CHAPTER 4

STUDY OF CHEMOKINE RECEPTOR EXPRESSION IN MONOCYTES TREATED WITH RECOMBINANT ANTIGENS (AMA1, MSP7 and WARP)

"The mind is everything. What you think you become"

-BUDDHA

Chapter 4

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INTRODUCTION

Innate immune cells of the monocyte lineage like monocytes, dendritic cells, and macrophages, play essential roles during malaria infection. As explained earlier, monocytes are derived from haematopoietic stem cells in the bone marrow and are released into the peripheral blood circulation upon maturation. Downstream signals leads to the differentiation of monocytes towards either dendritic cells or tissue macrophages (Chua et al., 2013). During malaria, macrophages in various organs are directly involved in the interaction with *Plasmodium*-infected erythrocytes, including macrophages in the liver (Kupffer cells), spleen (splenic macrophages), and bone marrow. Monocytes, dendritic cells and macrophages, use various cell surface and cytosolic receptors to sense and respond to infected erythrocytes (Liehl et al., 2012).

Chemoattractant cytokines or chemokines are key regulators of leucocyte trafficking (Ioannidis et al., 2014). Chemokine guides leucocyte migration to various lymphoid tissues and deployment of immune cells to peripheral sites of inflammation. As explained earlier, chemokines are a superfamily of low molecular weight polypeptides of about 8–14 kDa, which mediates their signal through seven-transmembrane spanning G-protein coupled receptors (GPCRs). Different chemokines share a significant degree of sequence homology and are sub divided into CC, CXC, CX_3C and C subfamilies based on the relative positions of conserved

cysteine residues near the N-terminus (Luster et al., 1998). Chemokines and chemokine receptors play an essential role in two distinct processes of leucocyte migration. The first is the migration and positioning of leucocytes within lymphoid organs as well as in peripheral tissues. In this process, the cells interact with the chemoattractant signals and follow a gradient of increasing concentration towards the source of the chemokine. The second trafficking process is mediated by chemokines and involves the arrest of migrating leucocytes on the vascular endothelium. This is followed by extravasation from the blood vessel into lymphoid and inflamed tissues (Chua et al., 2013). Chemokines play an important role not only in replenishing the tissue macrophages and DCs, but also mediate antimicrobial activity at the site of infection. During infection, monocytes and/or monocyte-derived cells can also enter draining lymph nodes and promote adaptive immune responses. $LY6C^{low}$ monocytes can be recruited to sites of infection even earlier than $LY6C^{hi}$ monocytes, and promote inflammatory response by releasing tumour necrosis factor (TNF) and chemokines. However, in most cases the recruitment of $LY6C^{hi}$ monocytes is more prominent and robust (Shi et al., 2011). Various bacterial, fungal and protozoal infections have been reported for the involvement of monocytes.

Listeria monocytogenes is the species of pathogenic bacteria that causes the infection listeriosis. It is a facultative anaerobic bacterium, capable of surviving in the presence or absence of oxygen. It can grow and reproduce inside the host's cells and is one of the most virulent foodborne pathogens, with 20 to 30% of clinical infections resulting in death (Ramaswamy et al., 2007). *L. monocytogenes* initially gains access to the body through the gastrointestinal tract but is capable of infecting the blood through monocytes, macrophages and polymorphonuclear leukocytes (Ghanem et al., 2012 and Drevets et al., 1999). *L. monocytogenes* lives as an intracellular pathogen inside these host cells, using the cell's own machinery to survive. The bacterium is first phagocytosed by these cells and secretes a pore-forming toxin called listeriolysin, which allows the bacterium to escape from the phagosome

(Schnupf et al., 2007). Inflammatory cell recruitment to the liver and spleen is necessary for the control of *L. monocytogenes* as these two organs are the major sites of infection. Deficiency in CCR2 reduces the recruitment of LY6C^{hi} monocytes to infected tissues. As *L. monocytogenes* is an intracellular pathogen TNF-and iNOS-producing (TIP) DCs plays an important role in the control of the disease (Kang et al., 2008). These cell population are derived from LY6C^{hi} monocytes that are recruited to the foci of infection. Recruitment of LY6C^{hi} monocytes into the spleen during *L. monocytogenes* infection is mediated in part by the expression of the chemokine receptor CX_3CR1 on monocytes and its ligand CX_3CL1 in the marginal zone and T cell areas of the spleen (Auffray et al., 2009).

Toxoplasma gondii is an obligate intracellular, parasitic protozoan that causes the disease toxoplasmosis. The life-cycle of *T. gondii* consists of: 1) a sexual stage that occurs only within cats and 2) an asexual stage that can occur within virtually all warm-blooded animals, including humans, cats, and birds. It has been reported that CCR2- or CCL2-deficient mice more susceptible to *T. gondii* infection due to diminished recruitment of LY6C monocytes (Robben et al., 2005 and Dunay et al., 2008). LY6C^{hi} monocytes are recruited to the lamina propria of the intestine, where they differentiate into major producers of iNOS and TNF. This could be observed in a CCR2- or its ligand CCL2 deficient mice, where TNF and iNOS production are markedly reduced in intestinal villi because of the retention of LY6C^{hi} monocytes in the bone marrow (Dunay et al., 2008).

In mice with *Plasmodium chabaudi*, LY6C^{hi} monocytes are recruited to the spleen and helps in phagocytosis of the parasite. Boring et al., (1997) used CCR2-/- mice to determine the role of MCP-1. They used peritoneal exudates from control and knockout mice for their ability to migrate in response to a chemotactic gradient of CCR2 ligands and observed that in CCR2-/- there was no chemotactic response towards CCR2 agonist. CCR2 also helps in controlling the blood stage malarial infection by regulating the recruitment and migration of monocytes from

bone to the site of inflammation. Sponaas et al., (2009) showed that in CCR2-/- mice treated with *P. chabaudi*, the number of inflammatory monocytes increased in bone marrow while the numbers were depleted in spleen. This suggests that the monocytes are generated in bone marrow and require CCR2 for their exit. Another observation made was, the levels of acute parasitemia could be lowered down to that of a wild type mice by adoptive transfer of monocytes from CCR2+/+ infected mice suggesting that CCR2 plays a role for these cells in controlling malaria infections. One of the major functions of spleen has been associated with the clearance of not only senescent erythrocytes but also intraerythrocytic parasites, such as *Plasmodium*. The stromal or reticular cells of spleen control this process by producing various chemokines, one of them is SDF-1 (Garnica et al., 2002). The infection causes a huge increase in spleen volume and cellularity. They have been shown to be responsible for splenic clearance of parasitized erythrocytes which occurs in later stages of malarial infection. Garnica et al., (2002) used groups of C57BL/6 mice treated with *P.chabaudi* parasitized RBCs and treated them with T-140 (antagonist to CXCR4) at ascending parasitemia. They observed that upon blocking CXCR4 adequate cell homing didn't take place which eventually led to a higher levels of parasitemia suggesting that CXCR4 plays an important role in the removal of parasitized RBCs in spleen.

A contrasting role for inflammatory monocytes has been observed in African trypanosomiasis, where monocytes have a pathogenic role. Accumulation of inflammatory monocytes is observed in the spleen, liver, and lymph nodes. CD11b Ly6C^{hi} inflammatory monocytes accumulates at the sites of infection and differentiate into inflammatory DCs expressing CD11c, iNOS, and TNF (Tip-DCs). Tip-DC populations in the liver cause liver necrosis/apoptosis, resulting in exacerbated disease (Bosschaerts et al., 2010.

Yeasts and moulds are an important class of pathogens that cause infections in mammalian hosts. *Cryptococcus neoformans* is an environmental fungus that causes pulmonary infections

on inhalation. CCR2 deficiency also resulted in decreased accumulation of $CD11b^{+}$ DCs in lung-draining lymph nodes and decreased recruitment of $LY6C^{hi}$ monocytes to the lung during *C. neoformans* infection . Thus, these studies suggest that $LY6C^{hi}$ monocytes are recruited in a CCR2-dependent manner and contribute to the accumulation of CD11b DCs in lung-draining lymph nodes during fungal infection (Guilliams et al., 2009).

In this chapter, we studied the effect of the malarial antigens cloned earlier in recombinant form, on the in vitro functionality of monocytes and to see if they had any role in controlling monocyte migration by means of any changes in chemokine receptor expression.

MATERIALS AND METHODS

STUDY POPULATION

Ten newly diagnosed malaria patients (from University Health Centre) were used to study the chemokine receptor mRNA levels. Three healthy individuals were also used to study effect of *P. vivax* recombinant proteins on the chemokine receptor mRNA levels. All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M. S. University of Baroda. Written informed consent was obtained from all subjects.

STIMULATION OF MONOCYTES FROM HEALTHY INDIVIDUALS

1×10^{6} PBMCs from three healthy individuals were seeded on a 24 well plate and kept it at 37ºC in a CO_2 incubator for 2 hours. After the incubation, the non-adhered mononuclear cell were removed and the adhered cells were washed twice with DPBS. The adherent cells were incubated with 5µg/ml of recombinant antigen in RPMI-1640 (Sigma) medium containing 10% fetal calf serum (Gibco), for 72 hrs at 37ºC in a CO_2 incubator. 3.75×10^{5} PBMCs/well from healthy individuals were also stimulated at the same concentration as above. After the

incubation, cells were lysed and total RNA was isolated. Unstimulated monocytes were used as a control.

RNA ISOLATION

RNA was isolated from adherent (monocytes) cells using total RNAiso Plus (Takara). All the glassware and plastic ware were first rinsed with chloroform to destroy any RNase present. All reagents used were prepared in diethyl pyrocarbonate (DEPC) treated autoclaved distilled water.

500µl of RNAiso Plus reagent (Takara) was added to each well, containing the monocytes, and mixed properly. The cells were transferred to a microfuge tube and left at room temperature for 10 minutes for lysis. To the lysate, 0.1 ml of chloroform was added and mixed until the solution turned milky. Following this the mixture was incubated at room temperature for 5 min and centrifuged at 12,000g for 15minutes at 4°C. The supernatant was then transferred into a fresh tube and equal volume of 100% isopropanol was added. The content was mixed and kept at -20ºC for 10 min for precipitation followed by centrifugation at 12,000g for 10 minutes at 4ºC. The supernatant was carefully removed without touching the pellet. 250µl of ethanol (75%) was added to the pellet and centrifuged at 7,500g for 5minutes at 4ºC and the supernatant was discarded. The pellet was dried at 42ºC for 10 minutes and dissolved in 15 µl DEPC treated water. RNA isolated were also quantified using a nano spectrophotometer.

cDNA SYNTHESIS AND CONFIRMATION OF cDNA QUALITY

For cDNA synthesis, one µg of each total RNA sample was mixed with anchored oligo dT in a 20 µl system using verso cDNA synthesis kit based on Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (Thermo Scientific) following manufacturer's instructions. Briefly, the RNA was mixed with oligo-dT, RT enhancer which contains DNAse I, dNTP mix and enzyme mix, followed by incubation at 50ºC for 60 min and then at 95ºC for 5 min to

inactivate the enzyme in a thermal cycler (Eppendorf). Each of the cDNA preparations was then amplified for 35 cycles in a thermal cycler with β actin-specific primers by taking 2 µl of cDNA in a 25 µl system. Amplification conditions were as follows: initial denaturation at 94ºC for 6 min, followed by 34 cycles of denaturation at 94ºC for 30 sec, annealing at 66 ºC for 30 sec, followed by extension at 72 ºC for 1 min and a final extension at 72 ºC for 10 min. PCR product were separated on a 0.8% agarose gel stained with ethidium bromide. Controls were set with amplification of the total RNA without reverse transcription which did not give any amplification (results not provided).

Table 4. 1 Reaction system for semi-quantitative RT-PCR

S. N0.	Name	Volume
1.	**5X cDNA buffer**	**4 µl**
2.	**dNTP mix (5mM)**	**2 µl**
3.	**RNA primer (500ng/ µl)**	**1 µl**
4.	**RT enchancer**	**1 µl**
5.	**Verso enzyme mix**	1 µl
6.	**cDNA template**	**x**
7.	**Autoclaved MilliQ water**	**Upto 20 µl**
	Total	**20 µl**

Table 4. 2 RT-PCR (Reverse transcriptase) conditions for cDNA synthesis

Temperature	Time
Take the template RNA	
70ºC	5 minutes
Add the other components to it	
50ºC (cDNA preparation)	60 minutes
95ºC (inactivation)	5 minutes

Table 4. 3 Reaction system for β-actin PCR

S. No.	Component	Volume (µl)
1.	Autoclaved MilliQ water	17
2.	DNA	2
3.	10X Buffer	2.5
4.	10 mM dNTPs	0.5
5.	Forward Primer (10 mM)	1.25
6.	Reverse Primer (10 mM)	1.25
7.	Taq Polymerase (2.0 U/ µl)	0.5
	Total	25

QUANTITATIVE REAL TIME PCR

q-PCR amplifications were performed in CFX96TM real-time thermal cycler (Bio-RAD) with specific primers for the chemokine receptors, CCR2, CCR5, CCR&, CX3CR1 and CXCR4. The amplification conditions were as follows:

Table 4. 4 System for Real time qPCR analysis of chemokine receptors

Steps		Temparature (ºC)	Time	No. of cycles
1.	Initial denaturation	94	3 minutes	
2.	Denaturation	94	10 seconds	
3.	Primer annealing	60	30 seconds	45
4.	Extension	72	1 minutes	

Each sample was run in triplicate and cycle threshold (C_t) was used for gene expression analysis. The transcripts expression of CCR2, CCR5, CCR&, CX3CR1 and CXCR4 in each sample was normalised to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript expression of the same sample. To quantify the data, the comparative Ct method was used.

Relative quantity was defined as $2^{-\Delta\Delta Ct}$, in which ΔCt=Ct (target)-Ct (reference), ΔΔCt=ΔCt (sample)-ΔCt (calibrator). The calibrator was the unstimulated monocytes' mRNA used for normalization. Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. Significant ANOVA was followed by Fisher's LSD test for different chemokine receptor levels compared to that of the control. The product specificity was confirmed by single peak in melt curve analysis (from 65ºC to 95ºC in 0.5ºC/5s increments). The negative controls were set with the total RNA without reverse transcription (data not provided).

Table 4. 5 Primers used for qRT-PCR analysis

Primer name	Primer sequence
CCR2 F (qRT- PCR)	5'-TTGTGGGTGGTTTGTGTTCCA-3'
CCR2 R (qRT- PCR)	5'-CTGTGGTCTTGAGGGCCTTG-3'
CCR7 F (qRT- PCR)	5'- AGAGGCTATTGTCCCCTAAAC-3'
CCR7 R (qRT- PCR)	5'- AGAGCTGGTCTGAGCATTTGA-3'
CX3CR1 F (qRT- PCR)	5'- GGCCTTGTCTGATCTGCTGTTTG-3'
CX3CR1 R (qRT- PCR)	5'- AATGCTGATGACGGTGATGAAGAA-3'
CXCR4 F (qRT- PCR)	5'- AGTGGGTCTTTGGGAATGCA-3'
CXCR4 R (qRT- PCR)	5'- CCGTCCTCCGTTTTAAAGCA-3'
GAPDH F (qRT- PCR)	F 5'- TGAGCACCAGGTGGTCTCC-3'
GAPDH R (qRT- PCR)	R 5'- TAGCCAAATTCGTTGTCATACCAG-3'
Beta Actin F (qRT- PCR)	FP - 5'AGCGGGAAATCGTGCGTGACA-3'
Beta Actin R (qRT- PCR)	RP - 5'-CGCAACTAAGTCATAGTCCG-3

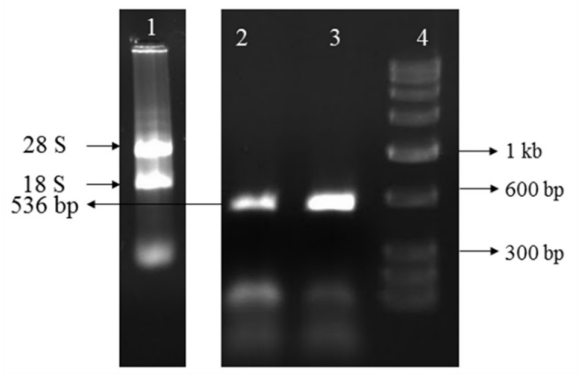
Table 4. 6 Reaction system for qRT-PCR

S. No.	Component	Volume (µl)
1.	DNA	0.5
2.	Forward Primer (10 mM)	0.5
3.	Reverse Primer (10 mM)	0.5
4.	2x SYBr mix	5.0
5.	Autoclaved MilliQ water	3.5
	Total	10

RESULTS

RNA ISOLATION AND SEMI-QUANTITATIVE RT-PCR

The total RNA was isolated from patients and healthy individuals' stimulated monocytes. The presence of 18S and 28S rRNA on the agarose gel shows the integrity of the RNA isolated. The quality of cDNA prepared by reverse transcription was confirmed by performing PCR with β actin specific primers (Figure 4.1-4.11). The cDNA prepared from these samples were further used to analyse the expression of chemokine receptors on the monocytes. RNA isolated from control monocytes stimulated with recombinant antigens were not prominently visible in the gel as the concentration was very less (data not shown). But the presence and stability of RNA was confirmed by β actin PCR (Figure 4.10-4.15).

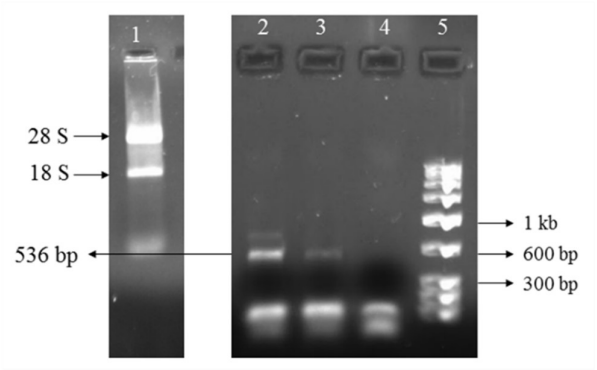


Lane 1: Total RNA

Lane 2 & 3: β-actin amplicon

Lane 4: Low Range DNA Rule

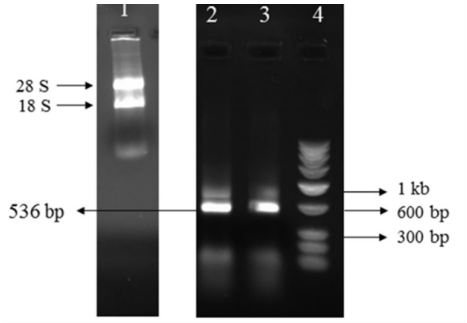
Figure 4. 1 Analysis of RNA and cDNA preparation of patient 1. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared from an individual infected with *P. vivax*.



Lane 1: Total RNA Lane 2-4: β-actin amplicon

Lane 5: Low Range DNA Rule

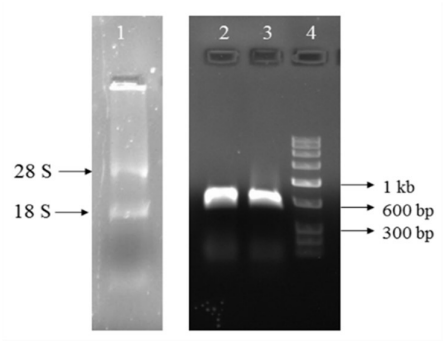
Figure 4. 2 Analysis of RNA and cDNA preparation of patient 2. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared of an individual infected with *P. vivax*



Lane 1: Total RNA Lane 2-3: β-actin amplicon

Lane 5: Low Range DNA Rule

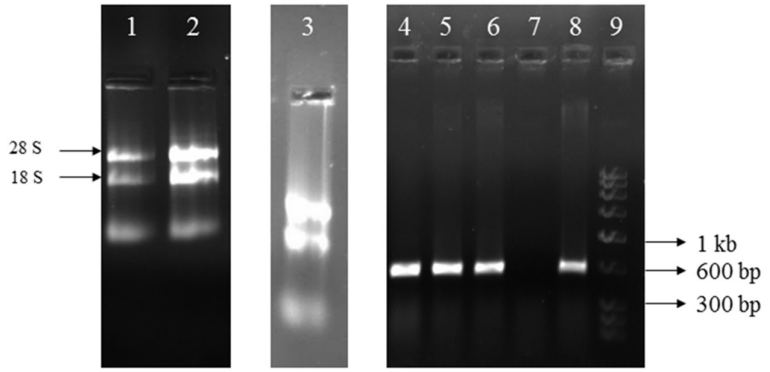
Figure 4. 3 Analysis of RNA and cDNA preparation of patient 3. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared from an individual infected with *P. vivax*.



Lane 1: Total RNA

Lane 2-3: β-actin amplicon

Figure 4. 4 Analysis of RNA and cDNA preparation of patient 4. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared from an individual infected with *P. vivax*.



Lane 1: Total RNA isolated from the individual 5

Lane 2: Total RNA isolated from the individual 6

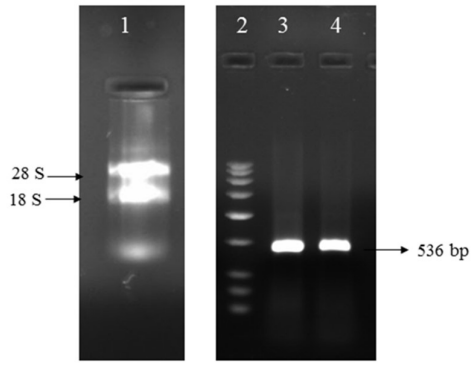
Lane 3: Total RNA isolated from the individual 7

Lane 4-6 & 8: β-actin amplicon

Lane 7: Negative control

Lane 9: Low Range DNA Ruler

Figure 4. 5 Analysis of RNA and cDNA preparation of patient 5, 6 and 7. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared from an individual infected with *P. vivax*.

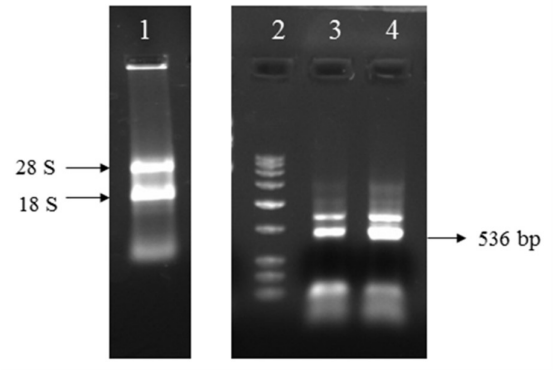


Lane 1: Total RNA

Lane 2: Low Range DNA Rule

Lane 3-4: β-actin amplicon

Figure 4. 6 Analysis of RNA and cDNA preparation of patient 8. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared from an individual infected with *P. vivax.*

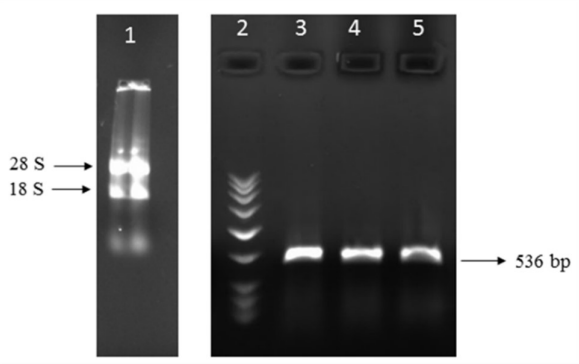


Lane 1: Total RNA

Lane 2: Low Range DNA Rule

Lane 3-4: β-actin amplicon

Figure 4. 7 Analysis of RNA and cDNA preparation of patient 9. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared from an individual infected with *P. vivax*.

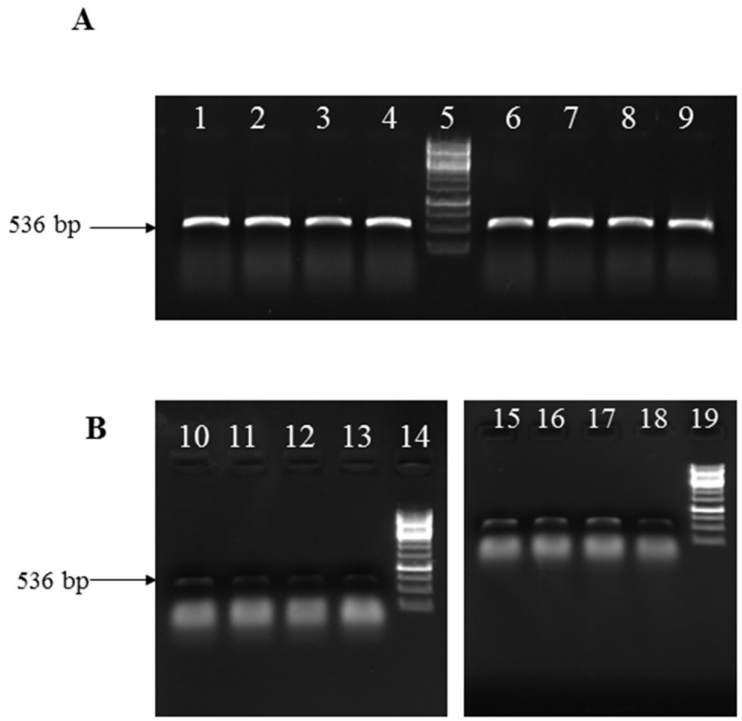


Lane 1: Total RNA

Lane 2: Low Range DNA Rule

Lane 3-5: β-actin amplicon

Figure 4. 8 Analysis of RNA and cDNA preparation of patient 10. Ethidium bromide stained 0.8% agarose gel electrophoresis of total RNA and β-actin amplicon from cDNA prepared from an individual infected with *P. vivax*.



Lane 1-2: Unstimulated monocytes

Lane 3-4: Monocytes stimulated with AMA1

Lane 5, 14 & 19: 1kb DNA ruler

Lane 6-7: Monocytes stimulated with MSP7

Lane 8-9: Monocytes stimulated with WARP

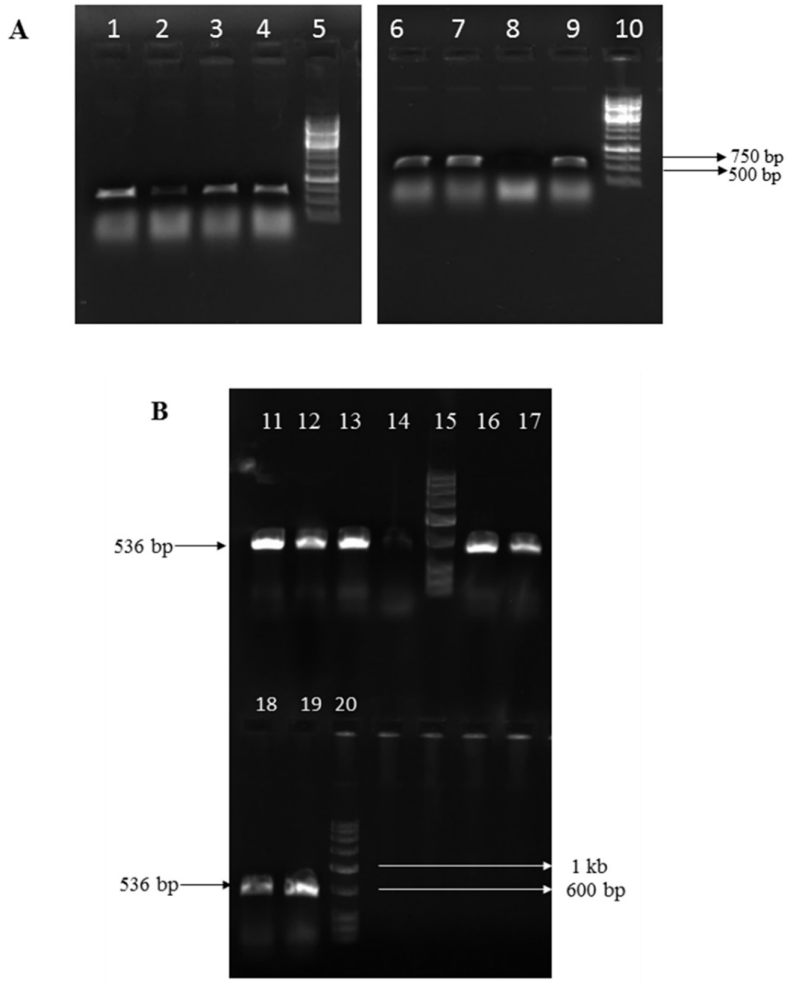
Lane 10-11: Unstimulated monocytes (from PBMCs)

Lane 12-13: PBMCs stimulated with AMA1

Lane 15-16: PBMCs stimulated with MSP7

Lane 17-18: PBMCs stimulated with WARP

Figure 4. 9 Analysis of cDNA prepared from healthy individual-1 (β actin PCR) after stimulation of (A) monocytes with recombinant antigens and (B) monocytes derived from PBMCs treated with antigens



Lane 1-2: Unstimulated monocytes

Lane 11-12: Unstimulated monocytes (from PBMCs)

Lane 3-4: Monocytes stimulated with AMA1

Lane 13-14: PBMCs stimulated with AMA1

Lane 5, 10: 1kb DNA ruler

Lane 15 & 20: Low Range DNA Ruler

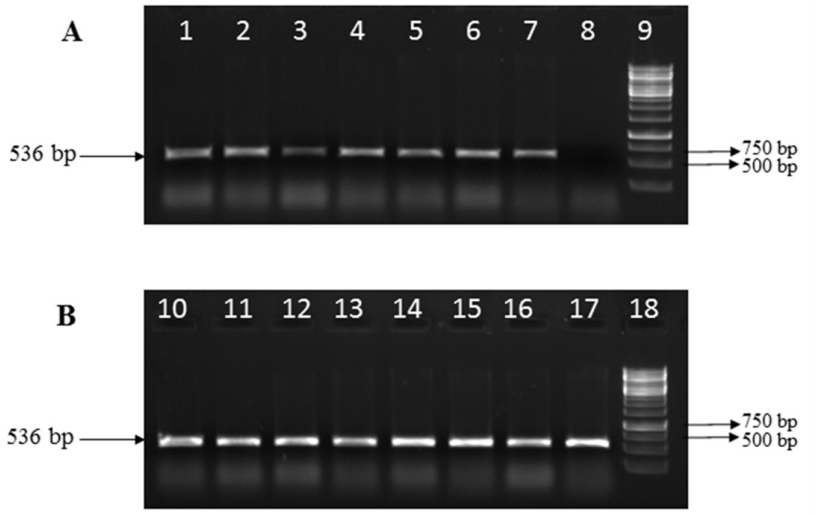
Lane 6-7: Monocytes stimulated with MSP7

Lane 16-17: PBMCs stimulated with MSP7

Lane 8-9: Monocytes stimulated with WARP

Lane 18-19: PBMCs stimulated with WARP

Figure 4. 10 Analysis of cDNA prepared from healthy individual-2 (β actin PCR) after stimulation of (A) monocytes with recombinant antigens and (B) monocytes derived from PBMCs treated with antigens



Lane 1-2: Unstimulated monocytes	Lane 10-11: Unstimulated monocytes (from PBMCs)
Lane 3-4: Monocytes stimulated with AMA1	Lane 12-13: PBMCs stimulated with AMA1
Lane 5-6: Monocytes stimulated with MSP7	Lane 14-15: PBMCs stimulated with MSP7
Lane 7-8: Monocytes stimulated with WARP	Lane 16-17: PBMCs stimulated with WARP
Lane 9 & 18: 1kb DNA ruler	

Figure 4. 11 Analysis of cDNA prepared from healthy individual-3 (β actin PCR) after stimulation of (A) monocytes with recombinant antigens and (B) monocytes derived from PBMCs treated with antigens of healthy individual 3

CHEMOKINE RECEPTOR PROFILE

CCR2, CCR7, CX3CR1 and CXCR4 expression was evaluated in monocytes isolated from patients by qPCR. Chemokine receptor profile in the patients suffering from *P. vivax* infection revealed that the fold expression of mRNA for CCR2 and CX3CR1 in these patients was higher than control while that for CCR7 and CXCR4 was not significantly different from the control (Figure 4.12). Figure 4.13A shows the the effect of in vitro exposure of monocytes to the recombinant *P. vivax* antigens AMA1, MSP-7 and WARP on the chemokine receptor profile. AMA1 and WARP stimulated monocytes showed a lower fold expression of CCR2, CX3CR1 and CXCR4, while there was no difference in the fold expression for CCR7. However MSP7 stimulated monocytes showed a reduced fold expression for all the chemokine receptors studied. The fold expression of mRNA for various chemokine receptors of monocytes derived from activated PBMC was also studied. As given in figure 4.13B, the fold expression of CX3CR1 was either unchanged (AMA1) or increased (MSP7 & WARP). The fold expression of other receptors (CCR2, CCR7 and CXCR4) were reduced compared to corresponding untreated monocytes.

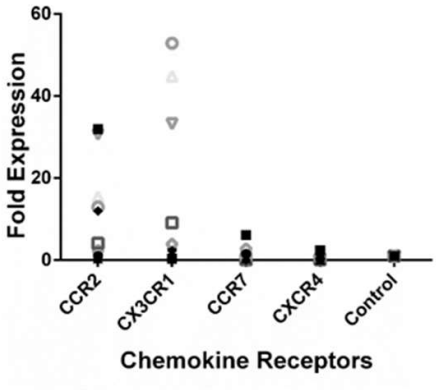


Figure 4. 12 Analysis of chemokine receptor expression in patients (n=10). Results were normalized to the expression of a housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

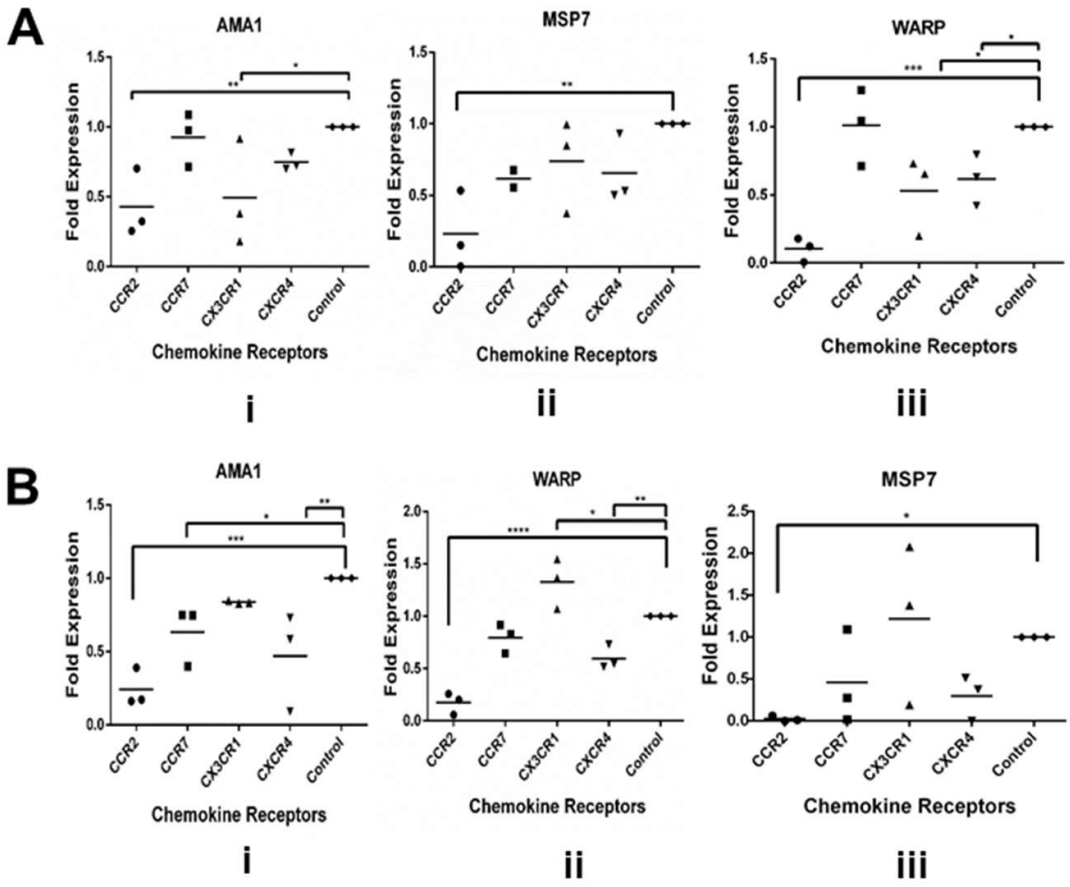


Figure 4. 13 Analysis of chemokine receptor expression: (A) Chemokine receptor levels in monocytes of healthy individuals (n=3) treated with recombinant antigens (B) Chemokine receptor levels in monocytes of healthy individuals (n=3) following treatment of peripheral blood mononuclear cells (PBMC) with recombinant antigens. Results were normalized to the expression of a housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *P < 0.05, **P< 0.01 and ***P< 0.001.

DISCUSSION

As explained earlier monocytes mediate host antimicrobial defence and are also involved in many inflammatory diseases (Serbina et al., 2008). Migration of monocytes plays an important role in the induction of immune response against any pathogen. During infection, the ability of monocytes to mobilize and traffic to the site of inflammation is central for their functions in promoting immune defence during infection and in driving inflammatory diseases. Chemokine receptors are responsible for bringing this about both by movement of monocytes from the bone marrow to the sites of inflammation, as well as to the regional lymph nodes where the activation of T cells takes place (Shi et al., 2011). In this present chapter, we studied the modulation of monocyte functions using recombinant malarial antigens.

The chemokine receptors studied here include CCR2, CCR7, CXCR4, and CX3CR1. The production of some of the bone marrow–derived cells, including monocytes, during a variety of infections depend on the chemokine receptor CCR2 for their migration out of the bone marrow. Spoonas et al., (2009) studied $CD11b^{high}Ly6C^{+}$ monocytes in mice infected with *P chabaudi* infection. After 8 days of infection, they observed that $CCR2^{-/-}$ mice infected with *P chabaudi* had significantly more $CD11b^{+}$ monocytes in the bone marrow and significantly fewer $CD11b^{high}Ly6C^{+}$ cells in the spleen than their infected WT counterparts. They also reported that $CCR2^{-/-}$ mice displayed significantly higher parasitemias with delayed reduction of the acute infection and transferring $CD11b^{high}Ly6C^{+}$ cells from day 7 infected WT mice to $CCR^{-/-}$ reduced the parasitemia level. Wang et al., (2009) studied the role of CXCR4 in the modulation of peripheral monocytes in the presence of a CCR2 antagonist. They reported that upon treatment of a CCR2 antagonist there was a reduction in the number of circulating $CD11b^{+}Ly6C^{hi}$ monocytes and 34% of bone marrow Ly6Chi monocytes expressed CXCR4 while no expression was observed on the blood monocytes. To validate the fact that CXCR4 plays a role in the decrease of blood monocyte a treatment of CXCR4 antagonist was given

prior to CCR2 antagonist. It was observed that upon pre-treatment of CXCR4 antagonist it significantly and dose dependently counteracted the effect of the CCR2 antagonist suggesting that CXCR4 recycles the monocytes back into the bone marrow. Auffray et al., (2007) demonstrated $CD16^{+}$ monocytes of *P. vivax* malaria patients expressed high levels of LFA-1 and CX3CR1. CX3CR1 is responsible for the ability of monocytes to patrol blood vessels in vivo suggesting that $CD16^{+}$ monocytes display enhanced capacity to adhere to endothelial cells in vitro may also contribute to the early inflammatory response observed during *P. vivax* infection. CCR7 mediated signal control the homing of immune cells to secondary lymphoid organs such as the lymph nodes and spleen. Upon activation the dendritic cells migrate towards the T zone of draining lymph nodes to present antigens to lymphocytes. Contact sensitization by using FITC skin paint is done to induce DC maturation and mobilization. Forster R et al 1999, used this method and observed that in $CCR7^{-/-}$ mice there a small amount of DCs residing in the lymph nodes as compared to the wild type mice.

Chimma et al., (2009) studied the phenotype of blood monocytes from healthy malaria-exposed individuals and that of patients with acute uncomplicated malaria by flow cytometry. Seventy six patients with uncomplicated falciparum malaria, and 10 healthy malaria-exposed individuals were enrolled in this study. The mean level of CCR2 and CX3CR1 expression on the total blood monocytes was three times higher in patients with acute uncomplicated malaria. Antonelli et al., (2014) studied the role of monocytes during *P. vivax* malaria. A total of 35 *P. vivax*-infected patients with uncomplicated malaria were enrolled in the study and flow cytometry was used to study various receptors levels in patients and in patients treated with chloroquine followed with primaquine. Monocytes from acute malaria patients displayed significantly lower levels of chemokine receptor CCR7. Our observations with patient monocytes agrees with the above reports. Patient monocytes showed elevated levels of both

CCR2 and CX3CR1 while the CCR7 mRNA levels in addition to CXCR4 levels, which is required for maintaining monocyte homeostasis, were reduced.

T cells can polarize into T_H1 and T_H2 effector cell types in response to distinct cytokines. T cells and cells presenting antigen to the T cells are themselves candidate sources of these polarizing cytokines. Sallusto et al., (1998) showed that TGF-β adds functionality to polarized Tcells by modulating the expression of chemokine receptors. CCR1 was expressed at low levels in Th1s and Th2s polarized under standard conditions. Addition of IFN-α resulted in a dramatic increase in CCR1 message, whereas addition of TGF-β resulted in an almost complete inhibition. In contrast, CCR7 was absent in Th1 and Th2 lines, but its expression was strongly upregulated by TGF-β. Suggesting that different cytokines produced under different conditions, may lead to differences in chemokine receptor expression. Our data agrees with the above report, control monocytes and monocytes derived from control PBMCs when treated with recombinant antigens showed a diverse pattern of antigen dependent CCR7 expression.

As explained earlier, monocytes are produced from their precursor cells in bone marrow and they home at spleen to carry out their protective function. This is mediated by the chemokine receptors namely, CCR2, CXCR4 and CX3CR1. Our data indicated a defect in the monocyte egress from the bone marrow to spleen since exposure of the control monocytes to the recombinant malarial antigens resulted in a reduction in the mRNA levels of CCR2 and CX3CR1, without any reduction of CXCR4 mRNA levels. This suggests that upon exposure with these antigens the monocytes level will also reduce in the circulation as well as in the spleen. Similar defect can also be observed in the case of monocytes derived from antigen exposed PBMCs as CCR2 and CXCR4 mRNA levels are reduced, whereas the CX3CR1 mRNA level is upregulated allowing monocyte homing to spleen. This therefore indicates that upon antigen exposure the expression of the chemokine receptors may be affected, which have a role in monocyte trafficking. There are various reports indicating that the recombinant

antigens used for vaccine did not had any desired therapeutic effect. As explained earlier, Thera et al., (2010) used recombinant AMA1 vaccine FMP2.1 and immunized 100 children between 1-6 years. It was reported that FMP2.1 induced high and sustained antibody levels in malaria-exposed children. But later on in 2011, the same group reported that the vaccine did not provide significant protection against clinical malaria. Our study thus throws light on the possible modulation of monocyte functions by recombinant malarial antigens, which may not have the desired therapeutic effect of combating the parasite and therefore could be one of the reasons for inconsistent results of malarial vaccines.