SUMMARY

"There is only one freedom of any importance, freedom of the mind"

-IRIS

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THESIS TITLE: Study of Malarial Antigenic Proteins on Monocyte Functionality Involved in Protection

Malaria, a pestilence to mankind affecting millions of people is caused by a unicellular, parasitic protozoa, plasmodium. In 2015, the global burden of malaria remained heavily concentrated in 15 countries, mainly in Africa. Together, these countries account for an estimated 80% of global malaria cases and 78% of deaths. Since 2000, progress in reducing malaria incidence in these high burden countries has lagged behind that of other countries globally. Around 22% of India's population resides in high transmission areas, 67% live in low transmission areas and 11% live in malaria-free areas. *P. vivax* is estimated to have been responsible for 13.8 million malaria cases globally in 2015, and to have accounted for about half the total number of malaria cases outside Africa.

There are five distinct species of plasmodium that infect humans under natural conditions. These are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Among them the most fatal and most prevalent is *P. falciparum* causing maximum number of deaths as it also leads to cerebral malaria. *P. vivax* is the most widely and frequently occurring malaria. While *P. malariae* is found worldwide but with low frequency. Both *P. vivax* and *P. ovale* form hypnozoite, a dormant liver stage, resulting in relapses of the infection.

Life cycle of *P. vivax* is similar to that of all of the other primate malaria species. It requires both an invertebrate and a vertebrate host for growth and survival; a female mosquito *Anopheles* and a primate. As the mosquito bites, it releases sporozoites into the blood circulation and enter the liver sinusoid vessels where they invade the Küpffer cells. Sporozoites

then penetrate a hepatocyte, eventually differentiating into a schizont. One antigen that helps in the invasion of the parasite into the hepatocyte is Apical membrane antigen -1. AMA-1 forms a ring-like junction with the hepatocyte cell surface and enters into the host cell by sliding through it into the intracellular vacuole. The next step is that a fully mature schizont is expected to have differentiated into thousands of individual single nucleated merozoites. Merozoite surface protein 7 (MSP7) helps in the attachment of the parasite to RBC. After the host cell membrane ruptures the merozoites are released and they invade new RBCs. The merozoites in some schizonts are programmed to differentiate into sexual-stage male or female gametocytes, respectively. Transmission blocking vaccines (TBVs) aims to induce immunity against the sexual stage providing protection to the immediate neighbourhood of the vaccinated individuals. WARP is a micronemal protein that helps the ookinete to attach the basal membrane of the mosquito midgut.

Monocytes are present in large multicellular organism like mammals, birds, amphibians, and fish and formed in bone marrow from haematopoietic cells. 10% of the total leukocyte population in human blood represents monocytes. Monocytes help to remove apoptotic cells and helps in maintaining homeostasis. They also represent a connecting link between inflammation and the innate defence against microorganisms to adaptive immune responses. Monocytes have a large number of receptors that can detect not only microorganisms, but also some lipid moiety. Once stimulated monocytes can release effector molecules involved in the defence against pathogen, and can differentiate into dendritic cell or tissue macrophages. The aim of the study was to analyse the monocyte functionality in vivax patients and healthy individuals, in response to recombinant antigens. To achieve this blood from *P. vivax* infected individuals was collected by the university health centre in vacutainer tube containing EDTA. Genomic DNA of *P. vivax* was isolated and the genes of Apical Membrane Antigen-1 (PvAMA1), Merozoite Surface Protein 7 (PvMSP7) and von Willebrand factor A (PvWARP)

were amplified using gene specific primers. The amplified products were gel purified to avoid any nonspecific bands and the ORF coding for PvAMA1, PvMSP7 and PvWARP was cloned into *E. coli* DH5α. The clones of these library were then screened for the presence of desired insert. Plasmids were isolated from the colonies using plasmid isolation kit (Xcelris). Inserts released, by restriction digestion, were then ligated with pET30a (+) vector (Invitrogen). Transformation of these ligated products in *E.coli* DH5α yielded in more than 100 colonies. Colonies were screened using double restriction digestion and PCR. The confirmed constructs were then transformed into expression host *E. coli* BL21 (DE3). Under given expression condition as 0.5 mM IPTG concentration, 37 °C temperature for 2 hours, the recombinant proteins were expressed. Hexahistidine tag on the recombinant protein was exploited for their purification by using nickel affinity chromatography. The eluted band corresponding to their molecular weight were distinctly visible on the coomassie and silver stained gels. Purified proteins were then precipitated by a chloroform, methanol method so as to remove the presence of any salt. The genes AMA1, MSP7 and WARP were successfully amplified from the genomic DNA of *P. vivax*. PCR products of 1338 bp (AMA-1), 1263 bp (MSP-7) and 888 bp (WARP) was observed on an agarose gel stained with EtBr. These amplicons were then cloned and subsequently cloned in pJET1.2 and pET30a(+), respectively. IPTG induction of the confirmed clones were analysed on SDS-PAGE. Bands corresponding to 49kDa (AMA1), 66kDa (MSP7) and 40 kDa (WARP) were observed. The recombinant protein hence was purified by nickel affinity chromatography and used further for diagnostic analysis and monocyte functionality in response to these recombinant antigens.

During malaria infection monocytes and macrophages are involved in interaction with the *Plasmodium* infected erythrocytes (IEs) and helps in its removal from the spleen. One of the effective ways of reducing parasitaemia is via phagocytosis. Phagocytosis rate of monocytes from healthy individuals were studied. Monocytes were treated the recombinant antigens and

incubated for 72 hours at 37ºC in a CO_2 incubator. Following this fluorescent latex beads (L3030, 2µm) were added to the monocytes and incubated for 60 minutes at 37°C, 5% CO_2 for 72 hours. Post phagocytosis trypan blue in PBS was added to each coverslip and the cells were examined using fluorescence microscopy. Since trypan blue quenches the fluorescence of extracellular beads, only intracellular (phagocytised) beads would fluoresce. The rate of phagocytosis of both, single bead as well as multiple beads in monocytes treated with rPvAMA-1 was reduced from 41.92% to 32.67% and from 24.29% to 17.29% respectively. Similarly, for rPvMSP7 the rate of phagocytosis was reduced to 30.17% and 14.03% for single and multiple beads respectively and for rPvWARP to 30.66% and 15.55%. The respiratory burst in the presence of the antigens was studied by the NBT assay, blue colour formation was more in the case of AMA-1 and MSP7 compared to that of the untreated and WARP treated monocytes.

Cytokines are small signalling molecules (proteins) produced by a vast number of cells like macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells. Chemokines are a type of cytokines that can induce chemotaxis in cells like neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts, and keratinocytes. Chemokines are a family of small cytokines, or signaling proteins secreted by cells. They mediate their interaction with the cells via a 7-transmembrane, G-protein–coupled receptor superfamily. During a parasite infection, chemokine receptor plays an important role in their pathology. Expression of CCR2 and CX3CR1 is reported to be three times higher in blood monocytes of patients infected with *P. falciparum*. CCR7 and CXCR4 signaling is reported for the generation of protective immune responses during *Toxoplasma gondii* infection. CCR2, CXCR4 and CX3CR1 are involved in the egress of monocyte from the bone marrow and homing to the spleen while CCR7 is required for the homing of monocytes to the lymph nodes.

The chemokine profile of monocytes in vivax patients and in healthy individuals treated with the recombinant antigens were also studied. Monocytes and PBMCs from three healthy individuals were isolated and incubated with 5µg/ml of recombinant antigen in RPMI-1640, for 72 hrs at 37ºC in a CO_2 incubator. After the incubation, cells were lysed and total RNA was isolated. Unstimulated monocytes were used as a control, and this was followed by RNA isolation and cDNA preparation. Similarly monocytes of 10 vivax patients were also isolated and used for the study. qRT-PCR was performed to study the chemokine profiles of CCR2, CCR7, CXCR4 and CX3CR1. Gene expression of CCR2 and CX3CR1 were upregulated while CCR7 and CXCR4 were downregulated in all the patients infected with *P. vivax*. GAPDH was used as internal control. Exposure of recombinant AMA-1 and WARP led to a reduction of CCR2, CX3CR1 and CXCR4, while no difference in the fold expression for CCR7. But upon exposure of MSP7 the monocyte showed a reduction in all the chemokine receptor levels. For the fold expression of mRNA for various chemokine receptors of monocyte derived from activated PBMCs, CX3CR1 was either unchanged (AMA-1) or increased (MSP7 and WARP). The fold expression of other receptors were reduced.

Malaria diagnosis involves identification of malaria parasites in patient blood. *Plasmodium* species in Giemsa-stained thick (for screening the presenting malaria parasite), and thin blood films (for species' confirmation) remains the gold standard for laboratory diagnosis. But this technique requires time and expertise for the correct diagnosis. Rapid diagnostic tests (RDTs) are new, simple, quick, accurate, and cost-effective diagnostic tests for determining the presence of malaria parasites. Many antigens are currently used for the diagnosis of malaria like histidine-rich protein II (HRP-II), Glutamate dehydrogenase, lactate dehydrogenase (LDH).

The recombinant antigens were blotted on a nitrocellulose membrane followed by treating the blot with serum from infected individuals. Serum from non-infected individual was used as a

control. For western blot analysis, AMA-1 reacted with 12 sera (85.71%), MSP-7 reacted with 10 sera (71.42 %) and WARP reacted with 14 sera (100%).

Conclusion: The rate of phagocytosis as well as the respiratory burst was reduced in monocytes from healthy individuals when treated with the recombinant antigens. NBT reduction was observed to be more in the case of AMA-1 and MSP7 compared to that of the WARP and untreated monocytes. Furthermore, the chemokine receptor gene expression studies also indicate that the efficacy of the recombinant antigens if used as vaccines may differ due to their ability to modulate monocyte functions.