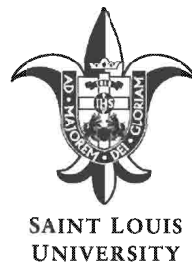


# **T cell mediated immunity in malaria and mycobacterial**

**infection:**

**A protective role for  $\gamma\delta^+$  T cells**

**Shewangizaw Worku**



**Stockholm, 2001**

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*TO MY FAMILY*

## SUMMARY

T cell mediated immunity is essential against intracellular infections. Studies of cell mediated immunity are important for the optimal design and development of effective vaccines. Identifying correlates of protective immunity will also enable measurement of vaccine efficiency. This thesis includes studies of T cell mediated immune protection against malaria and mycobacterial infections. A major focus of this work was the investigation of the role of  $\gamma\delta^+$  T cell responses.

In an initial study of lymphocyte subset compositions, a higher percentage of cytotoxic T cells were found in the peripheral blood of healthy adults from Ethiopia and Bangladesh than from Sweden. This suggested the involvement of environmental and/or genetic factors on the adaptation of the cellular immune system.

During acute malaria illness there was a complex pattern of changes in lymphocyte subset distribution and activation that appeared to be different in *P. falciparum* infection compared to *P. vivax*. During acute *P. falciparum* illness an increase in level and activation of  $\gamma\delta^+$  T cells, that was mostly due to increase in  $V\delta 1^+$  cells was found. However, during both infections increased numbers of  $CD4^+$ ,  $CD8^+$  and  $\gamma\delta^+$  T cells in peripheral blood were expressing the proliferation marker Ki-67. These results suggest that all T cells are activated and that lymphocyte redistribution and /or activation driven apoptosis may be the cause of the altered phenotypic profiles in peripheral blood.

An *in vitro* assay was developed to study the functional significance of  $\gamma\delta^+$  T cells. Generally activated  $\gamma\delta^+$  T cells of both  $V\delta 1^+$  and  $V\delta 2^+$  subsets but not similarly activated  $\alpha\beta$  T cells from non-malaria exposed individuals inhibited the *in vitro* growth of asexual blood stages of *P. falciparum* parasite. The inhibition was correlated to the number of  $\gamma\delta^+$  T cells and required cell-to-cell contact. Kinetic analysis suggested the likely targets to be the late infected erythrocyte (schizonts) or extracellular merozoites. These results suggest  $\gamma\delta^+$  T cells may have a protective role during malaria infection independent of previous exposure to malaria.

An *in vitro* assay was also developed to measure T cell mediated inhibition of mycobacterial growth. Both  $\alpha\beta^+$  and  $\gamma\delta^+$  T cells from PPD positive individuals inhibited intracellular growth of BCG, but only when activated by mycobacterial antigens. The mycobacterial growth inhibition capacity was up regulated by BCG vaccination and required cell-to-cell contact. These results suggest a role for  $\gamma\delta^+$  T cells in the memory responses against mycobacteria.

Expressions of proinflammatory cytokines and cytolytic molecules such as perforin, granzymes, granulysin and Fas/Fas ligand, were characteristic of both malaria and BCG growth inhibitory T cells. However, expression of these molecules in non-inhibitory activated cells were also seen, suggesting that growth inhibition requires restricted recognition of target cells by specific effectors.

Our results indicate that  $\gamma\delta^+$  T cells may represent an important component of the primary immune defense against *P. falciparum* infection and the memory immune defense against mycobacterial infection.

## ORIGINAL PAPERS

This thesis is based on the following papers,

- I Worku S, Christensson B, Bjorkman A, Islam D. Higher proportion of CD8<sup>+</sup> T cells in the blood in healthy adults from Ethiopia and Bangladesh compared with Sweden. *Trans R Soc Trop Med Hyg.* 1997. 91:618-22.
  
- II Worku S, Bjorkman A, Troye-Blomberg M, Jemaneh L, Farnert A, Christensson B. Lymphocyte activation and subset redistribution in the peripheral blood in acute malaria illness, distinct  $\gamma\delta^+$  T cell patterns in *Plasmodium falciparum* and *P. vivax* infections. *Clin Exp Immunol.* 1997. 108:34-41.
  
- III Worku S, Troye-Blomberg M, Christensson B, Bjorkman A, and Fehniger T, Activation of T cells in the blood of patients with acute malaria, Proliferative activity as indicated by Ki 67 expression. *Scand J Immunol*, In press.
  
- IV Troye-Blomberg M, Worku S, Tangteerawatana P, Jamshaid R, Soderstrom K, Elghazali G, Moretta L, Hammarstrom M, Mincheva-Nilsson L. Human  $\gamma\delta^+$  T cells that inhibit the *in vitro* growth of the asexual blood stages of the *Plasmodium falciparum* parasite express cytolytic and proinflammatory molecules. *Scand J Immunol.* 1999. 50:642-50.
  
- V Worku S, Hoft DF. *In vitro* measurement of protective mycobacterial immunity, antigen-specific expansion of T cells capable of inhibiting intracellular growth of Bacille Calmette-Guerin. *Clin Infect Dis.* 2000. Suppl 3:S257-61.
  
- VI Worku S, Hoft DF. Differential effects of antigen specific and control T cells on intracellular mycobacterial growth, *in vitro* models of protective immunity and mycobacterial persistence. Submitted for publication.

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## ABBREVIATIONS

APC	antigen presenting cell
BCG	Bacille Calmette-Guérin
C	constant segment of TCR gene
CD	cluster differentiation
CSF	cerebro spinal fluid
CTL	cytotoxic T lymphocyte
D	diversity segment of TCR gene
Di	double infection with Pf <i>and</i> Pv
FITC	fluorescein isothiocyanate,
hsp	heat shock protein
IFN	interferon
IL	interleukin
Ig	immunoglobulin
IPP	isopentenyl pyrophosphate
J	joining segment of T cell receptor gene
LAM	lipo-arabinomannan
MHC	major histocompatibility complex
MMRc	macrophage mannose receptor
Moab	monoclonal antibody
MPC	magnetic particle concentrator
NK cells	natural killer cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridin chlorophyll-A protein
PHA	phytohaemagglutinin
Pf	<i>Plasmodium falciparum</i>
Pv	<i>Plasmodium vivax</i>
RBC	red blood cell
iRBC	infected red blood cell
TCR	T cell receptor
Th	T helper cell
TH	tris-Hank
TGF	transforming growth factor
TNF	tumour necrosis factor
TT	tetanus toxoid
V	variable segment of TCR gene
WBC	white blood cells

## INTRODUCTION

### THE IMMUNE SYSTEM

Disease protection (immunity) is mainly achieved through adaptive immune responses, antigen specific B and T cell responses interacting through a complex network of soluble and cell associated molecules with effector mechanisms of the inflammatory system. A wide range of distinct cell types are involved. The T lymphocytes play a central role in the regulation of both T and B cell mediated immunity. Cell types of the innate immune system such as monocyte derived macrophages, dendritic cells, and Langerhans cells serve as antigen presenting cells (APC). Natural killer cells (NK cells), mast cells basophils and also macrophages interact with lymphocytes or their products and play critical parts in the mediation of immunological functions such as killing of microbial pathogens, secretion of cytokines and chemical mediators. The cells of the immune system are found in the blood and in peripheral organized lymphoid tissues, like the spleen and lymph nodes and in the mucosa and skin.

Two broad classes of lymphocytes are recognized, The B lymphocytes that are precursors of antibody secreting plasma cells and the T (thymus dependent) lymphocytes. T lymphocytes regulate the immune response and participate in direct effector functions. Precursor lymphocytes of the T and B cell lineage develop in the bone marrow. T cells undergo series of developmental steps in the process of maturation in the thymus whereas B-cell maturation takes place in the bone marrow.

#### T lymphocytes

T cells express antigen receptors on their surface membrane. These T cell receptors (TCR) are specific for determinants (epitopes) of antigens processed and presented in the context of MHC molecules present on APC. The T cells are committed to respond to a limited set of structurally related epitopes. Two types of TCRs exist, defined by their expression of heterodimeric complexes of  $\alpha\beta$  and  $\gamma\delta$  chains respectively. The ability of an organism to respond to virtually any non self antigen is achieved by the existence of very large number of different clones of lymphocytes each bearing receptors specific for a distinct epitope (1). The vast majority of T lymphocytes in peripheral blood (> 90%) express the  $\alpha\beta$  TCR whereas a minority (6-8 %) bear the  $\gamma\delta$  TCR (2).

### The $\alpha\beta^+$ TCR T Cells

The  $\alpha\beta^+$  TCR T cells express a disulphide linked heterodimer comprising  $\alpha$  and  $\beta$  chains each organized into a constant domain and a highly polymorphic variable domain similar to the Ig molecule. The  $\alpha\beta$  TCR heterodimer is anchored into the cell membrane by a hydrophobic trans-membrane region non covalently associated with the CD3 complex (3). Among the  $\alpha\beta$  TCR T cells two "sublineages" may be recognized.  $\alpha\beta$  TCR T cells, expressing the CD4 molecule ( $CD4^+$  T cells) recognize antigens presented by MHC class II molecules on APCs.  $\alpha\beta$  TCR T cells expressing CD8 ( $CD8^+$  T cells) recognize antigens presented by MHC class I molecules. Thus, the two cell types differ in their requirement of restriction molecules and appear to mediate different types of regulatory and effector functions (1). A small number of double negative  $CD4^-CD8^-$   $\alpha\beta$  T cells restricted by non-polymorphic MHC molecules have also been described (4).

$\alpha\beta^+$  T cell development, A wide variety of TCR is necessary for the immune system to cope with all antigens we encounter. The diversity of the  $\alpha\beta^+$  T cell receptor lies on processes of somatic rearrangements of the V, D and J gene segments encoding for the TCR chains  $\alpha$  and  $\beta$ . To date about 70 V $\beta$ , 2D $\beta$ , 13 J $\beta$ , 70 V $\alpha$  and 70J $\alpha$  gene segments have been described and sorted into families, based on homology at nucleotide level. This stochastic process of joining gene segments contributes to the huge variability in the structure of the antigen binding domain of the functional T cell receptor (5).

Lymphopoietic T cell precursors  $CD4^-CD8^-TCR^-$  T cells, first rearrange and express the TCR $\beta$  gene which select these cells for further expansion and maturation into  $CD4^+ CD8^+$  thymocytes and the rearrangement of TCR $\alpha$  gene will follow. Further maturation of  $CD4^+ CD8^+ \alpha\beta^+$  thymocytes requires positive selection. When  $\alpha\beta$  TCR binds MHC molecule in the thymus cells they will develop into functionally mature  $CD4^+8^-$  or  $CD4^+8^+$  precursors of helper and/or killer cells, respectively, a process called positive selection (6). Tolerance to self antigens is achieved by the process of negative selection where lymphocytes bearing TCR recognizing self antigens in the context of self MHC molecule undergo programmed cell death (apoptosis) (7).

### $CD4^+$ T lymphocytes

T cells bearing the CD4 molecule recognize exogenous peptides contained within the cleft of MHC class II molecules. Activated  $CD4^+$  T cells secrete cytokines that help the generation and maturation of T and B cells (8). On the bases of differential production of cytokines,  $CD4^+$  T cells can be further divided into at least two subsets (Th1 and Th2) capable of inducing

different kinds of immune responses (9). The  $CD4^+$  T helper 1 cells (Th1), predominantly produce the cytokines IL-2, IFN $\gamma$  and lymphotoxin. These cells mediate cellular immunity involving enhancement of microbiocidal activity by monocytes and macrophages and promoting the differentiation of cytotoxic lymphocytes, as well as regulating certain B cell responses. The  $CD4^+$  T helper 2 cells (Th2) produce cytokines IL-4, IL-5, IL-6, and IL-10 mainly promoting B cells to develop into antibody producing cells and inducing isotype switching from IgM/IgG to IgE (9). T cell clones capable of producing cytokine typical of both Th1 and Th2 cells have been obtained. These clones are designated Th0 cells (10). It is likely that the immune response to various pathogens *in vivo* may be predominantly either Th1 or Th2 type. It is well established from certain experimental models that the type of helper response will determine the outcome of the specific infectious challenge to the host (11-13).

### **$CD8^+$ T lymphocytes**

The classical cytotoxic  $CD8^+$  cells recognize antigens in the context of MHC class I molecule of an altered self cell, e.g. a virus infected cell. Cytotoxic T lymphocytes (CTL) are defined by their ability to kill target cells through direct cell-to-cell contact. In addition, they produce cytokines that are toxic for the target cells. Thus, CTLs play an important role in the defense against intracellular micro-organisms (14). CTLs usually depend on T helper cells for their differentiation and activation. The cytolytic granules in the cytoplasm of CTLs contain pore forming proteins called perforins and serine protease's called granzymes that mediate killing of target cells (15). Some T cells mediate suppression of cellular as well as humoral immunity. Most suppressor T cells seem to be  $CD8^+$  but differ from conventional  $CD8^+$  T cells in that they are restricted by MHC class II antigens and do not express the T cell costimulatory receptor CD28 (16).

### **$\gamma\delta^+$ TCR T cells**

The  $\gamma$  and  $\delta$  genes, The human  $\gamma$  gene found on the short arm of chromosome 7 consists of V gene clusters upstream to two J gene clusters each associated with distinct C gene regions. Fourteen  $V\gamma$  genes have been described, 6 of them being pseudo genes while 8 are potentially functional. Depending on the homology  $V\gamma$  genes are grouped in to  $V\gamma 1$  ( $V\gamma 1.1$ -  $V\gamma 1.8$ ),  $V\gamma 2$ ,  $V\gamma 3$  and  $V\gamma 4$ . Each of the two J clusters is followed by a down stream constant (C) gene segment. Rearrangements to the  $J\gamma 1$  gene will result in splicing to the  $C\gamma 1$  gene segment and rearrangement to  $J\gamma 2$  gene will result in splicing to the  $C\gamma 2$  gene when the mRNA transcript is produced. The  $C\gamma 1$  encodes for cystine residue allowing disulphide linkage to the TCR

$\delta$  chain. The C $\gamma$ 2 gene does not code for cystine residues, and thus, form  $\gamma\delta$  heterodimers without disulphide links (2,17).

The human TCR  $\delta$  gene is located within the TCR  $\alpha$  locus between the V $\alpha$  and J $\alpha$  segments. It consists of a single C $\delta$ , three J $\delta$  and three D $\delta$  gene segments. V $\delta$ 1, V $\delta$ 2 and V $\delta$ 3 are the commonly used V $\delta$  genes on  $\gamma\delta$  TCR. The TCR  $\delta$  are generated by rearrangement of dispersed germline gene segments during T cell development. Thus, a particular V $\delta$  gene segment is recombined to one or more of the D $\delta$  segments and one of the three J $\delta$  segments. During TCR  $\delta$  rearrangements a relatively large number of nucleotides are randomly incorporated in the junctions of rearranging gene segments. In addition, deletions of nucleotides from the joining ends of germline sequences occur. Both these mechanisms and/or imprecise joining appear to contribute to diversity of  $\gamma\delta$  TCR same as  $\alpha\beta$  TCR. The usage of up to three D segments in tandem is a striking feature of TCR  $\delta$  diversity (2,17,18).

### **$\gamma\delta^+$ T cell development and subsets**

In the thymus of both mice and humans different combinations of V $\gamma$  and V $\delta$  genes segments appear in waves during ontogeny. The first subset to be rearranged in the early fetal human thymus involves the joining of V $\delta$ 2 to D $\delta$ 3 and V $\gamma$ 8 and V $\gamma$ 9 to the upstream J $\gamma$  gene cluster associated with C $\gamma$ 1. This subset is usually referred to as the V $\delta$ 2 subset. Four to 6 months after birth there is a switch in the TCR- $\delta$  locus such that V $\delta$ 1 is joined to the upstream D $\delta$  gene segments and a switch in the TCR- $\gamma$  locus involving upstream V $\gamma$  (V $\gamma$ 2, 3,4,5 and 8) gene segments joined to downstream J $\gamma$  gene segments associated with C $\gamma$ 2. This subset is referred to as the V $\delta$ 1 subset. There is evidence supporting both positive and negative selection of  $\gamma\delta$  thymocytes in the mouse. Classically the development of T cells has been defined as occurring in the thymus. However, evidences point to that subsets of  $\alpha\beta$  and  $\gamma\delta$  intraepithelial lymphocytes can develop, at least partially, independent of the thymus (2,17,18).

Human  $\gamma\delta^+$  T cells can be broadly divided into V $\delta$ 1 and V $\delta$ 2 subgroups that can be distinguished by monoclonal antibodies (Table 1).  $\gamma\delta^+$  T cells in the peripheral blood have an extensive TCR junctional diversity and are largely polyclonal. The proportion of the V $\delta$ 2 subset among  $\gamma\delta^+$  T cells increases with age from about 25 % in cord blood to 70% in adult peripheral blood, while the V $\delta$ 1 subset decreases from 50% to 30%. In the post natal and adult thymus the V $\delta$ 1 subset represents the majority (80%) of all  $\gamma\delta^+$  T cells. In peripheral blood the majority of  $\gamma\delta^+$  T cells carry V $\delta$ 2 in combination with V $\gamma$ 9 and a single  $\gamma$  constant region, the disulphide linked C $\gamma$ 1. The V $\delta$ 1 subset in the blood predominantly bears a non disulphide linked  $\gamma\delta$  TCR, and does not seem to have a preference for pairing to any

particular V $\gamma$  chain (2,19). However, the majority of intraepithelial lymphocytes expressing V $\delta$ 1 also express V $\gamma$ 8 (20). Recently a high frequency of  $\gamma\delta$  T cells with dominance of the V $\delta$ 1 subset in the peripheral blood of healthy West African population different from Caucasians has been described (21).

### Characteristics of $\gamma\delta^+$ T cells

Several characteristics are shared between  $\alpha\beta$  and  $\gamma\delta^+$  T cells. Both express diverse rearranged antigen specific receptors and both express similar activation markers and adhesion molecules. Both secrete a similar array of cytokines and are capable of mediating cytotoxic effector functions. However,  $\gamma\delta^+$  T cells differ from  $\alpha\beta^+$  T cells in several features (2,18,22,23).

- $\gamma\delta^+$  T cells precede  $\alpha\beta^+$  T cells in ontogeny. TCR  $\gamma\delta$  gene rearrangements were detected in the human by 8 weeks of fetal development (24)
- $\gamma\delta^+$  T cells seem to preferentially home to certain anatomical locations, especially in epithelial tissues.  $\gamma\delta^+$  T cells are disproportionately abundant in the intestine, commonly found as intraepithelial lymphocytes (IEL). In humans the  $\gamma\delta$ , $\alpha\beta$  ratio among IELs is 1,5 compared with 1,50 in the lymph node.  $\gamma\delta^+$  T cells are also found in skin, uterine and vaginal epithelium (20,25,26).
- Most  $\gamma\delta^+$  T cells do not express CD4 or CD8 (around 25% may express CD8 on their surface very few of them express CD4) and recognize antigen largely unrestricted by classical MHC type I or II molecules (27).

**Table 1,** Monoclonal antibodies recognizing  $\gamma\delta^+$  T cells

Monoclonal antibody	Specificity	Used in study	Reference
TCR $\delta$ 1	C $\delta$ (all $\gamma\delta^+$ T cells)	-	(28)
TCR $\gamma/\delta$ 1	C $\gamma$ (all $\gamma\delta^+$ T cells)	I-VI	(29)
C $\gamma$ M1	C $\gamma$ (all $\gamma\delta^+$ T cells)	IV	(30)
$\delta$ TCS1	V $\delta$ 1-J $\delta$ 1, V $\delta$ 1-J $\delta$ 2	-	(31)
V $\delta$ 1(a)	V $\delta$ 1	II, IV	(27)
A13	V $\delta$ 1	IV	(32)
Ti $\gamma$ A	V $\gamma$ 9	II, IV	(33)
BB3	V $\delta$ 2	IV	(34)
B18	V $\gamma$ 8	II	(35)
23D12	V $\gamma$ 2,3,4	-	(36)
$\gamma$ V1.4	V $\gamma$ 4	-	(37)

### Specificity and function of $\gamma\delta^+$ T cells

Although the *in vivo* relevance of  $\gamma\delta^+$  T cells remains largely unknown, some important immunological functions such as cytotoxicity as well as the production of a wide array of cytokines have been shown in response to *in vivo* and *in vitro* stimulation (2,19).  $\gamma\delta^+$  T cells producing of IL-2 and IFN- $\gamma$  (Th1 like) and  $\gamma\delta^+$  T cells producing IL-4, IL-5, IL-6 and IL-10 (Th2 like) have been described (38). Cytotoxic activity is often seen upon activation of  $\gamma\delta^+$  T cells and is indicated by the release of cytoplasmic granules containing both perforin, granulysin and serine esterase's (39,40). Killing by  $\gamma\delta$  T cells is usually not restricted by polymorphic MHC molecules and the ligands are poorly characterized. In addition, both proliferative and cytotoxic responses of *in vitro* cultured  $\gamma\delta^+$  T cells against allo-MHC expressing cells have been observed.  $\gamma\delta^+$  T cells share with NK cells the expression of Fc-receptors that mediate antibody dependent cellular cytotoxicity (22).

A substantial fraction of human as well as mouse  $\gamma\delta^+$  T cells are stimulated by a number of microbial antigens and in particular by antigens derived from mycobacterial species (41). The  $\gamma\delta^+$  T cell response to mycobacteria is oligoclonal. In humans, the majority of the activated cells belong to the V $\gamma$ 9/V $\delta$ 2 subset although they exhibit extensive junctional diversity (42). Besides direct recognition of peptides, presentation of non-peptide low molecular mass products antigenic to V $\delta$ 2/V $\gamma$ 9 subset of human  $\gamma\delta^+$  T cells has been shown. These products were later identified to be several alkenyl or prenyl derivatives of phosphates, pyrophosphates or nucleoside triphosphates (43-48).  $\gamma\delta^+$  T cells recognizing alkylamines derived from microbes, edible plants and tea have also been described (49).

It has also been shown that  $\gamma\delta^+$  T cells may recognize microbial and self heat shock proteins. Interestingly, *in vivo* localization of  $\gamma\delta^+$  T cells together with hsp-expressing cells are often observed in a wide variety of pathological conditions (50). Hence, it has been suggested that  $\gamma\delta^+$  T cells function as a first line of defense against infectious pathogens by possibly eliminating infected or stressed autologous cells expressing heat-shock proteins. Recently, it was also shown that conventional antigen processing and presenting pathways are not required for the recognition of antigens by  $\gamma\delta^+$  T cells. Studies have demonstrated that non-polymorphic MHC class I like molecules such as CD1 can be important for recognition of antigens by  $\gamma\delta^+$  T cells (51,52).

*In vivo* depletion studies and transfer experiments have shown that  $\gamma\delta^+$  T cells have immunoregulatory capabilities (19,53). Some studies have also suggested that  $\gamma\delta^+$  T cells could play a suppressor or contra-suppressor role on B cell responses (54).  $\gamma\delta^+$  T cells have also been shown to function as helper cells for B cell inducing immunoglobulin secretion and isotype

switching in B cells *in vitro* (55). Conversely,  $\gamma\delta$  T cells are regulated by CD4  $\alpha\beta$  T cells (56). Altogether, these findings indicate that  $\gamma\delta^+$  T cells are important in the regulation of certain immune responses even though the mechanisms are yet largely unknown.

#### **$\gamma\delta^+$ T cells in different diseases**

Preferential localization of  $\gamma\delta^+$  T cells in peripheral blood and inflamed areas has been found in association with different diseases both in mice and humans. These cells also respond *in vitro* to stimulation with corresponding microbial antigens. A short summary of the observed  $\gamma\delta^+$  T cell responses *in vivo* and *in vitro* to microbial and inflammatory diseases is presented in Table 2



**Table 2** *In vivo* increase in number of  $\gamma\delta^+$  T cells in different diseases and *in vitro* responses to the disease related antigens in humans

Diseases	Specific disease	In vivo localization of increased cells	In vitro responses to antigen	References
Bacteria	<i>Mycobacteria Leprae</i>	Skin granuloma	+	(2)
	<i>Mycobacterium tuberculosis</i>	Lymphnode/peripheral blood	+, †, ‡	(2,199,200)
	BCG vaccination		+, †, ‡a	(59)
	<i>Listeria monocytogenes</i>	Peripheral blood	+, †, ‡	(60)
	Streptococci group A		+, †, ‡	Reviewed in (2)
	Staphylococci		+, †, ‡	
	<i>Salmonella typhi</i>	Peripheral blood	+, †, ‡	
	<i>Borrelia burgdoferi</i>	Synovial fluid	+, †, ‡	(61)
Virus	Epstein barr virus	Peripheral blood	+	Reviewed in (62)
	HIV/AIDS	Peripheral blood		
	Measles/SSPE	CSF		(63)
	Chronic viral hepatitis	Peripheral blood		(64,65)
Parasites	Cytomegalovirus	Peripheral blood		
	Leishmania (cutaneous)	Granuloma of skin		
	Schistosomiasis	Peripheral blood	+, †, a	(66)
	<i>Plasmodium falciparum</i>	Peripheral blood, spleen	+, †‡	(67)
	<i>Toxoplasma gondii</i>	Peripheral blood	+, †	(68)
	<i>Onchocerca volvulus</i>			
Chronic Inflammatory lesion	Rheumatoid arthritis	Synovial fluid		Reviewed in (2,19)
	Multiple Sclerosis	Brain lesion		
	Coeliac disease	Intestinal epithelium		
	Polymyositis	Muscle fibers		
	Atopic dermatitis	Peripheral blood		
	Pulmonary sarcoidosis	Blood and broncho alveolar lavage		
	Cancer	Tumor infiltrating lymphocytes	+, †, ‡	

+, proliferation to antigen stimulation. † production of cytokines. ‡, cytotoxic against infected cells. a, inhibition of parasite growth in vitro. b, after intra peritoneal infection.

### **Natural Killer cells**

NK cells constitute a heterogeneous sub population of peripheral blood mononuclear cells initially defined by their capacity to kill target cells without previous antigen exposure. They can be identified by expression of CD56, CD57 and/or CD16 antigens. NK cells are capable of lysing certain virus infected cells as well as tumor cells. This killing, as opposed to cytotoxicity by CTL, does not involve specific recognition via clonally distributed receptors and is not restricted by MHC products. NK cells appear to recognize and eliminate cells when the critical “self” proteins (like MHC class I molecules) are absent or expressed in reduced amount. Tumor cells, virus infected and transplanted cells may be recognized and killed by NK cells (57). Classical CTL and NK cells display morphological features of large granular lymphocytes (LGL) i.e. they have an extended cytoplasm and contain intracellular granules as well as multi-vesicular bodies. The killing by NK cells is mediated by perforin dependent or Fas-mediated pathways (58). NK cells may be induced to produce IFN $\gamma$  when stimulated.

### **Immunophenotyping of lymphocytes**

Immunophenotyping methods have been used to understand B and T cell differentiation, maturation and functional heterogeneity. Until recently, lymphocyte subset enumeration has been carried out using rosette methods or microscopic immunofluorescence. The recent availability of monoclonal antibodies in conjunction with flow cytometry has resulted in the wide spread phenotyping of human lymphocyte subsets using cell surface expressed molecules (69-73). A summary of lymphocyte cell surface molecules used as markers in our phenotypic analysis is presented in Table 3.

Immunophenotyping has been shown to be of diagnostic and prognostic value in the analyses of leukaemias, lymphomas and immunodeficiency conditions, such as AIDS (74). Furthermore, monitoring lymphocyte population levels in different phases of diseases both *in vivo* and *in vitro* may provide essential information on the level and the type of immune response elicited. However, technical and biological factors interfere with flow cytometric investigations making the definition of normal control values for blood leukocytes critical for the evaluation of the results (73).

Temperature, anticoagulant, support media, isolation, preparation, staining, cellular composition, reagents, gating, selected parameters to be acquired, methods of histogram analysis, fixation and cryopreservation appear to be important technical variables in the analysis of normal lymphocytes (75). Furthermore, several biological factors such as age, sex,

ethnic back ground, pregnancy, medications, hormones and nutritional status have been shown to influence the composition of lymphocyte subset populations in peripheral blood (73,76-79).

The understanding of the normal lymphocyte subset composition is largely based on studies from industrialized countries. So far there is limited information on lymphocyte phenotypic profile from populations living, in developing countries, i.e. areas with high prevalences of communicable infectious diseases. Distortions of the balance between lymphocyte subsets may follow antigenic or superantigenic challenges leading to the expansion of T cell subsets with a particular V $\beta$  expression and also antigen specific B cells. Moreover, both acute infection and more chronic exposure to environmental agents may also change the balance of the immune system and hence of lymphocyte subset distribution (21,80-82). Therefore different profiles of lymphocyte compositions may be expected in different populations living in different environments and with different genetic background.

**Table. 3,** Cell surface associated molecules used as markers of leukocyte differentiation and activation in flow cytometry

Surface molecule	Expressing cells	Function
CD3	All T cells	Multimeric cell membrane associated complex, primarily involved in signal transduction
CD4	MHC class II restricted (helper) T cells, monocytes, macrophages and dendritic cells.	Co-receptor for MHC class II. Receptor for HIV
CD8	MHC class I restricted cytotoxic/suppressor T cells, NK cells,	Co-receptor for MHC class I
CD14	Monocytes, macrophages and dendritic cells	Receptor for lipopolysaccharide, transduce signal leading to oxidative burst and synthesis of TNF $\alpha$
CD19	B cells	Part of B cell antigen receptor complex, potential binding site for CD77
CD25	Activated T cells, activated B cells, thymocytes, myeloid precursors, oligodendrocytes	$\beta$ chain of the receptor for T cell growth factor IL-2
CD45	All haematopoietic cells except RBC (leukocyte common antigen)	The intra cellular domain is tyrosine phosphatase required for signal transduction
CD45RA	B cells, subsets of naive T cells, monocytes	Isotype of CD45
CD45RO	Activated and memory T cell subsets, thymocytes	Isotype of CD45, functionally more potent than CD45RA and requires less stringent activation signal to be stimulated by low amount of antigen
CD57	NK cells, some T cells (especially CD8), few B cells and monocytes	Unknown function, CD57 <sup>+</sup> T cells mediate both cytotoxic and suppressor function
CD56	Mainly NK cells, nerve cells, subset of activated T cells also called neural cell adhesion molecule, NCAM-1	Plays a role in normal tissue development and alloreactive cytolytic function
HLA-DR	Antigen presenting cells, macrophages, activated B and T cells,	MHC class II antigen, important in antigen recognition and presentation to CD4 <sup>+</sup> T cells.

## MALARIA

### Epidemiology

Malaria is caused by a protozoan parasite. There are four generally recognized species of malaria affecting humans, *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. In addition to human malaria parasites there are other plasmodia, which infect apes, monkeys, birds, and rodents.

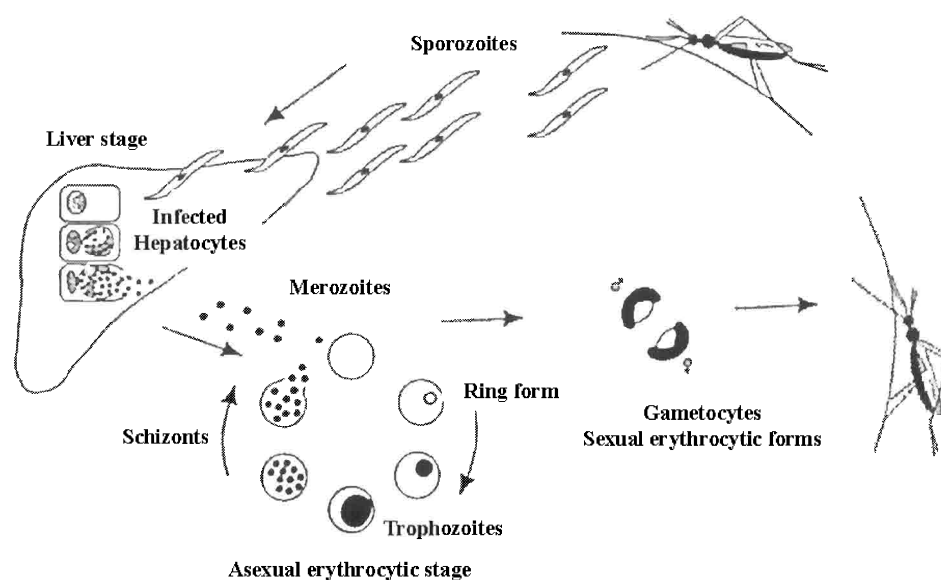
*P. falciparum* is a major cause of morbidity and mortality in humans (83). In tropical and sub tropical areas of the world every year more than 110 million cases of malaria occur of which more than 90 million are in Africa, south of the Sahara. WHO estimates that one to two million persons die from malaria each year. Most deaths occur in Africa in children aged less than five years. Malaria is thus one of the most serious and wide spread tropical diseases in the world today (84-86).

In Ethiopia 75% of total land mass is endemic for malaria and 64% of the population is at risk of contracting malaria. *P. falciparum* is the cause of malaria in 60% of the cases while *P. vivax* contributes to 40% of malarial illness and *P. malariae* causes less than one percent. Severe and complicated malaria is caused by *P. falciparum*, with a case fatality rate of about 10% and 33% in hospitalized adults and children less than 12 years old respectively (87).

### Parasite life cycle

The life cycle of malaria parasite is complicated and it involves several developmental stages with asexual reproduction in the human host and sexual reproduction in the *Anopheles* mosquito vector (Figure 1). The development of the parasite in the red blood cell brings certain changes such as enlargement, depolarization, pigment depositions and certain forms of stippling with distinct characteristics for each species of plasmodia (83).

**Figure, 1** The life cycle of *Plasmodium falciparum*



## Diagnosis

Light microscopy of Giemsa stained blood smears is the method most frequently used to diagnose malaria and to identify the infecting species. Examination of the blood films for multiply infected red blood cells, late stage asexual parasites (trophozoites and schizonts) or enlarged parasitized red blood cells are used to distinguish between *P. falciparum* and *P. vivax*. Lately polymerase chain reaction (PCR) based detection of parasite species has been shown to be a highly sensitive and specific assay for malaria diagnosis (88,89).

## Clinical Manifestations

Malaria infection causes an acute febrile illness. The severity and course of the malaria episode depends on the species and strain of the infecting parasite, the age, genetic constitution, state of immunity, general health and nutritional status of the patient and the possible delay and choice of treatment. *P. falciparum*, *P. vivax* and *P. ovale* normally cause an acute febrile illness whereas *P. malariae* only rarely cause acute illness in the normal host (83).

*P. falciparum* infection may be associated with severe disease. The incubation period of *P. falciparum* infection takes 9-14 days. The illness starts with headache, dizziness, pain in the back and limbs, malaise, anorexia, nausea, vague abdominal pain, vomiting and mild diarrhea, intermittent chills and fever, which may be continuous or remittent. The physical findings are non-specific and may include fever, prostration, postural hypotension, a tinge of jaundice and hepatosplenomegaly. In none immune people *P. falciparum* infection may cause severe illness. The severe illness in *P. falciparum* infection and fatal complications are mainly related to the development of micro vascular disease. The diffuse micro vascular disease resulting from large number of adherent and rosette-forming parasitized red cells exacerbated by the loss of red cell deformability produces functional obstruction to blood flow. These results in secondary organ dysfunction or damage from hypoxia and hypoglycaemia. Cerebral malaria (unrousable coma), severe normocytic anemia, renal failure, pulmonary edema, circulatory collapse and shock, spontaneous bleeding, hypoglycaemia and academia/acidosis are severe manifestations and complications of malaria (90). People living in endemic areas and frequently infected with the *P. falciparum* parasites acquire some immunity so could tolerate *P. falciparum* infections.

The incubation period of *P. vivax* malaria in the non-immune host is usually between 12-17 days but may be prolonged to 8-9 months or even longer. The classical febrile paroxysm with cold, hot and sweating stage is the main presenting feature

## **IMMUNITY TO MALARIA**

In areas of stable endemicity repeated exposure to the parasite leads to acquisition of specific immunity. However, even in people exposed to malaria for the first time there is a range of possible outcomes.

### **Natural resistance**

The plasmodia exhibit strict host specificity. Moreover, certain characteristics of the erythrocyte or the host cells increase the natural resistance. Absence of the Duffy blood group has been shown to affect penetration of *P. vivax* merozoite, hence protecting from *P. vivax* malaria. Genetic factors such as sickle cell trait, thalassaemia, glucose 6 phosphate dehydrogenase deficiency and ovalocytosis affect parasite survival and provide resistance to the host impeding the intracellular development of the parasite in the erythrocyte.

Non-specific phagocytosis and clearance of parasitized cells from the circulation by the action of the reticulo endothelial system in the liver and spleen also contributes to natural resistance (83).

### **Acquired immunity**

The severity and incidence of malarial illness decreases with increasing age. Antibodies transferred from immune mothers to infants provide protection from malaria during the first months of life. There are multiple experimental evidences for species and stage specific immunity in malaria showing that acquired immunity is of major importance in the protection against malaria. (91,92).

### **Humoral immunity**

Immunoglobulin levels in residents of highly endemic malarious areas are strongly elevated and the level of total anti-malarial antibodies increases with age. In addition, passive transfer of immune IgG has been shown to confer some protection. However, only few human studies found correlation between anti-malarial antibody levels and protective immunity, indicating that most of the anti-parasite antibodies have no protective effect (93). Protective antibody responses to the asexual blood stage of the parasites must be strain specific directed against variant antigenic determinants exposed on the surface of free merozoites or on the infected erythrocytes. It has been shown that children in malaria endemic areas largely do not have antibodies specific for the strains that are responsible for the acute malaria attacks (94,95). Antibodies may specifically bind to antigens on the merozoite surface thereby blocking invasion or to antigen on the surface of infected erythrocyte leading to destruction or elimination of parasitized cells by phagocytosis (96,97). Antibodies may also prevent cytoadherence of infected RBC to endothelial surfaces and prevent rosetting of infected RBC to non-infected RBC thereby preventing severe illness. Recently, plasma antibodies from malaria exposed multiparous women were shown to interfere with binding of *P. falciparum* to chondroitin sulfate (CSA) *in vitro*. Thus, acquisition of antibodies interfering with CSA specific parasite sequestration was shown to be critical element in resistance to pregnancy associated malaria (98,99). However for efficient production of protective anti-malarial antibodies an intact and functioning T cell system is required. (92,100-103).

Recently, elevated levels of total IgE as well as IgE anti-malarial antibodies have been reported in malaria patients (104). A possible pathogenic role of IgE in malaria was suggested by the finding of significantly higher levels in patients with cerebral malaria than in those



with uncomplicated *P. falciparum* infection. Furthermore, IgE elevation in severe malaria was parallel to an elevation of TNF $\alpha$  (104).

### **Cellular immunity**

Although antibodies clearly play a role in the control of the asexual blood stage, acquired resistance has been demonstrated in B cell deficient and depleted mice infected with certain Plasmodia species. Indeed CD4<sup>+</sup> deletion prevents clearance of infection and blocks protection against challenge. Both CD4<sup>+</sup> cells and a T helper 1 (Th1) CD4<sup>+</sup> clone adoptively transfer immunity in different murine malaria models (92). Cytotoxic CD8<sup>+</sup> T cells appear mainly to protect against the pre-erythrocytic stage directed against infected hepatocytes (105). A protective role for  $\gamma\delta$ <sup>+</sup> T cells against blood stage infection has also been proposed (106).

### **T cell control of blood stage infection**

Available evidence points to a major role of CD4<sup>+</sup> T cell in controlling blood stage infection. Immunity to plasmodia, as seen in *P. chabaudi*, is probably a two step process. CD4<sup>+</sup> T cells, which are activated during early acute phase of the infection, appear to be of Th1 type, producing predominantly IFN $\gamma$  and IL2. Later, when infection was controlled, the main antigen specific T cells were Th2 cells for antibody formation (12). Malaria specific Th1 cells are assumed to activate mononuclear phagocytic cells which kill or inhibit the parasite intra or extracellularly by a variety of mechanisms (107). IFN- $\gamma$  and TNF- $\alpha$  are important mediators in this defense. A key role by IFN- $\gamma$  against *P. falciparum* infection is supported by the finding that protected individuals have higher serum levels of IFN- $\gamma$  than non-protected individuals (108). The presence of a spleen and an intact splenic architecture is also essential for immunity (92). However, not only protection but also disease promotion appears to be associated with Th1 like CD4<sup>+</sup> T cell responses. Since RBC is devoid of MHC class I antigen cytotoxic cells are generally not believed to act against the asexual blood stage. However, CD8<sup>+</sup> cells can act as suppressor cells and have been suggested to be involved in regulation of the immunodeficiency observed during acute malaria (109).

Table 4 summarizes previous studies investigating human peripheral blood lymphocyte subpopulations following malaria infections, conducted in attempts to describe disease related lymphocyte profiles. Lymphopenia is a general finding with both reduced number and proportion of circulating T cells. Malaria induced lymphocyte destruction and temporary reallocation of peripheral T cells have been suggested to be the main reasons for this (92,110-115). Variable results have been obtained on subset composition of the reduced T cells.

However, an increase in the level of  $\gamma\delta^+$  T cells appears to be a consistent recent finding, despite reduced T cell population (116-119). These few studies were conducted predominantly in individuals from non-malaria endemic areas and the sensitivity of the immunophenotyping methods used were not satisfactory. In addition, the reasons for these alterations in lymphocyte subset compositions remain uncertain. Therefore, further studies investigating immuno phenotypic changes during malaria infections, especially in people from malaria endemic areas are warranted

**Table 4,** Summary of immunophenotyping studies of peripheral blood lymphocytes in malaria patients.

Observations	Subjects studied	Methods of Phenotyping	Authors
Decreased T cells number and percentages, increased null cells number and percentage increased percentage but not number of B cells	30 children and 3 adults, West Africa with rosetting technique acute Pf		1976, Wytler-DJ (120)
Decreased number and % of T cells, increased % of CD8 <sup>+</sup> T cells	20 adults, with acute Pf, Colombia	Immunofluorescence microscopy <i>et al</i> (121)	1983, Troye-Blomberg, <i>et al</i> (121)
Decreased number of CD4 T cells in less than 8 days Pf illness, and increased number of CD8 cells in more than 8 days Pf illness. Normal ratio in Pv. Increased level of HLA-DR expressing cells in all patients	26 patients with Pf malaria and 11 with acute Pv, 5 with short illness from Sweden rest from Colombia	Immunofluorescence microscopy <i>et al</i> (115)	1984, Troye Blomberg, <i>et al</i> (115)
No alteration in the number of circulating T cells and B cells	Thai patients with Pf malaria including cerebral malaria	Rosetting technique	1985, Brasseur (122)
Increased CD8% in all and decreased CD4 % only in cerebral malaria , Increased NK activity in uncomplicated Pf decreased activity in cerebral malaria	30 children 13 cerebral malaria, 17 uncomplicated Pf, Senegal	Immunofluorescence microscopy	1986, Stach, <i>et al</i> (114)
Normal CD3 cells in Pf reduced in Pv, reduced number and % of CD4 cells in both and decreased number of CD8 cells in Pv. No change in HLA-DR expression.	20 acute Pf, 18 Pv, Venezuela	Immunofluorescence microscopy	1986, Merino, <i>et al</i> . (113)
Lymphopenia, decreased CD8 cells and increased CD4,CD8 ratio	18 patients with acute Pf,	Flow cytometry	1988, Tongtawe <i>et al</i> (123)
Decreased number of T cells with no change on B cells, FC <sup>+</sup> and null cell numbers	59 Thai adults with Pf and Pv	Rosetting technique	1989, Wells <i>et al</i> (124)
Increase in the percentage and number of $\gamma\delta^+$ T cells during acute infection remains apparent for several weeks	12 adults, acute Pf, Thailand	Flow cytometry	1990, HO, <i>et al</i> (125)
Kinetic study, Decrease of T cells with high LFA-1 expression during acute infection, No increase of IL-2 (CD25) and ICAM-1 expression	9 adults, acute Pf, Sudan	Two color flow cytometry	1991, Hviid, <i>et al</i> (126)
Decreased % of CD3 <sup>+</sup> CD56 <sup>+</sup> cells, Decreased % of CD4 cells and increased % of CD8 and CD57 <sup>+</sup> cells in acute Pf.	35 Kenyan children and adults with acute Pf	Immunofluorescence microscopy	1991, Orago, <i>et al</i> (127)
Decreased lymphocyte counts but unchanged subset distribution. A very high percentage of T cells were activated, expressing HLA-DR, no relation to severity	16 acute Pf patients 8 Caucasian with primary exposure 8 Africans with history of exposure/Paris	Two color flow cytometry	1992, Chougnet <i>et al</i> (128)
Kinetic study, increased $\gamma\delta^+$ T cells, expanded subset were $V\gamma 9^+$ V $\delta 2^+$ and $V\gamma 9^+$ V $\delta 1^+$	One Caucasian man	Single color flow cytometry	1992, Chang, <i>et al</i> (118)

**Table 4. Continued**

Observations	Subjects studied	Methods of Phenotyping	Authors
Constant exposure to malaria does not lead to increase in $V\gamma\gamma$ T cells. Relatively high proportion of $V\delta 1$ T cells in residents of malaria endemic area	18 adults and 17 children in holoendemic area of Benin, west Africa, no clinical illness	Two color flow cytometry	1993, Goodier, <i>et al</i> (119)
Increased percentage and number of $\gamma\delta^+$ T cells during clinical paroxysms in none immune patients. Disease severity measured by clinical score specifically gastrointestinal symptoms was positively correlated to percentage of $\gamma\delta^+$ T cells.	18 primarily infected and 19 previously exposed with acute P. vivax infection. Sri-e Lanka	Immunofluorescence microscopy	1994, Perera, <i>et al</i> (129)
Lymphopenia and decreased CD4 and CD8 count but no change in ratio in clinical Pf malaria. Decreased % of CD4 cells in children with asymptomatic parasitemia and inverse relationship between parasitemia and percentage of CD4 T cells	312 children in Guinea-Bissau	Immunocytochemical staining	1994, Lisse, <i>et al</i> (112)
Decreased T cells, increased $\gamma\delta^+$ T cells, $Ti\gamma A^+$ ( $V\gamma 9$ ) cells	29 none immune Caucasian adults, with acute Pf, Paris	Two color flow cytometry	1994, Roussillon, <i>et al</i> (117)
Decreased number and % of T cells with high LFA-1 expression, particularly CD4 cells. Plasma levels of inflammation and activation markers were increased, some increase in CD25 expressing T cells	32 acute Pf. Endemic area residents, Sudan	Two color flow cytometry	1994, Elhassan, <i>et al</i> (130)
Increased percentage and number of $\gamma\delta^+$ T cells, increased HLA-DR expression, proportional expansion of $V\delta 1$ and $V\delta 2$ subsets, not related to severity	22 non immune adult patients Thailand.	Flow cytometry	1994, Ho <i>et al</i> (116)
Decreased number of platelets, WBC and lymphocytes (T, B and NK cells) at early phase. No changes in % of CD4 and CD8. $\gamma\delta^+$ T cells number increased post infection, increased CD69, HLA-DR and CD45RO expression in $\gamma\delta^+$ T cells, not in $\alpha\beta^+$ Cells	Two Australian experimentally infected adults	Two color flow cytometry	1996, Rzepczyk, <i>et al</i> (131)
No increase in $\gamma\delta^+$ T cells in children from endemic area. Europeans had increased levels, absolute number of $\gamma\delta^+$ T cells at day 7 post infection returned to normal at day 28	19 Ghanaian children, 7 cerebral malaria, 5 severe malaria anemia, 7, uncomplicated, 4 Europeans	Two color flow cytometry	1996, Hviid, <i>et al</i> (132)
Increased $\gamma\delta^+$ T cells mostly $V\delta 2V\gamma 9$ but also $V\delta 1$ in some, peak increase at 2 months, role in generating immune response?	5 Pf, 4 Pv. None immune patients, kinetic studies	Single color flow cytometry	1996, Schwartz, <i>et al</i> (133)
Lymphopenia, Increased number and percentage of $\gamma\delta^+$ T cells, mostly $V\delta 1$ cells in Pf and mixed. Reduced NK cells in Pv. Increased expression of HLA-DR, CD25 on $\alpha\beta^+$ T cells and $\gamma\delta^+$ T cells in all patients	39 patients, 7 mixed, 15 Pf and 17 Pv, Ethiopia	Three color flow cytometry	1997, Worku <i>et al</i> (study I)
Lymphopenia more severe with severe disease followed by re-emergence of lymphocytes after treatment. Suggested disease induced T cell re-allocation correlating with disease severity.	22 Gahanian children	Flow cytometry	Hviid et al 1997 (134)
Increased number of CD4 <sup>+</sup> , CD8 <sup>+</sup> and $\gamma\delta^+$ T cells expressing nuclear marker of proliferation ki-67 in peripheral blood. Malaria induced proliferation of lymphocytes	17 pf, 13 pv and 11 controls, Ethiopia	Immunocytochemical staining	Worku et al (Study III)

### ***In vitro* lymphocytes proliferation**

Occurrence of malaria specific  $CD4^+$  T cell subsets (Th1 and Th2) in humans, and involvement of Th2 type helper cells in the induction of some important *P. falciparum* specific antibodies has been demonstrated by determination of the cytokine profile of responding cells (34,135). Human lymphocytes, from donors primed by natural exposure to *P. falciparum* malaria, proliferate and/or release lymphokines after exposure *in vitro* to parasite extracts or to culture supernatants. Some of these antigen-specific responses can be detected years after initial *in vivo* exposure, reflecting a long lasting memory (115,121,136,137). In contrast, T lymphocytes from the blood of patients suffering from an acute attack of *P. falciparum* malaria generally respond poorly to antigen stimulation *in vitro*. Reduced T cell responsiveness *in vitro* in acute malaria may be due to redistribution of the activated T cells from the blood into lymphoid tissues (134,138). In some studies T cells from unexposed donors were also shown to respond to malaria antigen. This activation is assumed to be mitogenic rather than antigenic but some cells might be primed by cross-reacting environmental antigens.

### **$\gamma\delta^+$ T cells**

The levels of T cells expressing the  $\gamma\delta$  TCR are elevated in acute *P. falciparum* malaria. During acute infection  $\gamma\delta^+$  T cells have been shown to account for up to 30-40 % of the peripheral T cells (116-118,125,139). A highly significant increase in both the proportion and absolute number of  $\gamma\delta^+$  T cells has also been observed in non immune *P. vivax* patients during clinical paroxysms (129). Moreover,  $\gamma\delta^+$  T cells are increased in the spleen of humans who died of cerebral malaria and in the spleen of monkeys severely infected with *P. coatneyi* (140,141).

A protective role for  $\gamma\delta^+$  T cells in malaria has been suggested from animal studies. In mice infected with the avirulent strain *Plasmodium chabaudi adami* the degree of parasitemia was related to the number of  $\gamma\delta^+$  T cells in the spleen. Increases in parasitemia were followed by increase in  $\gamma\delta^+$  T cell number followed by subsequent lowering of the parasitemia (142). Exacerbated early parasitemia and chronic parasitemia maintained at significantly higher levels has been seen in  $\gamma\delta^+$  T cells deficient mice (143). Additionally, mice depleted of  $\gamma\delta^+$  T cells could not suppress *P. chabaudi adami* malaria in contrast to control mice that completely resolved the infections. In the same study mice depleted of CD4 cells also failed to resolve the infection (144). This indicates that activation of the  $\gamma\delta^+$  T cell subset requires the  $CD4^+ \alpha\beta^+$  T cells to be able to resolve the malaria infection. Similarly, the proliferation of  $\gamma\delta^+$  T cells in response to malaria antigen has been shown to depend on the presence of  $CD4^+$

$\alpha\beta^+$  T cells (145). Furthermore,  $\gamma\delta^+$  T cells were shown to control liver stage infection in mice (142,146).

A significant growth inhibition of *P. falciparum* *in vitro* by co-culturing infected RBC with human  $\gamma\delta^+$  T cells has also been reported (66). Additionally, *in vitro* stimulation of T cells with crude malarial antigen resulted in a selective proliferation of  $\gamma\delta^+$  T cells. These  $\gamma\delta^+$  T cells produced TNF $\alpha$  and IFN $\gamma$ . The elevated subset of the  $\gamma\delta^+$  T cells expressed the V $\gamma$ 9 V $\delta$ 2 heterodimer. The V $\gamma$ 9V $\delta$ 2 cells were also increased *in vivo* during acute infection (142,147). However, V $\delta$ 1 cell blasts were also seen after *in vitro* stimulation with malaria antigens and an increase in V $\delta$ 1 cells after acute infection has also been shown (116,133) indicating involvement of both subsets in anti-malarial response.

During acute *P. falciparum* infections the  $\gamma\delta^+$  T cell response is associated with products released when the schizonts ruptures (66). The parasite molecules recognized by  $\gamma\delta^+$  T cells and the restriction elements are not yet identified (142). Recently phosphorylated malaria compounds have been claimed to be responsible for the polyclonal activation of V $\gamma$ 9V $\delta$ 2  $\gamma\delta^+$  T cells (148).

In summary the evidences reported suggest that  $\gamma\delta^+$  T cells play a role in protective immunity against malaria. The mechanisms by which  $\gamma\delta^+$  T cells affect resolution of parasitemia in malaria remain to be elucidated. There is no single class of MHC molecule that is used as a restriction element by  $\gamma\delta^+$  T cells and a non-MHC restricted cytotoxic T cell recognition of merozoites or erythrocyte surface expressed antigens may contribute to the anti-parasitic effect mediated by these cells.  $\gamma\delta^+$  T cells in mice and humans are localized within the red pulp of spleen where they may interact with the circulating blood stage of the parasite (92,142). Thus,  $\gamma\delta^+$  T cell mediated killing of blood stage parasite was suggested to take place in the spleen. Lymphokine production by  $\gamma\delta^+$  T cell after antigen stimulation, contributing to activation of macrophages by these cells, may also play a significant role in primary immune response to blood stage parasites (38). Immunoregulatory functions and involvement in induction and maintenance of humoral immune responses by  $\gamma\delta^+$  T cells have been seen in non-malarial systems (55).

The  $\gamma\delta^+$  T cells could also play a role in pathogenesis of malaria. The cytokine profile (IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ ) of human peripheral blood V $\gamma$ 9 $^+$   $\gamma\delta^+$  T cells is compatible with a pro-inflammatory response and macrophage activation (147). These cytokines have been implicated in severe and cerebral malaria in humans and in experimental animal models. Furthermore, human  $\gamma\delta^+$  T cells are stimulated by schizont-infected erythrocytes or supernatants cultured after schizogony *in vitro*, a stage of the parasite associated with

pathogenesis of the disease (147). In addition,  $\gamma\delta^+$  T cell expansion in *P. vivax* coincides with the fever paroxysms (129) and  $\gamma\delta^+$  T cell expansion in the peripheral blood is pronounced in naive individuals undergoing their primary infection with *P. falciparum* or *P. vivax* i.e. those most susceptible to severe parasite induced disease (32,92,117,129).

## MYCOBACTERIA

### *M. tuberculosis* a challenge

Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of tuberculosis (TB) in 1882. *M. tuberculosis* is a major cause of morbidity and mortality worldwide resulting in 3 million deaths and over 8 million new cases of each year (149,150). Estimates are that roughly one third of the world population is infected with the bacillus. Human hosts commonly acquire TB infection by inhaling aerosolized bacteria. To a small extent tuberculosis is caused by *M. bovis*.

The virulence of the mycobacteria and the host response to *M. tuberculosis* play a major role in determining the clinical manifestations and the ultimate outcome of infection. The course of disease may range from self-limiting to fulminate disease with extensive tissue destruction. Only about 10% of infected individuals develop clinical illness. Some will develop active disease when the immune system is impaired such as that caused by infection with HIV, malnutrition or advanced malignancy (151) however, most cases of active TB disease occur in persons with no obvious defect in host immunity (149,150).

Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis* is the only vaccine available for prevention of human disease. The protective efficacy of BCG vaccination has been found to be variable. However, a recent metaanalysis estimated that BCG vaccination reduces the risk of pulmonary TB by 50% and leads to a decrease in TB related deaths by 71% (152,153). Moreover, the mechanism of immune protection by BCG has not been fully elucidated. Therefore, detailed knowledge of human immunity induced by BCG is vital for potential improvements in BCG use as a vaccine and for the development of new TB vaccines.

### Fate of infection with *M. tuberculosis*

Once *M. tuberculosis* has entered the lung they have four potential fates (154),

- 1) The initial host response can be completely effective killing the bacilli, such that the patient has no chance of developing TB at any time in the future,
- 2) The organism can grow and multiply immediately after infection, causing clinical disease known as primary TB,
- 3) Bacilli may become dormant and never cause disease at all resulting in latent infection manifested only as positive tuberculin skin test results or
- 4) The latent organism can eventually begin to grow with resultant clinical disease known as reactivation TB. In individuals with latent infections there is a lifetime 5-10% risk of



developing active disease (154,155). However, in severely immunocompromised hosts such as patients with HIV infection there is a 5-10% risk of developing active TB every year (156).

### **Mycobacteria macrophage interaction**

Mycobacteria are obligate aerobes generally characterized by a long replication time and a cell wall containing abundant lipids and waxes that provide hydrophobic character, acid fast properties and intracellular survival. Mycobacteria are facultative intracellular pathogens, which survive and multiply in the cells of monocyte-macrophage lineage. *M. tuberculosis* bacilli are thought to enter the macrophage via specific binding to several distinct cell surface molecules. *M. tuberculosis* can bind directly via complement receptors and the macrophage mannose receptor (MMRc) (157). IFN- $\gamma$  has been shown to down regulate MMRc expression. In contrast IL-4 and PGE<sub>2</sub> are potent enhancers of MMRc expression (158,159). The binding of virulent *M. tuberculosis* to MMRc may be mediated by mycobacterial lipids like ManLAM (160). Surfactant protein receptors (161) and CD14 (162) have also been implicated as alternative means for mycobacteria to enter the macrophages. Heat-labile serum components (complement component C3) and the complement receptors CR1, CR3, and CR4 also play important role in binding of *M. tuberculosis* to macrophage (163,164).

Following attachment and subsequent phagocytosis of *M. tuberculosis* sustained intracellular bacterial growth depends on the ability to avoid destruction by lysosomal enzymes, reactive oxygen and nitrogen intermediates. Viable and virulent mycobacteria have been shown to prevent fusion of mycobacterial phagosomes with lysosomes through retention of the TACO protein (165). *M. tuberculosis* can disrupt the normal functioning phagosome, preventing development into acidic hydrolase rich compartments. It is also assumed that the biochemical composition of mycobacterial phagosomes is altered preventing the delivery of mycobacteria into lysosomal compartments or blocking the association of phagosomes with host molecules that are harmful to the bacilli. Moreover, containment of viable *M. tuberculosis* within these specialized vesicles may reduce the capacity of mycobacterial antigens to be processed and presented in the context of MHC class II proteins (164,166).

### **IMMUNITY TO TUBERCULOSIS**

The importance of cellular immune responses in anti-mycobacterial immunity was demonstrated by adoptive transfer experiments that enabled transfer of both tuberculin hypersensitivity and resistance to tuberculosis (167,168). Studies in the mouse model of

tuberculosis infection have shown that lymphocytes and macrophages, which act in concert, control mycobacterial growth and invasion (169). Recent studies have presented evidence that CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ <sup>+</sup> and double negative CD4<sup>-</sup>CD8<sup>-</sup> T cells all are involved in anti-mycobacterial immunity(4,10).

### **CD4<sup>+</sup> T cells**

CD4<sup>+</sup> T cells play a central role in immune defense against tuberculosis. CD4<sup>+</sup> T cells recognize peptide antigens from mycobacteria degraded in phagolysosomal compartments and complexed with MHC class II molecules (5). Successful containment of primary infections is characterized by a strong cutaneous DTH response mediated by CD4<sup>+</sup> T cells. In addition CD4<sup>+</sup> T cells proliferate and produce IFN- $\gamma$  after *in vitro* stimulation with mycobacterial antigens (169). Further evidence for the importance of CD4<sup>+</sup> T cells in the control of *M. tuberculosis* infection in humans is obtained from studies of the clinical course of co-infection with HIV. Depletion of CD4<sup>+</sup> T cells during HIV infection increases susceptibility to primary and reactivation tuberculosis. As the CD4 count declines more severe disease including extra-pulmonary tuberculosis and mycobacteremia is more common (151). Local Th1 CD4<sup>+</sup> responses were also suggested to limit mycobacterial infection in tuberculosis pleuritis (170). Studies of mycobacterial specific proliferative responses and cytokine production of peripheral blood mononuclear cells have suggested that patients with tuberculosis mainly exhibit Th2 type of response while PPD positive healthy controls show Th1 responses (171,172). Others have suggested that the decreased proliferative and IFN- $\gamma$  producing responses in patients with tuberculosis are due to secretion by macrophages of cytokines such as TGF- $\beta$  and IL-10 which inhibit CD4<sup>+</sup> T cell function (173,174).

In murine models, CD4<sup>+</sup> T cells have been shown to have cytolytic effector function and to be the major source of IFN- $\gamma$ (196). In addition, adoptive transfer experiments and studies of gene knockout mice have confirmed the importance of CD4<sup>+</sup> T cells in resistance against mycobacteria (175,176). Furthermore production of cytokines such as IFN- $\gamma$  and TNF- $\alpha$  by CD4<sup>+</sup> Th1 cells that are directly involved in macrophage activation were shown to be important in the control of tuberculosis infection (177-179). CD4<sup>+</sup> T cells also enhance effector functions of macrophages and regulate the IL-2 mediated expansion of  $\gamma\delta$ <sup>+</sup> and CD8<sup>+</sup> T cells (180). Both CD4<sup>+</sup> T cell clones and mycobacterial antigen expanded CD4<sup>+</sup> T cells exhibit cytotoxic effector function against mycobacterial antigen pulsed or mycobacteria infected macrophages(181). Others and we have recently demonstrated that human CD4<sup>+</sup> T cells are directly involved in control of intracellular growth of mycobacteria through a cell contact dependent mechanism (182,183).

### CD8<sup>+</sup> T cells

Smith and Dockrell (184) recently review the role of CD8<sup>+</sup> T cells in mycobacterial infection. Mycobacterial antigens specific CD8<sup>+</sup> T cells are restricted by either MHC class I or CD1 molecules. Increased susceptibility to *M. tuberculosis* and BCG was evident in mice deficient of  $\beta$ -2 microglobulin, a component of both MHC class I and non-classical MHC class Ib molecule (185,186). Similarly, increased susceptibility to mycobacterial infections has been seen in mice with deficiency in TAP molecules. TAP molecules transport peptides from the cytosol to endoplasmatic reticulum for loading into MHC class I molecules (187,188). In addition, adoptive transfer of CD8<sup>+</sup> cells to recombinase activation gene deficient mice was shown to provide protection against BCG (176). Vaccination of mice with recombinant vaccinia virus or DNA plasmids expressing mycobacterial antigens were also shown to induce antigen specific CD8<sup>+</sup> CTL that conferred protection against subsequent challenge with *M. tuberculosis* (184). These findings confirm that CD8<sup>+</sup> T cells are required for control of tuberculosis. The mechanism mycobacterial antigens access the MHC class I pathway of antigen presentation is unclear.

In humans cytotoxic effector and IFN- $\gamma$  producing CD8<sup>+</sup> T cells were identified in the alveolar space of healthy tuberculin skin test positive persons (189). Mycobacterial antigen expanded CD8<sup>+</sup> T cells were shown to exhibit cytotoxic effector functions *in vitro* against mycobacteria infected or mycobacterial antigen pulsed targets (190-192).

### $\gamma\delta$ <sup>+</sup> T cells

A substantial amount of human and animal studies suggests that  $\gamma\delta$ <sup>+</sup> T cells play a significant role in host response to tuberculosis (193). Non-conventional MHC molecules like CD1 have been shown to present mycobacterial antigens to double negative  $\alpha\beta$ <sup>+</sup> T cells and  $\gamma\delta$ <sup>+</sup> T cells (52). In addition MHC-independent recognition of *M. tuberculosis* antigens has been shown to be characteristic of  $\gamma\delta$  T cells reacting to mycobacteria (194).

Expansion of  $\gamma\delta$ <sup>+</sup> T cells in mice exposed to mycobacterial antigens or live bacteria has been reported. The role of  $\gamma\delta$ <sup>+</sup> T cells in granuloma formation to *M. tuberculosis* was also demonstrated by studies of mice with combined immunodeficiencies. In these studies mice with sever combined immunodeficiency did not form granulomas and rapidly died after BCG infection. However, mice engrafted with co-isogenic lymphnode cells depleted of CD4<sup>+</sup> and CD8<sup>+</sup> T cells survived fatal BCG infection indicating the remaining  $\gamma\delta$ <sup>+</sup> T cells provided protection (195,196).

Mycobacterial antigens and live mycobacteria induce *in vitro* expansion of human  $\gamma\delta^+$  T cells especially the V $\gamma$ 9/V $\delta$ 2<sup>+</sup> TCR subset (4,37,42,194,197,198). Increased  $\gamma\delta^+$  T cell responses have been detected in healthy PPD positive and BCG vaccinated individuals (59,199,200). These cells are cytotoxic for monocytes pulsed with mycobacterial antigens and secrete cytokines that may be involved in granuloma formation (179,201). The activation of  $\gamma\delta$  T cells by mycobacterial antigens is dependent on antigen presenting cells. Alveolar macrophages can serve as efficient antigen presenting cells for  $\gamma\delta$  T cells suggesting that  $\gamma\delta$  T cells can be directly activated in the lung (194,202,203).

A number of protein and non-protein antigens that stimulate  $\gamma\delta$  T cells have been described. Phosphate containing molecules including the TUBags isolated from *M. tuberculosis*, isopentenyl pyrophosphate and related prenyl phosphates have been shown to stimulate  $\gamma\delta^+$  T cells. The functional role of  $\gamma\delta^+$  T cells in human responses to mycobacteria is uncertain (193). It is generally believed that  $\gamma\delta^+$  T cells are involved in primary immune defense. However, development of mycobacteria specific memory like  $\gamma\delta^+$  T cell immune responses secondary to vaccination with BCG has been reported (59). Recently, Dieli et al have reported that V $\gamma$ 9/V $\delta$ 2<sup>+</sup> T cell lines could inhibit intracellular mycobacteria (204).

### **Effector functions of T cells**

Memory immune T cells produce cytokines that activate macrophages, express cytolytic molecules and induce apoptosis of target cells. IFN- $\gamma$  activated murine macrophages are capable of inhibiting growth of mycobacteria (177,179,205-207). In murine macrophages inhibition of mycobacterial growth is mediated by toxic constituents in the phagolysosome such as reactive oxygen intermediates H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>, (208) and reactive nitrogen intermediates (RNI) such as NO and NO<sub>2</sub><sup>-</sup> (209). Cytokines are powerful modulators of murine macrophage NO synthesis via control over an enzymatic pathway involving inducible nitric oxide synthase (iNOS). TNF- $\alpha$  and IFN- $\gamma$  are potent activators of iNOS, while IL-4, IL-10 and TGF- $\beta$  suppress NO production (173,210,211). Growth inhibition of mycobacteria by cytokine stimulated murine macrophages strongly correlates with the generation of RNI. IFN- $\gamma$  deficient mice failed to produce RNI and therefore are unable to restrict the growth of *M. tuberculosis*, despite being able to develop granulomatous responses (177,178,212,213). However, IFN- $\gamma$  alone has not been found to mediate mycobacterial growth inhibition by human monocyte macrophages (214,215) suggesting intrinsic differences between human and murine cells. Human macrophages may require additional induction signals especially from T cells (183). The possible involvement of TNF- $\alpha$ , GM-CSF, and IL-12 has been suggested

from several studies (216-219). On the other hand the cytokines IL-1 and IL-6 have been reported to increase mycobacterial growth in human monocytes(220).

Cytotoxic T cells may directly kill intracellular mycobacteria by a perforin dependent mechanism resulting in the intracellular delivery of cytolytic T cell granule protein granulysin. Perforin, a granule protein that forms pores in the plasma membrane of target cells, is essential to mediate macrophage lysis and granulysin dependent killing of *M. tuberculosis* (15,221). Granulysin can kill *M. tuberculosis in vitro* (222) and the role of perforin in intracellular mycobacterial growth inhibition was demonstrated in a recent study (204). However, perforin deficient mice were not found to be highly susceptible to *M. tuberculosis* infection (223) indicating existence of other overlapping compensatory mechanisms that protect against tuberculosis. The other known granule independent mechanism of cytotoxicity involves apoptosis mediated by Fas-FasL interaction (221). cytokines, chemokines and interactions with effector cells have been shown to induce monocyte apoptosis affecting mycobacterial survival (224). Fas- FasL mediated lysis of *M. tuberculosis* infected macrophages has been demonstrated (225). Virulent mycobacteria have been shown to have the capacity to evade apoptotic mechanisms promoting their survival and replication (226).

## THE PRESENT INVESTIGATION

### SPECIFIC BACKGROUND

#### Study I.

Several biological and technical factors have been shown to influence results of immunophenotyping. The lymphocyte profiles in healthy control subjects from Ethiopia, Bangladesh and Sweden were investigated. Samples from the three study groups were handled and analyzed similarly using a standardized flow cytometric method. Thus, the possible interference of technical factors was minimized and possible differences in lymphocyte phenotypic profiles attributable to environmental factors and/or genetic background were evaluated

#### Study II.

Phenotypic characterization of lymphocytes has provided clues towards the pathogenesis and immune processes in different diseases. Only few studies that were based on less sensitive and less specific phenotyping methods have previously been performed in patients with malaria infections. In the few recent reports describing an increase in  $\gamma\delta^+$  T cells during acute malaria infection the concomitant changes in the remaining subset of lymphocytes were not studied. In this study three-color flow cytometry was used to evaluate the T cell subset characteristics and the level of activation, with special reference to  $\gamma\delta^+$  T cells, in patients with acute *P. falciparum* and *P. vivax* malaria infections from Ethiopia. A more accurate malaria species diagnosis was obtained by PCR analysis of blood samples

**Study III.** Lymphopenia and T cell subset changes have been reported in acute malaria. These changes were attributed to malaria induced activation and proliferation, lymphocyte redistribution and/or activation induced apoptosis. In this study the proliferation status of lymphocytes in peripheral blood were studied using immunoenzymatic staining for nuclear marker of proliferation Ki-67.

#### Study IV.

$\gamma\delta^+$  T cells have been found to be elevated and activated in acute malaria infection. The role of  $\gamma\delta^+$  T cells in protection and/or pathogenesis of malaria warrant further analysis. The non-MHC restricted cytotoxic  $\gamma\delta^+$  T cells could participate in malaria protection as component of primary immune defense. In this study the capacity of  $\gamma\delta^+$  T cells to inhibit the growth of the asexual blood stage *P. falciparum* parasites *in vitro* was investigated.

**Studies V & VI.** Clinical and *in vitro* experimental studies indicate existence of memory immune responses against mycobacterium infection. However, direct inhibition of mycobacterial growth by human memory immune T cells has not been demonstrated and surrogate markers of protective anti-mycobacterial immunity have not been identified. In addition, the relative contribution of memory T cell subsets and mechanisms of the growth inhibition of mycobacteria have not been studied. In these studies an *in vitro* mycobacterial growth inhibition assay was developed. Using this growth inhibition assay the role of antigen specific expansion, T cell subsets, and possible mechanisms involved in mycobacterial growth inhibition were studied.

## AIMS

- To investigate differences in peripheral blood lymphocyte profiles in different populations.
- To investigate the level, subset distribution and activation status of lymphocytes, with special reference to  $\gamma\delta^+$  T cells, in patients with acute *P. falciparum* and/or *P. vivax* malaria and to study the relationship between clinical and parasitological and immunological parameters.
- To investigate the *in vitro* interaction between  $\gamma\delta^+$  T cells and *P. falciparum* parasites.
- To develop an *in vitro* assay which enables measurement of protective T cell memory immune responses capable of inhibiting growth of mycobacteria.
- To study the role and mechanism of inhibition of mycobacterial growth by mycobacteria specific  $CD4^+$ ,  $CD8^+$  and  $\gamma\delta^+$  T cells.



## RESULTS AND DISCUSSION

### Differences in lymphocyte phenotypic profile in different populations (Study I)

**Differences in CD4<sup>+</sup> and CD8<sup>+</sup> T cells.** The mean percentage of CD4<sup>+</sup> cells was lower and the percentage of CD8<sup>+</sup> cells was higher in Bangladeshi and Ethiopian than in Swedish subjects. The levels for the Bangladeshi and Ethiopian subjects were also different from the previously published levels in North American and European subjects, whereas, the levels in the Swedish subjects were similar to those reported from the Western countries (72,227,228). Also in agreement with our finding was presence of higher levels of CD8<sup>+</sup> T cells and decreased CD4/CD8 ratios reported in Saudi male blood donors compared to Caucasian controls (79). Different in proportions and subset distributions of  $\gamma\delta$  T cells have also been reported between Caucasians and Africans (21)

**Differences in level of cytotoxic T cells.** The percentage of the (suggested) cytotoxic subset of CD8<sup>+</sup> T cells (CD8<sup>+</sup>CD57<sup>+</sup>) was twofold higher in the Ethiopian and Bangladeshi subjects than in the Swedish subjects. The level in Swedish subjects was similar to that reported for Europeans. The CD57<sup>+</sup> T cells have been shown to increase with increasing age (227). Repeated interactions with environmental agents have been proposed to contribute to the increase in this T cell subset. This age related changes could reflect an increase in immunocompetence (78). In environments with high incidences of bacterial and parasitic infections such as Bangladesh (229,230) and Ethiopia (231) our finding of higher levels of CD57<sup>+</sup> cells could therefore reflect an appropriate adaptation of the immune system to these multiple microbes. Previous exposure to different infectious agents and other environmental factors as well as nutritional status have been suggested to modulate the cellular constitution of the immune system as reflected by high level of immune activation in peripheral blood (80).

Altogether, the higher level of CD8<sup>+</sup> T cells and the suggested cytotoxic subset (CD57<sup>+</sup> CD8<sup>+</sup> T cells), the higher  $\gamma\delta$ <sup>+</sup> T cell level and a skewing in the TCR V $\beta$  repertoire usage in subjects from environments with high prevalence of infectious diseases could suggest that increased exposure to infectious agents may modulate the cellular immune responses towards a predominantly cytotoxic profile.

## Immune responses to malaria (Studies II & III)

**The study population (Study II),** Age and sex distributions and clinical and parasitological findings are summarized in Table 5.

**Table 5.** Characteristics of patients and control subjects in study I.

	Double infected	<i>P.</i> <i>falciparum</i>	<i>P. vivax</i>	Controls
Number (n)	7	15	17	16
Male (n)	5	13	13	12
Age ( mean $\pm$ SD )	25.3 $\pm$ 15.8	30.9 $\pm$ 9.8	25.6 $\pm$ 9.8	29.6 $\pm$ 5.2
Previous history of malaria (n)	6	12	13	4
Residence in endemic area (n)	7	9	17	3
Duration of illness (days )	4.2 $\pm$ 1.9	5.5 $\pm$ 3.6	3.6 $\pm$ 1.9	-
Fever (>37.5 <sup>0</sup> C) (n)	4	9	10	-
Splenomegaly (n)	4	7	4	-
Parasite density ( 10 <sup>3</sup> /ul ) <sup>†</sup>	2.73	3.98	1.40	-

<sup>†</sup> Geometric mean of parasite ( 10<sup>3</sup>/ul )

**Species diagnosis by PCR.** By nested PCR assay, 7 mixed infections and one miss diagnosis were detected. The observed lymphocyte phenotypic changes were attributable to the presence of the parasite only and independent of the parasite number. This emphasizes the importance of PCR based parasite species diagnosis when analyzing the immune response in malaria infections. The higher sensitivity and specificity of PCR assay in species diagnosis have also been shown previously (88,89).

**Increased  $\gamma\delta^+$  T cells in *P. falciparum* infection.** The phenotypic profiles in *P. falciparum*, *P. vivax* and double infected patients are summarized in Table 6. .  $\gamma\delta^+$  T cells were increased in the peripheral blood of *P. falciparum* infected patients but not in *P. vivax*. This finding corroborates previously reported data (116-118,125,139). In agreement with others we found no increase in the level of  $\gamma\delta^+$  T cells in *P. vivax* infected patients (129). A strong positive correlation between the percentage of CD4<sup>+</sup> CD8<sup>-</sup> and  $\gamma\delta^+$  T cells was found suggesting that the majority of  $\gamma\delta^+$  T cells were negative for CD4 and CD8. We also find an inverse correlation between the percentage of CD4<sup>+</sup> cells and  $\gamma\delta^+$  T cells. These results might indicate that CD4<sup>+</sup> cells are leaving the circulation while  $\gamma\delta^+$  T cells were expanding. Similar findings have previously been reported (117,232). The significantly elevated number of  $\gamma\delta^+$  T cells in the double infected patients in contrast to those in only Pv infected might indicate that *P.*

*falciparum* infection specifically stimulates the increase in blood  $\gamma\delta^+$  T cells. The increase in  $\gamma\delta^+$  T cells counts suggests either a proliferative response or a selective recruitment of  $\gamma\delta^+$  T cells to the circulation. Both proliferation and mobilization of  $\gamma\delta^+$  T cells from tissues have been shown previously (116,233,234). We have also shown peripheral blood lymphocytes are induced to proliferate during acute malaria (Study III).

**Table 6,** Summary table showing statistically significant differences in cellular phenotypic pattern in malaria infected patients compared to healthy controls.

	Double infected	<i>P. falciparum</i>	<i>P. vivax</i>
WBC	$\leftrightarrow^\dagger$	$\downarrow$	$\leftrightarrow^\dagger$
Lymphocytes	$\leftrightarrow^\dagger$	$\downarrow$	$\downarrow$
Anti.Pf IgG	$\uparrow^*$	$\uparrow^*$	$\uparrow$
T cells (CD3 <sup>+</sup> )	$\leftrightarrow^\dagger$	$\downarrow$	$\leftrightarrow^\dagger$
NK cells (CD56 <sup>+</sup> )	$\downarrow$	$\leftrightarrow^*$	$\downarrow$
Ratio, CD4 <sup>+</sup> ,CD8 <sup>+</sup>	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
CD57 <sup>+</sup> CD8 <sup>+</sup> /CD3 <sup>+</sup>	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
$\gamma\delta^+$ T cells	$\uparrow^*$	$\uparrow$	$\leftrightarrow$
V $\delta$ 1 $\gamma\delta^+$ T cells	$\uparrow$	$\uparrow$	$\leftrightarrow$
Ratio, V $\delta$ 1,V $\gamma$ 9/V $\delta$ 2	$\uparrow$	$\leftrightarrow^\ddagger$	$\leftrightarrow^\ddagger$
V $\gamma$ 8 $\gamma\delta^+$ T cells	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
CD25 $\gamma\delta^+$ T cell	$\uparrow$	$\uparrow$	$\uparrow$
CD25 CD3 <sup>+</sup>	$\uparrow$	$\uparrow$	$\uparrow$
HLADR <sup>+</sup> $\gamma\delta^+$	$\uparrow$	$\uparrow$	$\uparrow$
HLADR <sup>+</sup> CD3 <sup>+</sup>	$\uparrow$	$\uparrow$	$\uparrow$
CD45RO <sup>+</sup> $\gamma\delta^+$	$\leftrightarrow^\dagger$	$\downarrow$	$\leftrightarrow$
CD45RO <sup>+</sup> CD3 <sup>+</sup>	$\leftrightarrow$	$\uparrow$	$\leftrightarrow$

$\leftrightarrow$ , not significantly different,  $\uparrow$ , significantly increased,  $\downarrow$ , significantly decreased compared to controls,  $\dagger$  Decreased but not statistically significant  $\ddagger$  increased but not statistically significant compared to controls. \* Significantly different from patients with *P. vivax* infection

**V $\delta$ 1<sup>+</sup> cells are significantly increased.** The increase in V $\delta$ 1<sup>+</sup> cells was higher than that of V $\gamma$ 9/V $\delta$ 2<sup>+</sup> cells resulting in an increased V $\delta$ 1<sup>+</sup>,V $\gamma$ 9/V $\delta$ 2<sup>+</sup> ratio. Our finding is different from those previously reported (1,116-118). The increase in V $\delta$ 1<sup>+</sup>  $\gamma\delta$ <sup>+</sup> T cells in our study suggests that the V $\gamma$ 9/V $\delta$ 2<sup>+</sup> subset is not the only subset responding to malaria infection. These different findings may depend on host and/or parasite related factors. Most of previous studies involved patients from non malaria endemic areas. Our results may therefore indicate that  $\gamma\delta$  T cell responses in malaria immune individuals may be different than in non-immune individuals as previously suggested (132).

A number of samples were stained for V $\gamma$ 8<sup>+</sup>  $\gamma\delta$ <sup>+</sup> T cells using the Moab B18 kindly provided by K. Söderström (MTC, Karolinska Institute). No difference was found in the level of V $\gamma$ 8<sup>+</sup>  $\gamma\delta$ <sup>+</sup> T cells between patients and controls in contrast to reports in other diseases (50).

**$\gamma\delta$ <sup>+</sup> and non- $\gamma\delta$ <sup>+</sup> T cells are activated *in vivo*.** Expression of the receptor for the T cell growth factor IL-2 is necessary for autocrine and/or paracrine growth of T cells in the early stage of T cell activation. The expression of HLA-DR on T cells is considered as a late activation marker.

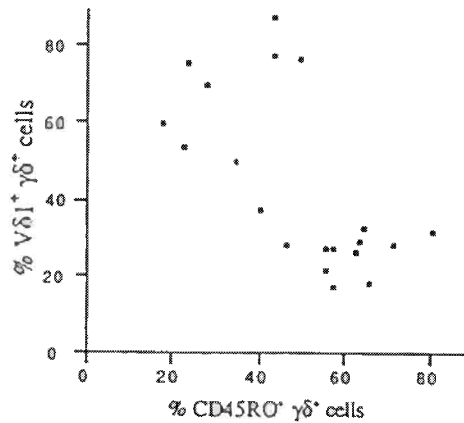
Both  $\gamma\delta$  and non  $\gamma\delta$ <sup>+</sup> T cells were shown to be activated as shown by increased percentage of cells expressing CD25 and HLA-DR. High levels of soluble IL-2R in plasma during acute malaria infection have been reported, but no increase in IL-2R expressing cells has previously been described (126,235,236). The reason for this difference is not clear. Possibly a higher sensitivity of three color flow cytometry and /or analysis of more samples and from a different geographical area may have enabled us to detect the CD25<sup>+</sup> T cells. Activated HLA-DR<sup>+</sup> CD8<sup>+</sup> cells have been described to contain cytotoxic effector cells (69,237). However, their functional contribution in malaria remains to be determined.

A higher fraction of the  $\gamma\delta$ <sup>+</sup> T cells than non  $\gamma\delta$ <sup>+</sup> T cells was activated as shown by a five fold increase in CD25 expressing cells and a more than two fold increase in HLA-DR expressing  $\gamma\delta$ <sup>+</sup> T cells. These findings suggest a more prominent activation of  $\gamma\delta$ <sup>+</sup> T cells than of non- $\gamma\delta$ <sup>+</sup> T cells in malaria. However, it might also indicate that activated non- $\gamma\delta$ <sup>+</sup> T cells are preferentially sequestered out of circulation as previously suggested (126) or that  $\gamma\delta$ <sup>+</sup> T cells are mobilized into the blood.

Our finding that the percentage of CD45RO<sup>+</sup>  $\gamma\delta$ <sup>+</sup> T cells was lower in *Pf* patients and was negatively correlated to percentage of V $\delta$ 1 cells (Figure 2) could also imply that naive V $\delta$ 1 cells were mobilized to the circulation. However, expansion of these cells cannot be excluded since activation of V $\delta$ 1 cells without CD45RO expression has been reported. Moreover,

CD45RO expression has been shown to be a later event in cellular activation (238,239). It has previously been described that the majority of  $V\delta 2^+$  cells expressed  $CD45RO^+$  whereas only a minority of  $V\delta 1$  cells are  $CD45RO^+$  (240,241). The presence of a dominant  $V\delta 1$  subset of  $\gamma\delta^+$  T cells in *Pf* patients might explain the lower percentage of  $CD45RO^+$   $\gamma\delta^+$  T cells in these groups.

**Figure 2,** Negative correlation of the percentage of  $\gamma\delta^+$  T cells expressing  $CD45RO^+$  and percentage of  $V\delta 1^+$  cells in *P. falciparum* (Pf) and double infected (Di) patients ( $R_s = -0.45$ ,  $P = 0.026$ ).



$CD57^+CD8^+$  cell subsets have been reported to contain cytotoxic cells (242). In our study no difference was seen in the percentage of  $CD57^+CD8^+$  T cells between patients and controls, implying that there is no selective recruitment or proliferation of these cells during malaria infections. This is not surprising in view of the fact that malaria infected blood cells do not express the restriction molecules needed for  $CD8^+$  T cells mediated cytotoxicity. Thus, these cells probably are less important for control of the asexual blood stages of the malaria infection.

**Increased *in vivo* lymphocyte proliferation (III).** Subset changes and un-responsiveness of lymphocytes to *in vitro* stimulation have not been fully explained (111). We also found complex pattern of changes in phenotypic profile of lymphocytes during acute malaria (Study II). We therefore sought to study the contribution of lymphocyte proliferation towards these changes. A two-color immunoenzymatic staining method was designed to evaluate the proliferation status of peripheral blood lymphocytes in patients with acute *P. falciparum* and

*P. vivax* diseases. Proliferation status was assessed by expression of proliferation associated nuclear antigen, Ki-67 that is exclusively expressed in proliferating cells (243,244).

Our data show that *P. falciparum* and *P. vivax* patients have higher percentages peripheral blood lymphocytes in state of proliferation (Ki-67<sup>+</sup>). These findings corroborate the result of ours and of previous studies where significant activation of lymphocytes in peripheral blood was reported (Study II)(126,235,236). We can now state that lymphocytes during acute malaria were not only activated but were also induced to proliferate. We found a proportional increase in the percentage of Ki-67 expressing cells between both the CD4<sup>+</sup> and the CD8<sup>+</sup> T cell sub-populations. In few samples, increased number of proliferating  $\gamma\delta$  T cells were also detected. However, no difference in the proportions of T cells subsets within the Ki-67<sup>+</sup> cells was seen between patients and controls (Table 7). This may suggest non-selective, overall indiscriminate stimulation of PBMC during malarial illness. Malaria induced stimulation of cells resulting in chemokine/lymphokine secretions might act as bystander effects on the majority of circulating PBMC as shown previously (245,246).

Interestingly the increase in Ki-67<sup>+</sup> lymphocytes was more prominent in patients with first time malaria infection. These might indicate that increased proliferation was mediated by parasite factors. In non-immune individuals where there is limited control of parasite replication the high parasite burden may induce more immune activation (247). The association of increased Ki-67 expression with splenomegaly might mirror immune response undergoing in the spleen (248).

Increased T cell activation and increased inflammatory cytokine such as TNF- $\alpha$  during acute malaria have been implicated in pathogenesis of malaria (247,249). However, our findings of increased activated cells driven to differentiation and proliferation taken together with findings of immune responses of potential importance in parasite clearance such as T cell activation (107,111), increased Th-1 cytokines, IFN- $\gamma$  and IL-2, production (245,246) and increased  $\gamma\delta$  T cells (Study II) (125,139) which were shown to inhibit growth of *P. falciparum* parasites *in vitro* (Study IV) (66) may suggest that immune activation is primarily essential in host defense against blood stage malaria.

**Table 7.** Proportions of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells within the Ki-67<sup>+</sup> mononuclear cells in peripheral blood<sup>a</sup>

	CD3 <sup>+</sup> Ki-67 <sup>+</sup> / total Ki-67 <sup>+</sup>	CD4 <sup>+</sup> Ki-67 <sup>+</sup> / total Ki-67 <sup>+</sup>	CD8 <sup>+</sup> Ki-67 <sup>+</sup> / total Ki-67 <sup>+</sup>
<i>P. falciparum</i>	68 (46 - 87)	30 (13 - 49)	20 (11 - 26)
<i>P. vivax</i>	64 (44 - 73)	41 (26 - 47)	26 (19 - 35)
Controls	61 (42 - 69)	41 (50 - 73)	34 (19 - 45)

<sup>a</sup> The results represent medians and inter quintile ranges (25%-75%)

**$\gamma\delta^+$  T cells inhibit *P. falciparum* growth *in vitro*.** We have found that freshly isolated  $\gamma\delta^+$  T cells activated with PHA inhibit the growth of *P. falciparum in vitro*. The parasitemia was reduced by 10-79%. Under similar experimental conditions  $\alpha\beta^+$  T cells showed no inhibitory effect. The growth inhibition by  $\gamma\delta^+$  T cells was well correlated with the number of  $\gamma\delta^+$  T cells used in the assay. Similarly, inhibition of the parasite growth by crude *P. falciparum* antigen activated  $\gamma\delta^+$  T cells was seen. Furthermore, both V $\delta$ 1<sup>+</sup> and V $\gamma$ 9V $\delta$ 2<sup>+</sup>  $\gamma\delta^+$  T cell clones were found to inhibit *in vitro* parasite growth but no inhibition was seen by  $\alpha\beta^+$  T cell clone. Similar findings have also been reported using human  $\gamma\delta^+$  T cell clones activated with crude *P. falciparum* antigens (66).

When cultures were followed over time, the levels of parasitemia remained relatively constant during the first 24-38 hr, and the intracellular parasite development from ring to trophozoite and to schizont stages was not affected. Inhibition of the parasite growth was seen during the second replication at 46hr. This indicates that the  $\gamma\delta^+$  T cells hindered the merozoites to reinvade fresh erythrocytes. Hence, the likely target for the inhibitory activity of  $\gamma\delta^+$  T cells was the extracellular merozoites or late infected erythrocyte (schizont).

No growth inhibition was observed with cell free supernatants collected from activated  $\gamma\delta^+$  T cell cultures alone or from co-cultures with infected RBC. This implies that the parasite inhibition is not mediated by toxic products or by soluble factors released by the  $\gamma\delta^+$  T cells. Thus, there is a need for cell-to-cell contact as described previously(66). The cytokine profile

and expression of cytotoxic molecules in these inhibitory and non-inhibitory T cell lines and clones were analyzed. In general similar mRNA levels for IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , IL-5, IL-6, and IL-8 were detected in all activated clones. IL-1, IL-2, IL-4, IL-10 and GM-CSF were variably expressed. Similar to previous report (147) mRNA for the cytolytic molecules Perforin, granzyme A, Fas and Fas ligand that might directly be involved in the killing of extracellular parasites were also detected. These findings indicate that both activated inhibitory and non-inhibitory cells are equipped with the machinery that enables the killing of the parasite. Hence, we can suggest that the difference between this inhibitory  $\gamma\delta^+$  T cells and non-inhibitory  $\alpha\beta^+$  T cells depends not on the level of activation but on the ability to recognize parasite antigens or infected erythrocytes.  $\gamma\delta^+$  T cells have been shown to recognize antigens with out MHC restriction or antigen processing contrary to  $\alpha\beta^+$  T cells (19). Therefore  $\gamma\delta^+$  T cells can recognize and kill extracellular parasites or infected erythrocytes that do not express MHC molecules while the  $\alpha\beta^+$  T cells may require additional restriction molecules. The parasite antigens these  $\gamma\delta^+$  T cells recognize and the *in vivo* relevance of the *in vitro* growth inhibition still remains to be elucidated.

Our findings provide direct evidence that  $\gamma\delta^+$  T cells inhibit growth of *P. falciparum* parasites *in vitro* and, thus, may play an important role in human primary immune responses against malaria. The role of  $\gamma\delta^+$  T cells in primary immune defense against malaria has been demonstrated in animal models (143).

**NK cells are reduced in *P. vivax* and double infected patients.** Our finding of a reduced level of NK cells in *Di* and *Pv* patients but not in *Pf* patients may suggest that this change is associated with *P. vivax* infection and may thus indicate that NK cells play distinct roles in *P. falciparum* and *P. vivax* infections. The development by the parasite derived mechanisms for down regulation of NK cells could contribute to parasite survival. A role for NK cells against erythrocytic stage malaria parasites has been suggested in a number of studies. In acute *P. falciparum* malaria infections both NK activity and IFN- $\gamma$  levels were reported to be raised and NK cells from immune donors were shown to have cytotoxic activity against schizont infected erythrocytes (114,250). A rise in NK cells in peripheral blood in acute uncomplicated malaria but a depression in cerebral malaria has also been seen (127).

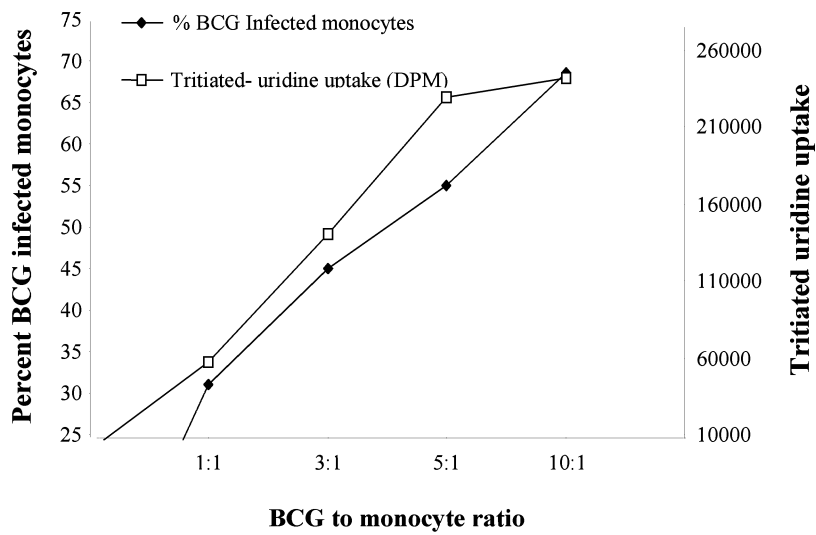


### **In vitro inhibition of mycobacterial growth (Studies V&VI)**

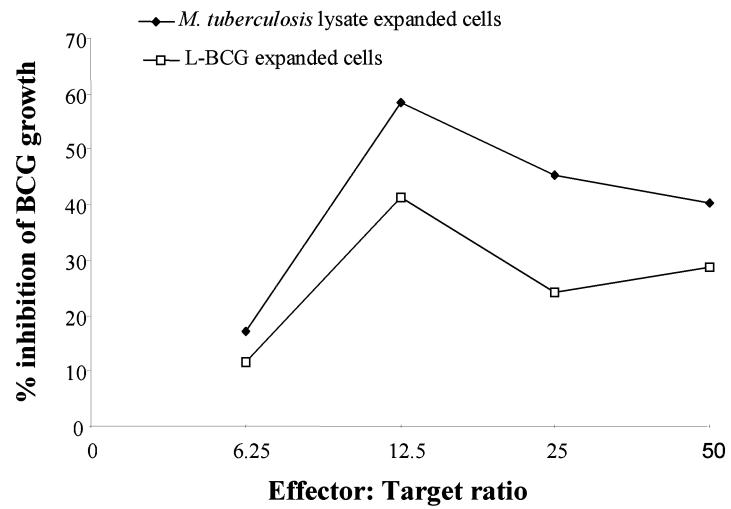
**Development of mycobacterial killing assay.** Mycobacteria replicate within macrophages in the human host. Cell mediated immunity is required for recognition and elimination of these intracellular pathogens. Most studies of mycobacterial immunity have focused on T cell proliferation, cytokine production and cytolytic activity, but the effect of these *in vitro* responses on intracellular viability and growth of mycobacteria have not been adequately investigated.

We hypothesized that activated mycobacterial specific memory T cells from PPD positive or BCG vaccinated individuals are capable of inhibiting mycobacterial replication in monocyte macrophages. We stimulated PBMC with whole lysate of *M. tuberculosis* or live BCG to activate and expand memory cells as effectors. We used BCG infected autologous monocytes as targets. Growth of mycobacteria in experimental and control co-cultures was measured by CFU counting and/or tritiated uridine uptake. Optimal BCG dose for monocyte infection, effector to target cell ratios and duration of co-culture were initially studied.

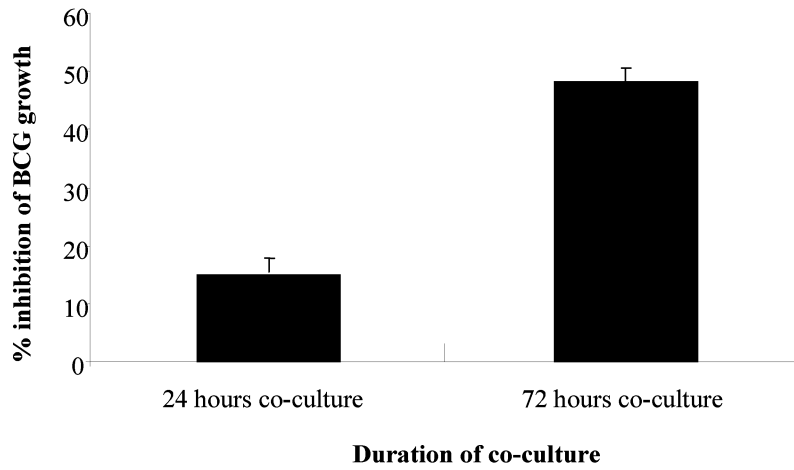
To determine the efficiency of monocyte infection, 6 day old adherent monocytes prepared in slide chambers were infected with increasing numbers of BCG overnight, washed and stained for acid fast bacilli (AFB). At BCG to monocyte infection ratios of  $> 5:1$ , more than 50% of monocytes were infected with BCG (Figure 3). When infected monocytes were cultured for 72 hours optimal intracellular BCG growth in monocytes was also attained with ratios of 3 - 5 BCG per monocyte (Figure 3). To determine optimal Effector cell to Target cell ratios *M. tuberculosis* lysate expanded and live BCG expanded effector cells were co-cultured for 72 hours with BCG infected autologous monocytes at different Effector:Target cell ratios. Optimal inhibition of BCG growth was attained at E:T ratios of 12.5-25:1 (Figure 4). Enhanced inhibition of BCG growth was detected when co-cultures of effector and target cells were extended for 72 hours, as compared to 24 hours, at optimal effector to target cell ratios (Figure 5).



**Figure 3. Optimal infection of monocytes and intracellular BCG growth was achieved with a ratio of 5 BCG for every monocyte.** Adherent monocytes prepared in slide chambers were infected with increasing numbers of BCG overnight, washed and stained for acid fast bacilli (AFB) to determine the percent infected (—◆—). Other cultures of BCG infected monocytes were allowed to grow for 72 hours in the presence of media rested T cells lysed with 0.2% saponin and BCG growth was measured with tritiated uridine uptake (—□—). At BCG to monocyte infection ratios of 5:1 more than 50% of monocytes were infected with BCG and maximal uptake of tritiated-uridine



**Figure 4. Inhibition of BCG growth depends on the ratio of effector to target cells.** *M. tuberculosis* lysate (—◆—) and live BCG (—□—) expanded effector cells were co-cultured with BCG infected monocytes at increasing E:T ratios. Efficient inhibition of BCG growth was attained at 12.5:1 and 25:1 ratios.



**Figure 5. Inhibition of BCG growth was enhanced when the co-culture time of effector and target cells was extended for 72 hours.** *M. tuberculosis* lysate expanded effector cells and media incubated controls were co-cultured with BCG infected monocytes for either 24 or 72 hours. Growth of BCG was measured by tritiated uridine uptake. The percentage inhibition of BCG growth by *M. tuberculosis* lysate expanded cells was calculated in comparison with growth in medium rested cells. Increased inhibition of BCG growth was evident when the co-culture period was extended to 72 hours. Results shown represent mean ±SD from two experiments.

**Activated memory T cells inhibit mycobacterial growth.** Using the standardized assay we demonstrated that mycobacterial antigen specific T cells could inhibit growth of intracellular mycobacteria. The presence of mycobacterial specific cytolytic T cells has been reported before (180,190,221). However, inhibition of intracellular mycobacterial growth by these antigen specific cells has not been shown, rather some studies suggested that cytolytic cells release intracellular mycobacteria leading to reinvasion of fresh monocytes enhancing mycobacterial replication (251,252). These previous studies were based on short term co-cultures of effector cells to mycobacteria infected targets and concentrated on specific T cell clones and lines expanded with a limited set of antigens. Silver et al have shown that non-adherent cells from both PPD positive and PPD negative individuals can inhibit intracellular growth of mycobacteria (182). However, it is unclear if the responses being measured in this assay represent memory immunity. In our assay media rested control cells as well as T cells activated by the non-mycobacterial antigen tetanus toxoid did not inhibit mycobacterial growth confirming that only mycobacterial specific activated T cells can inhibit intracellular BCG growth. Furthermore, we studied the BCG growth inhibiting ability of PBMC obtained from adults before vaccination and 2 and 6 months after BCG vaccination. We found a significant increase in intracellular BCG growth inhibiting capacity by mycobacterial specific T cells post BCG vaccination indicating that our assay measures protective memory immune responses.

**Mycobacterial growth inhibition is intracellular.** First no significant BCG was detected in the supernatants harvested from the co-cultures. Second, when we supplemented co-cultures with fresh monocytes no change was seen in BCG recovered from co-cultures with mycobacterial antigen expanded effectors. However, increased levels of BCG were recovered from co-cultures in monocytes alone or with media rested control cells. These results suggest that BCG growing in monocytes alone or in the presence of media rested cells could infect the freshly added monocytes enhancing overall replication, but BCG in co-cultures with mycobacteria specific effectors was either impaired or killed in the primary monocytes and no BCG was available to invade the freshly added monocytes. All together our results indicate growth inhibition occurred intracellularly.

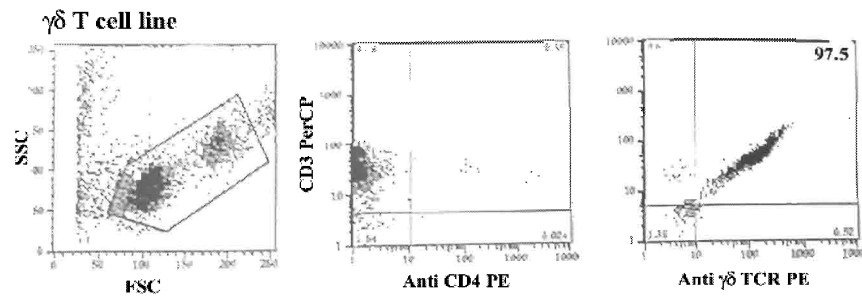
**$\gamma\delta^+$  T cells inhibit intracellular mycobacterial growth.** Development of memory like responses by  $\gamma\delta^+$  T cells after BCG vaccination or mycobacterial exposure has been previously reported (59, 199). However, the functional significance of this immune response has not been defined. We found significant expansion of  $\gamma\delta^+$  T cells when PBMC from PPD positive donors were expanded with *M. tuberculosis* lysate, live BCG or IPP + IL-2 as seen previously (59,193). We found significant inhibition of intracellular BCG growth by effector cells expanded with mycobacterial antigens but not with effector cells expanded with IPP +

IL-2 or TT. Considering that  $\gamma\delta$  T cells were a markedly expanded T cell subset in the mycobacterial antigen expanded effector cell population we predicted the involvement of these  $\gamma\delta^+$  T cells in intracellular growth inhibition. In addition, when mycobacterial antigen expanded effector cells were co-cultured either with autologous or allogeneic infected monocytes comparable levels of intracellular growth inhibition were seen. This evidence suggested to us that non-classical MHC restricted effector cells such as  $\gamma\delta^+$  T cells were likely to play a role in inhibition of mycobacterial growth. Mycobacterial antigen reactive  $\gamma\delta^+$  T cells restricted by non-MHC molecules have been described (52,194).

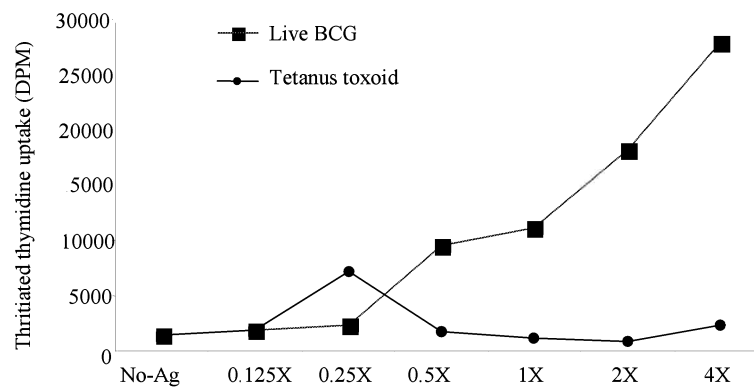
For direct confirmation of these predictions we used  $\gamma\delta^+$  T cells enriched from mycobacterial antigen expanded or TT expanded PBMC for effector cells in the *in vitro* mycobacterial killing assay. We found that  $\gamma\delta^+$  T cells from mycobacterial antigen expanded PBMC but not from TT expanded PBMC were able to inhibit intracellular growth of BCG. Enriched mycobacterial specific  $CD4^+$  and  $CD8^+$  T cells also inhibited BCG growth. In summary we present evidence that mycobacterial antigen specific  $\gamma\delta^+$  T cells can inhibit intracellular growth of mycobacteria. Therefore,  $\gamma\delta^+$  T cells could be important for protective immunity against tuberculosis. Similar to our findings Dieli *et al* have reported that  $V\gamma9/V\delta2^+$  T cell lines could inhibit intracellular mycobacteria.

To further study the  $\gamma\delta^+$  T cells we generated BCG specific  $\gamma\delta^+$  T cell lines from PPD positive individuals using a protocol described by Balaji and Boom (203). These cell line constituted >98%  $\gamma\delta^+$  T cell and are specific to live BCG (Figure 6.). However, the specific antigenic determinants, from live BCG, inducing these T cell lines are not yet characterized. To our surprise these  $\gamma\delta^+$  T cell lines did not exhibit intracellular mycobacterial killing. We also found that phosphoantigen (IPP + IL-2) expanded  $\gamma\delta^+$  T cells did not inhibit mycobacterial growth. The fact that phosphoantigen (IPP + IL-2) expanded  $\gamma\delta^+$  T cells and this  $\gamma\delta^+$  T cell lines were not able to inhibit mycobacterial growth might indicate antigen specificity of inhibitory  $\gamma\delta^+$  T cells. Phosphoantigen expanded  $\gamma\delta^+$  T cells may not recognize molecules presented by infected macrophages. It has previously been shown that phosphoantigens are not stably associated with antigen presenting cells (203). Alternatively, it could also be possible that activation with phosphoantigen alone may not sufficiently activate inhibitory functions of  $\gamma\delta^+$  T cells. Phosphoantigens selectively expand  $\gamma\delta$  T cells whereas the *M. tuberculosis* lysate and live BCG also expand both  $CD4^+$  and  $CD8^+$  T cells that may produce co-factors that sufficiently activate  $\gamma\delta$  T cells. The fact that  $CD4^+$  cells may provide help to augment  $\gamma\delta$  T cell responses has been described (56). Therefore, lack of cofactor producing  $CD4^+$  and/or  $CD8^+$  T cells at the induction phase of mycobacteria specific  $\gamma\delta^+$  T cell responses both in the phosphoantigen stimulated  $\gamma\delta^+$  T cells and in pure  $\gamma\delta^+$  T cell lines might explain lack of killing ability.

A.



B.



**Figure 6. Mycobacteria specific  $\gamma\delta^+$  T cell lines were generated from PPD positive individual.** PBMC from a PPD positive individual were initially expanded with live BCG.  $\gamma\delta^+$  T cells were then purified by immunomagnetic negative selection (Miltenyi) and stimulated with live BCG in the presence of irradiated allogeneic PBMC as APC, and IL-2. These  $\gamma\delta^+$  T cell lines were maintained with biweekly addition of BCG, irradiated PBMC and IL-2. Shown in 6.A. are 2 parameter dot plots from flow cytometric studies of the live BCG specific  $\gamma\delta^+$  T cell line. The cell lines were >97% pure  $\gamma\delta^+$  T cells. Shown in 6.B. are the proliferative responses measured by  $^3\text{H}$ -thymidine incorporation for  $10^5$  BCG specific  $\gamma\delta^+$  T cells stimulated for 48 hours with titrated dose of antigens (BCG 1X= 2000CFU/well or Tetanus toxoid 1X = 10LF/ml). The  $\gamma\delta^+$  T cell lines proliferated only when stimulated with BCG. In separate experiments the  $\gamma\delta^+$  T cell lines did not proliferate when stimulated with canarypox infected PBMC as well.

**Effector cell to target cell contact is required.** Contact requirements were studied by culturing target BCG infected monocytes with effector T cells either in direct contact or separated by transwell inserts. Inhibition of BCG growth was seen only when effector cells were in direct contact with targets. In addition, transfer of co-culture supernatants to cultures with BCG infected monocytes did not affect mycobacterial growth. These results prove evidence that effector cells recognize BCG infected target cells using cell surface associated molecules. Our results corroborate findings from previous studies that showed soluble mediators alone do not lead to intracellular killing of mycobacteria (183,214,215,253).

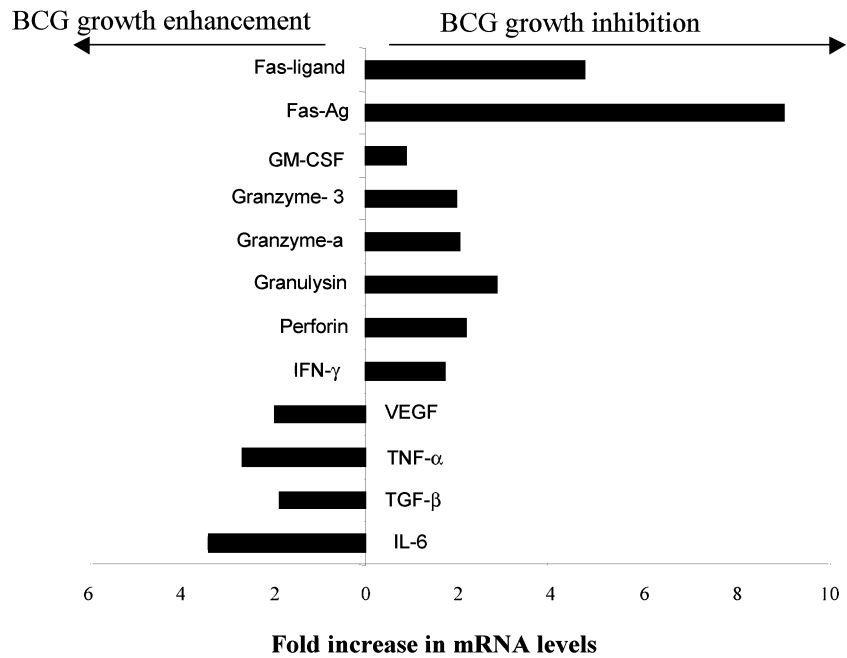
**Cytokines, cytolytic molecules and direct microbicidal agents.** In an attempt to understand the mechanisms of intracellular growth inhibition we studied 1) protein and mRNA levels of cytokine and 2) mRNA levels for molecules associated with cytotoxicity, apoptosis and direct microbicidal effects.

Protein levels of cytokines were determined using ELISA and mRNA levels were determined by RNase protection assays. Comparisons of mRNA levels in BCG growth inhibitory mycobacterial antigen expanded co-cultures and in BCG growth enhancing control T cells are summarized in Figure 7. Higher levels of mRNA for IFN- $\gamma$ , GM-CSF, perforin, granzysin, granzymes, Fas and FasL were detected in the co-cultures with inhibitory mycobacterial specific T cells. In contrast, higher expression of mRNA for TNF- $\alpha$ , TGF- $\beta$ , IL-6 and VEGF were characteristic for co-cultures with BCG growth enhancing media rested cells. No significant expression of any of this mRNA was seen in non-infected or BCG infected monocytes.

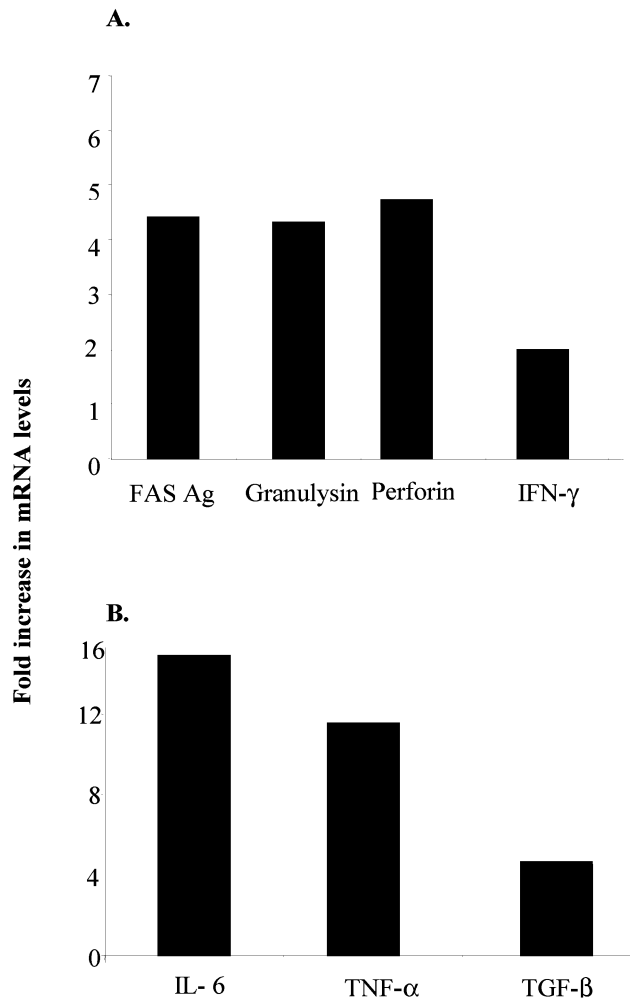
The levels of mRNA expression in BCG expanded effector cells or media rested cells before co-culturing was also studied. mRNA levels were increased more than two fold after 24 hours of co-cultures than before co-cultures. These results indicate differential increases in mRNA levels were induced during co-culture with BCG infected monocyte targets (Figure 8).

Similar to mRNA levels, the cytokine protein levels studied by ELISA showed significantly higher levels of IFN- $\gamma$  and lower levels of TNF- $\alpha$  in the supernatants from inhibitory co-cultures than in supernatants from non-inhibitory co-cultures. mRNA for granzysin and perforin were detected in non-inhibitory TT expanded co-cultures, but these cells did not inhibit intracellular BCG growth, confirming the need for antigen specific recognition





**Figure 7. Differential expression of mRNA for cytokine, cytolytic molecules and microbicidal agents.** RNA was prepared from 24 hour co-cultures of BCG expanded effector cells or media rested control effector cells with BCG infected monocytes. mRNA levels were determined using RNase protection assay. mRNA levels in inhibitory co-cultures were compared to the mRNA levels in BCG growth enhancing co-cultures. Approximately  $\geq 2$  fold higher levels of mRNA for Fas-ag, GM-CSF, granzymes, granulysin, perforin and IFN- $\gamma$  were found in inhibitory co-cultures. Two fold lower levels of mRNA for VEGF, TNF- $\alpha$ , TGF- $\beta$  and IL-6 were detected in the inhibitory co-cultures .



**Figure 8. Increase in mRNA levels are induced by co-cultures of effector cells with BCG infected monocytes.** RNA was harvested from BCG expanded or media rested effector cells before co-culturing and after 24 hours of co-culturing with BCG infected monocytes. In **A.** is shown about 2 fold or more increases in mRNA levels of Fas Ag, Granulysin, Perforin, and IFN- $\gamma$  detected in RNA isolated from co-cultures of BCG expanded effector cells with infected monocytes, than from effector cells alone. In **B.** Are shown increase in mRNA levels of IL-6, TNF- $\alpha$  and TGF- $\beta$  in co-cultures of media rested control cells with infected monocytes than from control cells alone.

Previous studies have suggested associations between the expression of IFN- $\gamma$ , perforin, granulysin, granzymes and Fas/Fas-L effector molecules and control of mycobacterial infections (15,177,204,212,221,222,224). Experimental and clinical evidence suggesting TNF- $\alpha$ , TGF- $\beta$  and IL-6 may augment mycobacterial growth and may be central to the immunopathogenesis of tuberculosis (173,174,220). Based on our results we hypothesize that infected macrophages are recognized by activated mycobacterial specific T cells and intracellular mycobacterial growth is inhibited through mechanisms involving cell to cell contact in the presence of high levels of IFN- $\gamma$ , perforin, granzymes and granulysin which may mediate effector functions. High levels of expression of Fas/Fas-L also suggest involvement of apoptotic mechanisms in control of intracellular mycobacterial growth. However, in the absence of activated mycobacterial specific memory cells, as in PPD negative individuals, resting cells may be susceptible to mycobacterial driven immune modulation. Viable and replicating mycobacteria may induce cells to produce mediators such as, TGF- $\beta$ , IL-6 and TNF- $\alpha$  that augment mycobacterial growth and suppress T cell activation and immune responses promoting development of disease.

## 5. CONCLUSIONS

- $\gamma\delta^+$  T cell appear to play an important role against blood stage malaria infection
  - Increase in the level and activation as well as changes in the subset redistribution of  $\gamma\delta^+$  T cell are found in the peripheral blood of patients acutely ill from *P. falciparum* infection.
  - These changes in cellular subset composition are partly due to malaria-induced activation of T cells resulting in T cell proliferation.
  - *In vitro* experiments provide direct evidence for *P. falciparum* growth inhibition capacity of activated  $\gamma\delta^+$  T cells
    - *P. falciparum* antigens activate growth inhibitory  $\gamma\delta^+$  T cells .
    - Inhibitory  $\gamma\delta^+$  T cells express cytolytic and proinflammatory mediators
    - Late schizont or extracellular merozoites of parasite are targets
    - Inhibition requires cell to cell contact
- Mycobacterial antigen specific  $CD4^+$ ,  $CD8^+$  and  $\gamma\delta^+$  T cells appear to play an important role against mycobacterial infection.
  - Mycobacterial antigen specific  $CD4^+$ ,  $CD8^+$  and  $\gamma\delta^+$  T cells are expanded to *in vitro* stimulation with live BCG or whole lysate of *M. tuberculosis*
  - Mycobacterial antigen expanded but not control antigen expanded effector cells inhibit intracellular growth of BCG
    - $CD4^+ \alpha\beta^+$ ,  $CD8^+ \alpha\beta^+$ , and  $\gamma\delta^+$  T cells exhibit mycobacterial growth inhibiting capacity
    - Growth inhibition is up regulated by BCG vaccination
    - Effector cell to target cell contact possibly involving restricted recognition is required to execute inhibitory functions
    - Increased levels of IFN- $\gamma$ , granzymes, perforin, granulysin and Fas/Fas-L expressions are associated with mycobacterial growth inhibition whereas increased levels of TGF- $\beta$ , TNF- $\alpha$ , IL-6 and VEGF are associated with enhancement of mycobacterial growth
- Differences in peripheral blood lymphocyte profiles between populations groups were observed, that probably reflect differences in past exposure to infectious agents. Hence, environmental and/or genetic background is important bias factors to be considered in any immunophenotyping studies.

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