por CDs. Observamos que las CDs que expresan sialoadhesina captan activamente *HuRex* y, además, los *HuRex* siguen la misma ruta intracelular que las partículas de VIH-1, como ya había sido descrito con otros exosomas.

C.4 Discusión

Esta tesis ha tenido como objetivo principal explorar el potencial de las VEs en el descubrimiento de antígenos y la mediación de respuestas inmunes celulares, que pueden ser cruciales para el control de *P. vivax*. A pesar de que estamos lejos de una aplicación clínica, este trabajo proporciona valiosa información para el desarrollo de vacunas contra la malaria causada por *P. vivax*.

Dada la falta de un cultivo *in vitro* continuo de *P. vivax* (Noulin et al. 2013), el estudio de *Rex* derivados de reticulocitos infectados con *P. vivax* habría sido un desafío. Tratamos de superar esta limitación con un análisis de VEs derivados del plasma de pacientes infectados con *P. vivax* y de *Rex* derivados de reticulocitos humanos. Estos análisis nos han dado una idea sobre la potencial composición de los *Rex* derivados de los reticulocitos infectados. Es importante destacar que demostramos la presencia de moléculas de clase HLA en estas vesículas, lo que sugiere que los *Rex* pueden tener la capacidad de mediar las respuestas inmunes.

Además, con los estudios de la composición de las VE circulantes de pacientes, hemos descubierto múltiples proteínas de parásitos asociadas a las VEs, lo que refuerza el uso de VEs en la búsqueda de potenciales antígenos. Por otro lado, este estudio nos ayuda a descubrir posibles funciones que pueden tener las VEs en las infecciones naturales por vivax, las cuales aún no habían sido exploradas.

C.4.1 *PvVEs como fuente de posibles antígenos*

Se ha demostrado que los proteomas de VEs derivados de glóbulos rojos infectados con malaria están enriquecidos en proteínas de merozoitos como MSP-1 (Abdi et al., 2017; Mantel et al., 2013; Martín-Jaular et al., 2016) y proteínas de parásitos exportadas como *P. falciparum*PTP2, miembro de la subfamilia PHISTc (Regev-Rudzki et al., 2013). En *Pv*VEs hemos detectado proteínas de merozoito MSP-1 (PVP01_0728900) y MSP-3.1. Curiosamente,

*P. vivax*MSP1 y algunos miembros de la familia *P. vivax*MSP3 están siendo investigados como posibles candidatos a vacunas (França et al., 2017; Mourão et al., 2012). También hemos encontrado una proteína PHISTc (PVP01_0119200), ortólogo de *P. cynomolgi*PHIST / CVC-8195, que se había visualizado en los complejos caveolares de vesículas en los glóbulos rojos infectados con *P. cynomolgi* (Akinyi et al., 2012). Se ha demostrado que PHISTc es altamente inmunogénico al evaluar respuestas humorales a la infección por *P. vivax* (Lu et al., 2014; Wang et al., 2016). Dado que las respuestas de células T citotóxicas podrían representar un mecanismo de control para la infección por vivax en etapa sanguínea (Burel et al., 2016; Junqueira et al., 2018), dirigimos nuestra investigación para predecir los epítopos citotóxicos de células T dentro de MSP-3.1 y PHISTc. Se pueden encontrar valores más bajos de rango medio (unión media más alta) para las secuencias predichas de PHISTc que para las de MSP3.1, lo que de alguna manera sugiere que PHISTc podría tener más epítopos T que MSP3. De manera esperable, este análisis mostró uno de los mayores obstáculos para diseñar estrategias de vacunación basados en células T: el polimorfismo de las moléculas de HLA.

Sin embargo, la identificación de las proteínas de parásito asociadas a *Pv*VEs circulantes es aún bastante limitada. Es probable que los VEs derivados de células infectadas representen una pequeña proporción de las VEs totales en circulación. Podríamos mejorar la identificación de componentes del parásito en las *Pv*VEs tratando de enriquecer en VEs que vengan de células parasitadas, como, por ejemplo, del reticulocito, mediante técnicas de inmuno-aislamiento contra el receptor de transferrina, principal componente de los *Rex*. De todas formas, esto siempre sería un enriquecimiento, ya otras células pueden expresar este receptor y además éste también se puede encontrar en forma soluble en circulación. Por esta razón y, sobre todo, por tener una idea lo más aproximada a la situación *in vivo*, decidimos realizar una descripción proteómica inicial de *Pv*VEs lo menos sesgada posible, utilizando *Pv*VEs enriquecidos por SEC. En un futuro, se debería realizar una reevaluación de la composición de los *Pv*VEs utilizando otro tipo de aislamiento VEs para validar que el proteoma aquí presentado.

C.4.2 *Explorando la participación de PvVEs en la activación de respuestas inmunes*

La mayor cantidad y la alterada composición de los *Pv*VEs en comparación con los *h*VEs, sugiere que las *Pv*VEs podrían mediar múltiples interacciones huésped-patógeno en el curso de una infección de *P. vivax*. Las proteínas de parásito que portan podrían servir como antígenos, y la presencia de otras moléculas relacionadas con las respuestas inmunitarias indica que los *Pv*VEs podrían contribuir a la regulación inmune durante la infección por malaria y

podrían ser de interés para el desarrollo de vacunas.

En esta tesis, evaluamos específicamente su interacción con el bazo humano y la capacidad de estimular células T ya que la prueba de concepto de vacunación con *Rex* en el modelo de *P. yoelii* demostró que la protección era dependiente del bazo y estaba asociada con células T de memoria (Martín-Jaular et al., 2016). Nuestro trabajo muestra que varias poblaciones leucocitarias del bazo humano tienen una significativa mayor interacción con los *Pv*VEs que con *h*VEs. En próximos experimentos sería interesante dilucidar el mecanismo de captación de estas VEs y estudiar los posibles efectos que tienen las células receptoras. Sin embargo, nuestros intentos en explorar la estimulación de respuestas T por VEs en estas células se han visto limitados, entre otros factores, por la baja disponibilidad de bazos humanos, y la incapacidad de obtener CPAs con suficiente pureza y viabilidad para llevar a cabo experimentos de presentación antigénica. Sin embargo, estamos seguros de que futuros estudios con esplenocitos humanos pueden ayudar al desarrollo de novedosas estrategias para diseccionar respuestas inmunes a antígenos de vivax. Por ejemplo, un modelo de ratón humanizado con esplenocitos humanos ya ha sido utilizado para seleccionar candidatos a vacunas contra la malaria (Ghosn et al., 2018).

Paralelamente, queríamos evaluar la estimulación por *Pv*VEs de células T de sangre periférica en individuos expuestos a malaria y en inviduos naive, especialmente por la importancia que podrían tener las células T citotóxicas en el control de la etapa sanguínea de la infección de vivax (Burel et al., 2016; Junqueira et al., 2018) y porque la mayoría de las respuestas inmunitarias activadoras de las VEs se han centrado también en su capacidad para estimular las respuestas de las células T (Robbins & Morelli, 2014). Resultados preliminares en individuos naive indican la posibilidad de que los VEs circulantes puedan contribuir a una mayor activación de células T CD8+ durante las infecciones de vivax. Futuras investigaciones reevaluarán estos hallazgos, y tratarán de determinar si los posibles antígenos transportados en las *Pv*VEs son diana de las respuestas inmunes adquiridas naturalmente. Además, sería altamente informativo identificar los péptidos de vivax que podrían ser reconocidos. Para ello la combinación de estrategias experimentales como la elución de péptidos unidos a moléculas de HLA y su identificación por espectrometría de masas, así como las predicciones de epítopos T *in silico* acelerarían la descripción del inmunopeptidoma de *Pv*VEs.

Sin embargo, todavía necesitamos una mejor comprensión de la inmunidad contra *P. vivax*. Esto es fundamental para dilucidar cuáles son los mecanismos de protección que deberían promover las estrategias de vacunación. En esta tesis nos hemos centrado en evaluar el potencial de los *Pv*VEs para promover la activación de células T, pero puede a ver otros mecanismos fundamentales, como las respuestas humorales, para el control de vivax. Hay que tener en cuenta que la inmunización con *Rex* de las infecciones por *P. yoelii* también

podía promover respuestas humorales (Martin-Jaular et al., 2011). Por lo tanto, podría ser interesante extender nuestros estudios sobre la inmunogenicidad de *Pv*VEs a otras células inmunes, como las células B.

C.4.3 *Rex como una plataforma de vacunas contra malaria vivax*

Habiendo demostrado que los *Rex* protegen contra un parásito propenso a infectar reticulocitos (Martín-Jaular et al., 2016; Martin-Jaular et al., 2011) y teniendo en cuenta que los reticulocitos son el objetivo preferencial de las etapas sanguíneas de *P. vivax* (Kitchen, 1937), los *Rex* humanos que portasen antígenos de vivax podrían representar una prometedora plataforma de vacuna contra la fase sanguínea de la malaria vivax. Nuestra proteómica sobre *HuRex* ha extendido el limitado conocimiento que había sobre la composición de estas vesículas (Blanc & Vidal, 2010). Comparando este nuevo proteoma con proteomas de reticulocitos humanos (Chu et al., 2017; Gautier et al., 2016; Wilson et al., 2016) y eritrocitos maduros (Bryk & Wiśniewski, 2017; D'Alessandro et al., 2017; Pasini et al., 2006; Wilson et al., 2016), encontramos muchas proteínas comunes, especialmente de la membrana plasmática y el citosol. Estos datos están de acuerdo con la idea de que los exosomas reflejan una "biopsia celular", pero al mismo tiempo están enriquecidos en una carga específica (Raposo & Stoorvogel, 2013; Yáñez-Mó et al., 2015). Además, está disponible el proteoma completo de *Rex* de ratas (Carayon et al., 2011) y su comparación con el proteoma de *HuRex* reveló más de 200 proteínas conservadas. Por lo tanto, este conjunto de datos representa un valioso recurso para realizar diferentes estudios. Sorprendentemente, varios receptores y proteínas involucrados en la entrada del parásito en la célula huésped, como CD98 (Malleret et al., 2017), CD71 (Gruszczyk et al., 2018), banda 3 (Alam et al., n.d.) y banda de eritrocitos 7 (Hiller et al., 2003) se eliminan selectivamente en *HuRex*. Nuestro estudio amplía el conocimiento de la biología de la célula que *P. vivax* invade preferentemente, y sugiere una posible explicación parcial del fracaso de los sistemas de cultivo *in vitro*. Además, este proteoma podría ayudar a identificar mecanismos de patología mediados por VEs y biomarcadores no invasivos para otras enfermedades que afectan a los glóbulos rojos aparte de la malaria (Hebbel & Key, 2016).

La identificación de moléculas HLA clase I refuerza nuestra hipótesis del potencial de *Rex* para facilitar la inducción de respuestas celulares citotóxicas. La interacción de *HuRex* con las CDs que expresan Siglec-1 enfatiza aún más la contribución de *HuRex* a la presentación de antígenos mediante la transferencia de antígenos o complejos de antígeno/HLA transportados en *HuRex*. En el futuro será muy relevante evaluar la inmunogenicidad de *HuRex* portando epítopos de vivax que se hayan descubierto en las *Pv*VEs.

En conjunto, los resultados de esta tesis indican que las VEs abren una prometedora vía para controlar la malaria vivax. Nuestro trabajo respalda

más estudios sobre el uso de las VEs, particularmente *Rex*, como fuente de nuevos antígenos y como una nueva plataforma de vacuna contra la malaria por *Plasmodium vivax*.

C.5 Conclusiones

1. Las VEs que se encuentran en circulación durante infecciones naturales por P. vivax (*Pv*VEs) presentan una alterada composición respecto a las VEs de donantes sanos (*h*VEs) según el estudio proteómico comparativo por espectrometría de masas.

2. Las *Pv*VEs contienen proteínas del parásito que podrían funcionar como antígenos. De hecho, dos de las proteínas identificadas presentan prometedores epítopos T citotóxicos de acuerdo a predicciones in silico. Estos resultados promueven el uso de *Pv*VEs en el descubrimiento de antígenos.

3. Las *Pv*VEs están compuestos por un repertorio menos variado de proteínas humanas que los *h*VEs. Sin embargo, hay proteínas exclusivamente asociadas a los *Pv*VEs relacionadas con funciones inmunes según el análisis de enriquecimiento en términos de ontología génica.

4. La presencia de proteínas comunes en *Pv*VEs y en exosomas derivados de reticulocitos tales como el receptor de transferrina, y la identificación de proteínas del parásito en *Pv*VEs sugiere que al menos una parte de las VEs que se encuentran en circulación puede derivar de reticulocitos infectados con P. vivax.

5. La detección de antígenos leucocitarios humanos de la clase I y de proteínas del parásito en los *Pv*VEs destaca la posibilidad de que los *Pv*VEs representen vesículas portadoras de antígenos y puedan promover respuestas inmunes. En particular, la presencia de moléculas de HLA de la clase I indica que estas vesículas pueden estar involucradas en la estimulación células T citotóxicas.

6. Existe una interacción mayor entre monocitos, células B y T del bazo humano, y los *Pv*VEs en comparación con los *h*VEs.

7. Resultados preliminares indican que puede haber una activación de células T citotóxicas por *Pv*VEs.

8. El análisis proteómico por espectrometría de masas de *Rex* humanos reveló que más de 300 proteínas están asociadas a estas vesículas, entre ellas están las moléculas de HLA de la clase I. Estos datos representan

un recurso valioso para mejorar nuestra comprensión de la eritropoyesis humana y una oportunidad para desarrollar futuros estudios sobre patologías eritrocitarias.

9. Células dendríticas que expresan sialoadhesina captan activamente *HuRex*. Estos resultados refuerzan el papel de *Rex* en la comunicación intercelular en respuestas inmunes y su potencial como plataforma de vacunación contra malarias propensas a invadir reticulocitos humanos como la malaria vivax.

Appendix

D Published Journal Articles

D.1 Article 1

Title: Proteomics study of human cord blood reticulocyte-derived exosomes.

Authors: *Míriam Díaz-Varela*, Armando de Menezes-Neto, Daniel Perez-Zsolt, Ana Gámez-Valero, Joan Seguí-Barber, Nuria Izquierdo-Useros, Javier Martinez-Picado, Carmen Fernández-Becerra & Hernando A. del Portillo

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Proteomics study of human cord blood reticulocyte-derived exosomes

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Míriam Díaz-Varela[1], Armando de Menezes-Neto[1,6], Daniel Perez-Zsolt[2], Ana Gámez-Valero[1,7], Joan Seguí-Barber[1], Nuria Izquierdo-Useros[2,3], Javier Martinez-Picado[2,4,5], Carmen Fernández-Becerra[1,3] & Hernando A. del Portillo[1,3,5]

Reticulocyte-derived exosomes (*Rex*), extracellular vesicles of endocytic origin, were initially discovered as a cargo-disposal mechanism of obsolete proteins in the maturation of reticulocytes into erythrocytes. In this work, we present the first mass spectrometry-based proteomics of human *Rex* (*HuRex*). *HuRex* were isolated from cultures of human reticulocyte-enriched cord blood using different culture conditions and exosome isolation methods. The newly described proteome consists of 367 proteins, most of them related to exosomes as revealed by gene ontology over-representation analysis and include multiple transporters as well as proteins involved in exosome biogenesis and erythrocytic disorders. Immunoelectron microscopy validated the presence of the transferrin receptor. Moreover, functional assays demonstrated active capture of *HuRex* by mature dendritic cells. As only seven proteins have been previously associated with *HuRex*, this resource will facilitate studies on the role of human reticulocyte-derived exosomes in normal and pathological conditions affecting erythropoiesis.

Research on exosomes is gaining momentum as these vesicles of endocytic origin act in intercellular communication and represent novel therapeutic strategies and non-invasive biomarkers of disease[1–3]. During their maturation to erythrocytes, reticulocytes selectively remove proteins, noticeably the transferrin receptor (TfR or CD71), as well as membrane-associated enzymes, through the formation of multivesicular bodies which after fusion with the plasma membrane release intraluminal vesicles, termed exosomes[4–6].

Studies on the protein cargo composition of reticulocyte-derived exosomes (*Rex*) are mostly limited to animal models and validations through non high-throughput technologies[7–9]. These studies clearly established that *Rex* represent a selective cargo disposal mechanism in the terminal maturation of reticulocytes into erythrocytes[7]. More recently, mass spectrometry (MS)-based proteomic analysis of *Rex* from phenylhydrazine-treated rats[10] and from mice infected with rodent malaria parasites with a unique tropism to reticulocytes[11], described the proteome of *Rex* in these species. Results reinforced the view that *Rex* have selective cargo and demonstrated for the first time that *Rex* from malaria-infected reticulocytes contain parasite antigens involved in antigen presentation and capable of modulating immune responses[11,12]. To our knowledge, however, only seven proteins (TfR, Stomatin, Flotillin-1 and 2, CD55, CD58 and CD59) have been previously reported in human *Rex*[7,13,14].

In this work, we present the first MS-based proteomic profile of human reticulocyte-derived exosomes (*HuRex*). *HuRex* were isolated from cultures of human cord blood using two different culture conditions, absence/presence of exosome-depleted serum, and two different exosome isolation techniques, size-exclusion chromatography (SEC) and ultracentrifugation (UC). The proteome consists of 367 proteins most of which have not been previously reported. Immunoelectron microscopy validated the presence of the transferrin receptor, a major *Rex* component, and comparative analysis with the MS-datasets from reticulocytes[15–17] and mature red blood cells[17–20], rendered a selected list of *HuRex* plasma membrane and cytosol proteins. In addition, we identified proteins involved in antigen presentation and observed an active capture of *HuRex* by mature dendritic cells. These

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D.2 Article 2

Title: Spleen-Dependent Immune Protection Elicited by CpG Adjuvanted Reticulocyte-Derived Exosomes from Malaria Infection Is Associated with Changes in T cell Subsets' Distribution

Authors: Lorena Martín-Jaular, Armando de Menezes-Neto, Marta Monguió-Tortajada, Aleix Elizalde-Torrent, *Míriam Díaz-Varela*, Carmen Fernández-Becerra, Francesc E. Borras, Maria Montoya & Hernando A. del Portillo

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Spleen-Dependent Immune Protection Elicited by CpG Adjuvanted Reticulocyte-Derived Exosomes from Malaria Infection Is Associated with Changes in T cell Subsets' Distribution

*Lorena Martín-Jaular[1†], Armando de Menezes-Neto[1†], Marta Monguió-Tortajada[2], Aleix Elizalde-Torrent[1], Miriam Díaz-Varela[1], Carmen Fernández-Becerra[1,2], Francesc E. Borras[2], Maria Montoya[3,4] and Hernando A. del Portillo[1,2,5]**

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Reticulocyte-derived exosomes (*rex*) are 30–100 nm membrane vesicles of endocytic origin released during the maturation of reticulocytes to erythrocytes upon fusion of multivesicular bodies with the plasma membrane. Combination of CpG-ODN with rex obtained from BALB/c mice infected with the reticulocyte-prone non-lethal *P. yoelii* 17X malaria strain (*rexPy*), had been shown to induce survival and long lasting protection. Here, we show that splenectomized mice are not protected upon *rexPy*+CpG inmunizations and that protection is restored upon passive transfer of splenocytes obtained from animals immunized with *rexPy*+CpG. Notably, *rexPy* immunization of mice induced changes in PD1$^-$ memory T cells with effector phenotype. Proteomics analysis of *rexPy* confirmed their reticulocyte origin and demonstrated the presence of parasite antigens. Our studies thus prove, for what we believe is the first time, that *rex* from reticulocyte-prone malarial infections are associated with splenic long-lasting memory responses. To try extrapolating these data to human infections, *in vitro* experiments with spleen cells of human transplantation donors were performed. Plasma-derived exosomes from vivax malaria patients (*exPv*) were actively uptaken by human splenocytes and stimulated spleen cells leading to changes in T cell subsets.

Keywords: reticulocyte-derived exosomes, vaccine, malaria, spleen, PD-1 cells, effector memory T-cells, pD1-cells

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INTRODUCTION

Extracellular vesicles are cell-secreted lipid bilayer structures that can be classified based on their size and subcellular origin in two major types: exosomes and microvesicles (Raposo and Stoorvogel, 2013). Exosomes are small membrane vesicles (30–100 nm) of endocytic origin; thus, an internalized segment of plasma membrane generates multivesicular bodies containing small