Vγ9Vδ2 T cells - response to P. falciparum-derived phosphoantigens and potential for use in colon cancer immunotherapy

CHENXIAO LIU
Vγ9Vδ2 T cell is the dominant circulating γδ T cell subset in humans, can expand massively upon malaria infection and are cytotoxic to cancer cells. Vγ9Vδ2 T cells are stimulated by phosphoantigens, primarily isoprenoid pyrophosphates like isopentenyl pyrophosphate and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). Vγ9Vδ2 T cell from human blood were studied for proliferation, response to blood-stage malaria parasites and during colon cancer progression. Vγ9Vδ2 T cell proliferation was stimulated by media from *P. falciparum*-infected erythrocytes from all asexual blood stages - rings, trophozoites, schizonts and rupturing schizonts as well as sexual stage gametocytes assessed by the protocols we developed to obtain pure cultures of all stages. Further, we demonstrated that the molecules that stimulated the Vγ9Vδ2 T cell proliferation are phosphoantigens that are released from intact infected erythrocytes. This does not require schizont rupture. Interestingly, the parasites consumed all the iron ion of hemoglobins during their development from the ring to the rupturing schizont stage. We found that an *Anopheles gambiae* immune cell line responds to HMBPP by activation of MAPK and PI3K signaling pathways. Moreover, transcription of dual oxidase and nitric oxide synthase was upregulated by addition of HMBPP in the midgut of *Anopheles gambiae* which increases cell tolerance to oxidative stress. A range of small isoprenoid pyrophosphates were found to stimulate proliferation of Vγ9Vδ2 T cells from PBMCs as was the isoprenoid monophosphate DMAP. However other isoprenoid monophosphates and alcohols did not. We found that cryopreserved unexpectedly increase the proliferation ability of HMBPP–stimulated PBMCs. To test the cytotoxicity of Vγ9Vδ2 T cells against adherent colon cancer cell lines, a flow cytometry-based assay was developed. Using the assay we found that proliferated Vγ9Vδ2 T cells are cytotoxic to various cancer cells and that HMBPP increases cytotoxicity towards adherent colon cancer cells. In a clinical study we found that Vγ9Vδ2 T cells could not always be proliferated from colon cancer patients and that the inflammatory homing receptor CXCR3 was expressed at higher levels in colon cancer patients than the control group. Moreover, at cancer stadium 4 a lower frequency of Vγ9Vδ2 T cells was more common than in the other groups.

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Abbreviations

AMP  Antimicrobial peptide
APC  Antigen presenting cell
ATP  Adenosine triphosphate
BTN3A1 (CD277)  Butyrophilin 3A1
CAR  Chimeric antigen receptor
CCR  C-C chemokine receptor
CD277  Cluster of differentiation 277
CD3  Cluster of differentiation 3
CDP-ME  4-diphosphocytidyl-2-C-methylerythritol
CSFE  Carboxyfluorescein succinimidyl ester
CXCR  CXC chemokine receptor
DMAPP  Dimethylallyl pyrophosphate
DMSO  Dimethyl sulfoxide
DOXP  1-deoxy-D-xylulose 5-phosphate
DUOX  Dual oxidase
EC50  Half maximal effective concentration
EtOH  Ethanol
FACS  Fluorescence-activated cell sorting
FMO  Fluorescence minus one
FOXO  Forkhead box class O
FPP  Farnesyl pyrophosphate
GPP  Geranyl pyrophosphate
HMBPP  (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HMG-CoA  3-hydroxy-3-methylglutaryl-CoA
IFN-γ  Interferon-γ
IL  Interleukin
IPP  Isopentenyl pyrophosphate
JNK  c-Jun N-terminal kinase
MAPK/ERK  Mitogen-activated protein kinases/extracellular signal-regulated kinases
MEcPP  2-C-methyl-D-erythritol 2,4-cyclopyrophosphate
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>MEP</td>
<td>2-C-methylerythritol 4-phosphate</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NBP</td>
<td>Nitrogen-bisphosphate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>P. falciparum</td>
<td><em>Plasmodium falciparum</em></td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;cell</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;EM&lt;/sub&gt;cell</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;EMRA&lt;/sub&gt;cell</td>
<td>Effector memory RA T cell (terminally differentiated cells)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>T&lt;sub&gt;N&lt;/sub&gt;cell</td>
<td>Naïve T cell</td>
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<tr>
<td>TNM</td>
<td>Tumor, the number of regional lymph nodes and distant metastasis</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor-related apoptosis-inducing ligand</td>
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Introduction

T lymphocytes

αβ T cells, a type of lymphocyte of the adaptive immune system, are the majority of T cells in humans. αβ T cells are classified to CD4+ T helper cell and CD8+ cytotoxic T cells. αβ T cells are activated by specific short peptides presented by MHC (major histocompatibility complex) of antigen presenting cells. MHC I can mediate a cytotoxic response of CD8+ T cells and MHC II presentation stimulates CD4+ T cells [1]. CD28 signalling provides the co-stimulation for the activation. The other subset of T lymphocytes in humans are γδ T cells that have features of both adaptive immune response and innate immune response [2]. MHCs are not antigen presenting molecules to γδ T cells. Two major groups of γδ T cells are Vδ1+ T cells and Vδ2+ T cells [3]. Vγ9Vδ2 T cells (it was once named Vγ2Vδ2 T cells) is the dominant population in circulating γδ T cells in humans [4]. The range is 3 to 5 % in percentage of Vγ9Vδ2 T cells in circulating γδ T cells [5, 6]. Circulating γδ T cells and/or Vγ9Vδ2 T cells respond to various microbial infections of bacteria and protozoal parasite including *Plasmodium falciparum* (*P. falciparum*) infection [7]. γδ T cells can reach 30-40% of T lymphocytes in peripheral blood during acute *P. falciparum* infection [8]. The subset is found in higher primates exclusively but the Vγ9 gene and/or Vδ2 genes exist in various Placentalia [9].

T lymphocyte differentiation

αβ T lymphocyte differentiation in blood

αβ T cells differentiate through naive T cells (T\textsubscript{N}), central memory T cells (T\textsubscript{CM}), effector memory T cells (T\textsubscript{EM}) to terminally differentiated T cells (T\textsubscript{EMRA}) in human peripheral blood. T\textsubscript{N} cells have not encountered the cognate antigen. After stimulation of antigen, T\textsubscript{N} cells differentiate to T\textsubscript{CM} cells that respond to cognate antigens faster and stronger than T\textsubscript{N} cells [10, 11]. T\textsubscript{CM} cells differentiate into T\textsubscript{EM} cells that produce much IFN-γ and CD8+ T\textsubscript{EM} cells produce perforin with stimulation of antigens [11]. The CD8+ T\textsubscript{EM} can differentiate into terminally differentiated T cells also known as effector
memory RA (CD45RA) T cells (T_{EMRA}). The T_{EMRA} cells produce most perforin and granzyme by stimulation of antigens [11]. CD27, a member of tumor necrosis factor receptor superfamily, is expressed much in the surface of T_N cells and T_{CM} cells. CD45 is a protein tyrosine phosphatase, receptor type C, that is expressed in all leucocytes and plays an important role in controlling T cell receptor signalling [12]. CD45RA, an isoform of CD45, is expressed on T_N and T_{EMRA}.

Chemokines directs the cells move to the necessary site. For instance, they attract memory T cells to inflammatory site. C-C chemokine receptor type 7 (CCR7), a homing receptors that induces the cell to migrate to secondary lymph nodes, is highly expressed in T_N cells and T_{CM} cells [13]. When the naive CD8+ T cells develop to memory T cells, the CCR7 that is expressed on T cells is down-regulated but the inflammatory chemokine receptors, CCR5, CXCR3 (CXC chemokine receptor 3) and CXCR6 are up-regulated [14].

**Vγ9Vδ2 T lymphocyte differentiation**

The classification for differentiation of αβ T cells can also be used for Vγ9Vδ2 T cells. The Vδ2 T_N phenotype cells start to proliferate at low concentration of IPP while the T_{CM} phenotype cells need higher concentration for proliferation [15] Much IFN-γ was found in Vδ2 T_{EM} phenotype cells and much perforin was found in Vδ2 T_{EMRA} phenotype cells collected from inflammatory sites [15]. The pattern of expression of chemokine receptors (CCR2, 5, 6, 7 and CXCR3) in the 4 phenotypes is similar to it of αβ T_N, T_{CM}, T_{EM}, T_{EMRA} in lymph nodes and inflammatory site [15].

**Antigens for Vγ9Vδ2 T cells**

Vγ9Vδ2 T cells respond to a group of non-peptidic antigens - phosphoantigens that are organic phosphates. Two typical phosphoantigens are isopentenyl pyrophosphate (IPP) that is an intermediate in both the mevalonate and the non-mevalonate pathway and (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) that is an intermediate in the non-mevalonate pathway for isoprenoid synthesis (Figure 1). IPP and HMBPP are five-carbon isoprene unit providers in isoprenoid synthesis. The mevalonate pathway exists in most eukaryotes, some bacteria and archaea [16-18], the non-mevalonate pathway, an alternative pathway for isoprenoid synthesis, is found in many bacteria, apicomplexan protozoa, chloroplasts (of plants) and photosynthetic eukaryotes [18] (Figure 2).
Figure 1. The structures of IPP and HMBPP (Created with ACD/3D Viewer software)

Figure 2. Isoprenoid synthesis. The mevalonate pathway (right with purple arrows) is a metabolic pathway in humans and provides precursors for isoprenoid synthesis. The pathway begins from acetyl-CoA. Through acetocacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), the HMG-CoA reductase catalyzes the HMG-CoA to yield mevalonate. The reaction is the rate-limiting step of the pathway. Mevalonate is converted to 5-phosphomevalonate by catalysis of mevalonate kinase that is the second essential enzyme in the pathway. After phosphorylation of mevalonate, the 5-carbon IPP is yielded by decarboxylation of mevalonate-5-phosphate. IPP is converted to 5-carbon dimethylallyl pyrophosphate (DMAPP). 1 IPP and 1 DMAPP are synthesized to 10-carbon geranyl pyrophosphate (GPP) and 1 GPP and 1 IPP are synthesized to 15-carbon farnesyl pyrophosphate (FPP) [19]. The non-mevalonate pathway (left with green arrows) begins from pyruvate and glyceraldehyde-3-phosphate to 1-deoxy-D-xylulose 5-phosphate (DOXP). In the second step, DOXP is
converted to 2-C-methylerythritol 4-phosphate (MEP). This reaction is the committed step [20] so the non-mevalonate pathway is known as MEP pathway. MEP is converted to 4-diphosphocytidyl-2-C-methylerythritol (CDP-ME). Through 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-MEP) and 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate (MeEP), HMBPP is synthesized. HMBPP is reduced to IPP or DMAPP. (Adapted from author’s licentiate thesis).

Artificially synthesized bisphosphonates and bacteria-produced 2 to 5-carbon alkyl chain alkylamines with a single primary amine induce accumulation of intracellular IPP by inhibition of farnesy pyrophosphate synthase in the mevalonate pathway of the humen cell cytoplasm. The accumulated IPP and other pyrophosphates activate Vγ9Vδ2 T cells [21-23] (Figure 1).

Antigen presentation to Vγ9Vδ2 T cells
A lot is unknown about the activation of Vγ9Vδ2 T cells. In 1995, a study showed the cell contact is necessary for phosphoantigen-media activation [24]. However, traditional antigen presenting molecules, MHCs, are not required for TCR signalling [17, 24]. In 2012, it was reported that the transmembrane protein Butyrophilin 3A (BTN 3A) was involved in the activation of Vγ9Vδ2 T cells [25]. Later, it was shown that BTN3A1 acts as the antigen presenting molecule but the extracellular portion of it binds to phosphoantigens with low affinity [26]. The intracellular B30.2 domain high-affinity binds to phosphoantigens directly and induce activation of Vγ9Vδ2 T cells [27]. By nuclear magnetic resonance (NMR) and X-ray crystallography, it has been shown phosphoantigens bind to theB30.2 domain selectively. Some other negatively charged molecules also can bind to the domain but only phosphoantigens induce the conformation of B30.2 domain [28]. It is believed that some proteins contributing to the BTN3A1 signalling have not yet been discovered [29, 30]. In summary, more research is needed for the role of BTN 3A1 in antigen presentation.

Moreover, it has been reported accumulated intracellular IPP is exported to out of cells by the ATP-binding cassette transporter in dendritic cells [31]. It has been reported HMBPP can be uptake into cells by some energy-consuming way [32].

The role of interleukins in activation and differentiation and of Vγ9Vδ2 T cells
Interleukins are a group of molecules acting as signals for communication among populations of leukocytes [33]. Interleukins (ILs) induce proliferation and differentiation of Vγ9Vδ2 T cells in the presence of phosphoantigens. IL-2 induces proliferation of Vγ9Vδ2 T cells. IL-2 is considered a T cell growth factor [34] but has been found IL-2 suppresses CD4+ and CD8+ T cells by interaction with T regulatory cells [35]. IL-2 can be produced by αβ
T cells, natural killer cells and dendritic cells [35]. Based on our results (Paper IV) and other studies, Vγ9Vδ2 T cells develop to effector memory T cells with stimulation of phosphoantigens and IL-2 but cannot develop to terminally differentiated cells [3, 15].

IL-15, a T cell growth factor with similar structure to IL-2, is another cytokine for Vγ9Vδ2 T cells [36]. The IL-2 receptor and IL-15 receptor share 2 subunits of 3, as a result, they have several similar functions in stimulation of cells but also play some distinct roles in the adaptive immunity response [37]. One of the two subunits, the common cytokine receptor γ-chain, is shared by IL-2, IL-4, IL-7, IL-15 and IL-21 receptors [38]. It has been reported that IL-15 helped Vγ9Vδ2 T EM cells to differentiate to T EMRA [39]. IL-12, a proinflammatory cytokine strengthens the Vγ9Vδ2 T cells activation by phosphoantigens and IL-2 or IL-15. IL-21, expressed in T helper cells and natural killer T cells mainly, is a cytokine for polarization of Vγ9Vδ2 T cells to central memory follicular B helper T (T FH) like cells in the presence of phosphoantigens [40, 41]. IL-4 polarizes Vγ9Vδ2 T cells to T helper type 2 like cells with the help of anti-IL-12 antibody and IL-2 [42]. Vγ9Vδ2 T cells can be polarized to terminally differentiated T helper type 17 like cells by addition of IL-1, IL-6, IL-23 and transforming growth factor beta (TGF-β) [43] and T regulatory like cells by addition of TGF-β and IL-15 [44].

The mosquito as a host for malaria parasites

The Anopheles gambiae is a crucial host of P. falciparum. The parasites develop and fertilize in the mosquitoes. Briefly, the mosquito takes gametocytes into the midgut by blood meal. In the midgut lumen, gametocytes develop to gametes. Gametes fertilize and develop to zygotes. Further, the zygotes transform to ookinetes and the ookinetes invade and pass through the epithelium of the midgut. Ookinetes transform to oocysts once the parasite reach the basement membrane of the epithelium. The oocysts begin to produce sporozoites and release sporozoites to the hemolymph. The sporozoites migrate to the lumen of salivary gland and reach the humans by mosquitoes biting [45].

The mosquitoes defence against the parasite through both humoral immunity and cell-mediated immunity. The humoral immunity includes complement-like immune response and release of antimicrobial peptides (AMPs). The cell-mediated immunity includes phagocytosis. Nitric oxide and oxidative-mediated damage is also important in the defence [45, 46]. The hemocytes are crucial and play multiple roles such as phagocytosis, nitric oxide and oxidative-mediated damage and release of AMPs in insects immune system [47]. The hemocytes are classified to 3 types of cells - granulocytes, oeno-
cytoids, and prohemocytes according to different functions in mosquitoes [48].

Malaria parasites and Vγ9Vδ2 T cells

Introduction in malaria
Malaria is a deadly disease caused by infection of mosquito-born parasitic protozoans. There were 212 million cases and 429 000 death due to malaria globally in 2015 according to World Malaria Report 2016 [49]. 99% of the deaths were caused by *P. falciparum* so *P. falciparum* is the most dangerous among parasitic protozoans. Though some special drugs such as artemisinin, chloroquine, and primaquine have been found, many drug resistance mutations have also been found [50-54]. Interestingly, the fitness of drug-resistant parasites to the hosts and no drug environment is less than that of wild-type [55].

The symptoms of malaria include fever, trembling, respiratory distress, joint pain and headache [56]. Periodic fever is the typical symptom of malaria and period in infection of *P. falciparum* is approximately 48h. The erythrocytes rupture when merozoites are released in the blood cell cycle (Figure 2). Due to synchronizing development of most parasites, following the burst of many erythrocytes, the fever occurs soon [57].

Vγ9Vδ2 T cells activation by *P. falciparum* infected erythrocytes
HMBPP, IPP and DMAPP are synthesized in the apicoplast of *P. falciparum* by the non-mevalonate pathway and this is the only isoprenoid synthesis in the parasite [58]. The lysate from *P. falciparum* in the asexual blood cycle activated Vγ9Vδ2 T cells or γδ T cells. Phosphoantigens released from lysed parasites were suggested to induce the activation [59-61]. It has been reported that schizont rupture-released molecules stimulate γδ T cells [62] or Vγ9Vδ2 T cells [63, 64]. Later, the molecules are suggested to be phosphoantigens released due to schizont rupture [65]. In addition, the stimulation of γδ T cells by proteins released from gametocytes has been shown [66].

The life-cycle of *P. falciparum* in humans
The life cycle of *P. falciparum* is complicated. Briefly, female *Anopheles gambiae* transmit sporozoites to humans. In the human host, the sporozoites develop to merozoites in liver cells and are released to human peripheral
blood. The merozoites infect erythrocytes and develop. After passing the ring stage and the trophozoite stage, the parasite develops from a merozoite to a schizont. The schizont ruptures and releases 16 merozoites [67]. Soon the infected erythrocyte ruptures and merozoites are released to human blood. The development from one merozoite to sixteen merozoites is called as asexual stage (asexual cycle). It has been reported that the period of the asexual blood cell cycle is approximately 48h in parasites [61] (Figure 3). At the ring stage, some parasites redirect to the sexual stage by development to mature micro (male) and macro (female) gametes through 5 stages. The mosquito takes gametes into the midgut by a blood meal.

Figure 3. Blood cell cycle of *P. falciparum*. The cycle starts from the invasion of a merozoite to an erythrocyte. After the invasion, the parasite is at the merozoite stage. The merozoite becomes flat and develops to the ring stage. With Giemsa staining, the parasite shows a ring form. The parasite at the ring stage develops to the trophozoite stage. The trophozoite becomes bigger and has irregular shape. In the stage, the number of free ribosomes increases and it means the synthesis of proteins increases. The trophozoite develops to the schizont. In the schizont, the division of nucleus is visible and the parasite has more free ribosomes, mitochondria, plastids and lipid vacuoles. 16 merozoites are released after schizont rupture and the merozoites are released from erythrocyte. During the ring stage, some parasites redirect to micro (male) and macro (female) gametocytes. (Adapted from author’s licentiate thesis).
Transmembrane transport in infected erythrocytes

Compared with uninfected erythrocytes, the permeability of plasma membrane in infected erythrocytes increases a lot for many small molecules such as amino acids, purines and some vitamins [68]. The transport of the molecules is due to expression of parasite-associated channels in the plasma membrane of infected erythrocytes [69]. Whether the channels transport pyrophosphates is unknown.

Possibility of transmembrane export of pyrophosphates in *P. falciparum* and infected erythrocytes

IPP, DMAPP and HMBPP are synthesized in apicoplast by the non-mevalonate pathway in the parasites. It is very possible that IPP and/or DMAPP are exported from the apicoplast to the parasite cytoplasm for further isoprenoid synthesis but the mechanism is unknown [70]. It has been reported that intracellular IPP was transported out from nitrogenbisphosphate (NBP)-treated dendritic cells by adenosine triphosphate (ATP)-binding cassette transporter A1 [31]. ATP-binding cassette transporters were found in the plasma membrane of *P. falciparum* [71]. The ATP-binding cassette transporter A1 is expressed in human mature erythrocytes [72]. Therefore, the IPP is likely to be transported from apicoplast to blood plasma. But whether HMBPP and other pyrophosphates can be exported is unknown. (Figure 4)
Figure 4. Release of phosphoantigens from an infected erythrocyte. IPP, DMAPP and HMBPP are synthesized in the apicoplast by the non-mevalonate pathway. Some scientific evidence supports IPP is exported through membranes of apicoplast, parasite and infected erythrocyte. But whether HMBPP or other pyrophosphates can be released from through membranes of apicoplast, parasite and infected erythrocyte is unknown. In this schematic diagram the proportions have been distorted.

Vγ9Vδ2 T cells recognition of cancer cells and cytotoxicity to cancer cells

Activated Vγ9Vδ2 T cells produce and secrete cytokines including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) to kill cancer cells [73]. Moreover, activated Vγ9Vδ2 T cells produce tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [73, 74] and perforin/granzymes [73, 75, 76] for the killing.

The mevalonate pathway is up-regulated in various cancer cells [77, 78] including colon cancer cells [79] by dysregulation of HMG-CoA reductase
and other enzymes of the mevalonate pathway in cancer cells [77, 78]. As a consequence, the level of the pyrophosphates, intermediates of the mevalonate pathway, is high in the cancer cells.

It has been reported the mevalonate pathway-upregulated cell line - Daudi cell stimulated Vγ9Vδ2 T cell receptor (TCR) [80]. In addition, Vγ9Vδ2 T cells did not respond to Daudi cells and a breast cancer cell line by inhibition of the synthesis of intracellular isoprenoid pyrophosphates [81]. TCR-mediated cytotoxicity was also shown in a melanoma cell line [82, 83]. To amplify the signal from the mevalonate pathway, cancer target cells were treated with NBPs for accumulation of intracellular isoprenoid pyrophosphates [73, 84-86]. These studies show Vγ9Vδ2 T cells are cytotoxic to various NBPs-treated cancer cells. In addition, the cytotoxicity to NBP-treated target cells is mediated by the Vγ9Vδ2 TCR [73].

Natural killer group 2D (NKG2D), a transmembrane protein, is found to be expressed in γδ T cells, natural killer cells and activated CD8+αβ T cells in humans [87]. By binding to NKG2D ligands, NKG2D recruited DNA-activating protein of 10 kDa (DAP10) to activate the phosphatidylinositol-3-kinase (PI-3K) pathway and Grb/Vav1 signalling in cells [88]. The activation induces cytotoxicity. NKG2D ligands are rarely expressed in healthy cells but are expressed under cellular stress such as malignant transformation and infection [87]. NKG2D–mediated cytotoxicity in Vγ9Vδ2 T cells has been shown to be either co-stimulated by TCR mediated signalling or kill target cells independent of TCR mediated signalling [73, 82, 89]. Vγ9Vδ2 T cells were shown to be cytotoxic to colon cancer cells [89] and colon cancer stem cells [73].

Immunotherapy using Vγ9Vδ2 T cells to treat cancer focusing on colon cancer

Introduction to colon cancer

Cancer is one of the mortal diseases in worldwide. There were 14.1 million new cases and 8.2 million death related to cancer in 2012 [90]. Colon cancer is also known as colorectal cancer. It is the third most common cancer in both sexes (9.7% of total cases) and about 690 000 patients died worldwide in 2012 according to the world cancer report 2014 [90]. In Europe, colon cancer is the second most common cancer in both genders and there are around 210 000 death in 2012 [90]. Chromosomal instability, CpG (cytosine nucleotide phosphate guanine nucleotide) island methylator phenotype, and microsatellite instability are causes of colon cancer at the level of genes [91].
Colon cancer tumor grows *in situ* (intraepithelial) and invades into mucosal lamina propria, submucosa until the tumor penetrates through entire colon and reaches other organs. According to the invasion of primary tumor (T), the number of regional lymph nodes (N) where the tumor tissue metastasizes and distant metastasis (M) (TNM classification), the colon cancer is classified to 4 stages nowadays [92](Table 1). Tumor deposits is another considered factor in the TNM system. The previous Dukes classification that was created decades ago is still used for description of many colon cancer cell lines that were collected decades ago.

Table 1. The TNM classification [92]

<table>
<thead>
<tr>
<th>Site of invasion of primary tumor (T)</th>
<th>Furthest site or tissue of invasion</th>
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<tbody>
<tr>
<td>TX</td>
<td>Primary tumor can not be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>Primary tumor can not be found</td>
</tr>
<tr>
<td>Tis</td>
<td>Intraepithelial or lamina propria</td>
</tr>
<tr>
<td>T1</td>
<td>Submucosa</td>
</tr>
<tr>
<td>T2</td>
<td>Muscularis propria</td>
</tr>
<tr>
<td>T3</td>
<td>Pericolerectal tissues</td>
</tr>
<tr>
<td>T4a</td>
<td>Visceral peritoneum</td>
</tr>
<tr>
<td>T4b</td>
<td>Nearby organs or other tissues</td>
</tr>
</tbody>
</table>

Regional lymph nodes (N) | The number of metastasis regional lymph nodes
---|---
NX | The number can not be assessed
N0 | Not any
N1a | 1
N1b | 2-3
N1c | 0 but there are tumor deposits in tissues
N2a | 4-7
N2b | Above 7

Distant metastasis (M) | Number of distant metastasized organs/sites
---|---
M0 | 0
M1b | 1
M1 | Above 1

Table 1.2. TNM classifications

<table>
<thead>
<tr>
<th>Stages</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Dukes stages</th>
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<tr>
<td>0</td>
<td>Tis</td>
<td>0</td>
<td>0</td>
<td></td>
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<td>I</td>
<td>1</td>
<td>0</td>
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<td>A</td>
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19
Immunotherapy using Vγ9Vδ2 T cells

Immunotherapy means therapy by activation or inhibition of the immune response to treat disease. Fever-induced tumor regression is not a new finding and this phenomenon has been known for several hundred years at least. In the 19-century, some formal reports showed several cases in this field. In some very early reports, tumors regressed in malaria parasites infected patients [93]. William Coley invented several inactivated bacteria vaccines (Coley’s toxins) to induce fever and inflammation in cancer patients in experimental treatments [94-96]. Killed Serratia marcescens is one of the bacteria [95] and it has the entire set of enzymes of the non-mevalonate pathway [97]. This suggests the vaccine can stimulate Vγ9Vδ2 T cells by bacteria-produced HMBPP. However, the efficacy of the toxins cannot be verified. The idea that using human immunity resists cancers has been developed. CAR (chimeric antigen receptor) T cell treatment to cancer is a breakthrough in immunotherapy. Using gene technology, patient autologous αβ T cells can be engineered to anti-cancer cells by introducing anti-cancer CAR. The engineered T cells are then adoptively transferred to patients for treatment [98]. By now, CAR T cell immunotherapy has been successfully used in some cancers treatment [98, 99]. Other immunotherapies for instance immunotherapy by inhibition of immune checkpoints such as anti-PD1 (programmed death 1) is used for patients [98, 100].

Vγ9Vδ2 T cells kill a wide range of cancer cells. The adoptive human Vγ9Vδ2 T cells suppress tumour growth in murine cancer models with administration of NBPs [84, 101-103]. Many infiltrated Vγ9Vδ2 T cells were
found in tumours of general primary carcinoma [85] and renal cell carcinoma [76]. Zoledronate, one of the NBPs, is medically used for treatment of osteopenia including bone loss and fracture due to metastasis of tumors or/and due to hormonal therapy for breast and prostate cancers [104, 105]. BTN3A1, the potential antigen presentation molecule, was not downregulated in colon cancer tumor tissue while some other BTN families were downregulated [106]. This suggests that colon cancer tumor does not have reduced capacity to activate Vγ9Vδ2 T cells through BTN3A1.

Based on the findings described above, adoptive immunotherapy of Vγ9Vδ2 T cells with the combination of zoledronate is potent for treatment of cancer. The source Vγ9Vδ2 T cells or γδ T cells can be acquired not only from the patients themselves but also from haploidentical donors [107]. It has been shown that the immunotherapy using Vγ9Vδ2 T cells is effective for some cancer patients [108, 109]. Based on a meta study, the therapy is not correlated with severe adverse effect and has favorable effect on patients [110]. An adoptive immunotherapy-Immunicell®, is evaluated at phase II and III currently.

Interestingly, 22 subsets of leukocytes were identified in solid cancer tumors including colon cancer. Higher level of γδ T cells correlated with longer survival time with the most statistic significance among the 22 subsets [111].
Aims and questions

1) To investigate whether *Anopheles gambiae* immune system responds to phosphoantigens likely to be present in *P. falciparum*-infected blood.

2) To investigate whether *P. falciparum*-infected erythrocytes release phosphoantigens that can induce proliferation of $V\gamma 9V\delta 2$ T cells at all blood cycle stages.

3) To develop a protocol for simultaneous collection of pure cultures at all stages of *P. falciparum*-infected erythrocytes.

4) To investigate what small isoprenoid pyrophosphates, isoprenoid monophosphates and isoprenoid alcohols stimulate proliferation of $V\gamma 9V\delta 2$ T cells.

5) To design a cryopreservation procedure for $V\gamma 9V\delta 2$ T cells and tests if cryopreservation influences the proliferation.

6) To design a FACS (fluorescence-activated cell sorting) cytotoxicity assay for adherent target cells.

7) To characterise the frequency, proliferation capacity, differentiation and inflammatory homing potential of $V\gamma 9V\delta 2$ T cells from colon cancer patients compared with age-matched healthy donors.
Methodological considerations

*P. falciparum* culturing and synchronization

The NF54 and FCR3 strains of *P. falciparum* were cultured by infection of type O erythrocytes in growth media with type AB serum. Type O erythrocytes are compatible with serum collected from all types (A, B, AB and O) of blood [112] and type AB serum lack anti-type A or B antibodies so it is compatible with all types erythrocytes. 5% hematocrit was kept in the cultures verified by Giemsa stained smear checking. Cultures were tested for mycoplasma infection regularly.

Plasmion is a kind of gelatin solution. Plasmion is used for synchronization of the parasite-infected erythrocytes asexual stages because infected cells at the late trophozoite sediment slower than infected cells at the ring and young trophozoite stages and uninfected cells. However, Plasmion is only useful in knob+ *P. falciparum* strains [113]. Knob-like protrusions are expressed in plasma membrane of knob+ *P. falciparum* infected erythrocytes and help cells to attach in endothelium of capillaries [114]. Sorbitol ruptures trophozoite and schizont infected erythrocytes by osmotic pressure because sorbitol diffuses into cells via the plasmodial erythrocyte surface anion channel expressed by infected cells [115, 116]. By the combination of Plasmion and sorbitol protocols, highly pure synchronized cultures at the rupturing schizont stage, ring stage, trophozoit stage and schizont stage can be acquired (Paper II Figure 2 and Supplementary Tables 1,2 and 3). The purity was checked by microscopy.

Peripheral blood mononuclear cells preparation

Peripheral blood mononuclear cells (PBMCs) were collected from peripheral blood or buffy coats by Histopaque density centrifugation (Figure 5). PBMCs include lymphocytes, monocytes and macrophages. It has been reported that the neutrophil, a kind of multi-lobed nuclei cell, inhibits activation and proliferation of Vγ9Vδ2 T cells by generation of reactive oxygen species [117] and 3 serine proteases [118]. The 3 serine proteases reduces the expression of CD25 and BTN3a1. Reactive oxygen species downregulate the expression of CD25 and CD69. As a result, removing neutrophils is good for expansion of Vγ9Vδ2 T cells.
Purified $V\gamma 9V\delta 2$ T cells ($V\gamma 9V\delta 2$ T cell lines) that are maintained by phytohaemagglutinin (PHA) and IL-2 [119] can proliferate with stimulation of phosphoantigens and IL-2 but the proliferation increases much in the presence of PBMCs as antigen presenting cells [24]. In addition, a subset of memory $\alpha\beta$ T helper cells has been reported to improve the proliferation of $V\gamma 9V\delta 2$ T cells [120].

Selection of PBMCs samples

Samples of PBMCs were isolated from blood of local healthy donors (under 67 years old except one) or from buffy coats (donors’ age from 18 to 60) from the local blood center. The donors were from both genders. Immunosenescence is found in the $\gamma\delta$ T cells or $V\delta 2$ T cells [6, 121, 122]. The absolute number of $V\delta 2$ T cells and $\alpha\beta$ T cells per unit of blood decreases by age (donor’s ages are from 20) but $V\delta 1$ T cells do not [121, 122]. The expansion of $V\delta 2$ T cells from old people (75-89) and very old people (100-104 years old) is lower than that from young people [121]. Gender may be an impact of frequency of $V\delta 2$ T cells [122, 123]. Interestingly, one paper shows the number of $V\delta 2$ T cells in females is significantly lower than it in male in Japanese donors [122]. However, another one shows the percentage of $V\gamma 9V\delta 2$ T cells in CD3+ cells of female is significantly higher than it in male in American donors [123]. As a result, selection of age and gender-matched samples is crucial for some studies.
Figure 5. PBMCs isolation and expansion of Vγ9Vδ2 T cells. Buffy coats were diluted at the ratio of 1:4 to 1:6 with RPMI-1640. Blood was diluted at the ratio of 1:1. 20-36 mL of diluted buffy coat or blood was added into 50mL Falcon tube under 10-13mL of Histopaque 1077. Samples were centrifuged at 400g, at room temperature for 30 min. A white ring, the PBMCs, was extracted between plasma and Histopaque. HMBPP solution at final concentration of 80 pM was added into a culture container and kept until the EtOH: NH₃ solvent evaporated entirely to exclude the impact of the solvents to PBMCs. The PBMCs were washed in RPMI-1640 and added into culture container. IL-2 was added days 3, 5 and 7 (and day 9 in patient samples). On days 11-12, the cells were harvested or analyzed. (Adapted from author’s licentiate thesis).

Vγ9Vδ2 T cells culture

Phosphoantigens partly activate the MAPK/Erk (mitogen-activated protein kinase/extracellular signal-regulated kinases) and PI-3K signalling pathways of Vγ9Vδ2 T cells [124]. IL-2 was not added to PBMCs until to 3 days of incubation (phosphoantigen is added on day 0) to restrict proliferation of other types of cells. (Figure 5) 25U /mL IL-2 was chosen for proliferation to mimic the IL-2 concentration in PBMCs with stimulation of PHA [125]. Compared with some other protocols where they used 100-1000U/ml IL-2 [126-129], 25U /ml IL-2 is a more physiological concentration.
Cytotoxicity assay

Cytotoxicity assay based on FACS have been developed and improved for many years [130-133]. Compared with other typical methods (Table 2), the advantages of this method are quantitative analysis by counting absolute number of cells with multiple markers labelling in samples.

The methods were designed for suspension target cells but some adapted methods were applied for adherent target cells [134]. For our study on the cytotoxicity of Vγ9Vδ2 T cells, we developed a new assay based on the old methods. 96-well, U-bottom plate was applied for the assay. Briefly, purified γδ T cells were mixed with CSFE-labelled colon cancer cells at different ratios in 96-well plates. After incubation, the mixed cells were transferred to FACS tubes and the dead cells were labelled with 7-AAD. The live and dead cancer cells were counted with a flow cytometer.

Surprisingly, we find even using tissue-culture treated surface polystyrene; colon cells cannot adhere on the surface or adhere poorly during four hours of the incubation (Paper III).

Table 2. Comparison of cytotoxicity assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Brief description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr51 assay</td>
<td>Target cells are labelled with Cr51. The percentage of killed cells is estimated by measurement of radioactivity due to leakage from dead cells.</td>
<td>Suitable for both adherent and suspension cells.</td>
<td>Risky on radioactivity. Cannot count absolute number of killed cells. Cannot label other markers.</td>
</tr>
<tr>
<td>MTT* assay</td>
<td>A colorimetric assay where the purple formazan reduced from MTT catalyzed by NAD (P)H*-.</td>
<td>Suitable for both adherent and suspension cells.</td>
<td>Cannot count absolute number of killed cells Cannot label other markers. Cannot differ-</td>
</tr>
</tbody>
</table>
In our study, we have characterized the frequency, differentiation status and tumour targeting potential of Vγ9Vδ2 T cells in patient samples. To identify the Vγ9Vδ2 T cells from PBMCs and acquire the frequency, anti-CD3, anti-γ9 and anti-δ2 antibodies were applied. To acquire the differentiation status, anti-CD27 and anti-CD45RA were applied. Anti-CXCR3 and anti-CCR5 are used to estimate the tumour targeting potential. The samples were stained with 7-fluorophore conjugated antibodies. Due to spectral overlap, fluorescence compensation was set according to fluorophore single staining controls [136]. Fluorescence minus one (FMO) controls means that the sample is stained with all fluorophores except one. FMO controls are used for determining gates and quadrants because compensation and other factors may cause the scatter of compensated background fluorescence of stained samples spreads wider than the scatter of unstained sample (Figure 4B). It has been reported that there was no distinct border between CD45RA+ and CD45RA- Vγ9Vδ2 T cells [137]. We also find the border between the two populations is not clear as CD3+Gamma9-Delta2- population (Figure 6, A).
Figure 6. Gating for analysis of the PBMCs sample A. Comparison between CD45RA-population and CD45RA+population in Vγ9Vδ2 T cells and non-Vγ9Vδ2 T cells. PBMCs were labelled with antibodies to multiple cell surface markers. Vγ9Vδ2 T cells (Gamma9+Delta2+CD3+) cell and non-Vγ9Vδ2 T cells (Gamma9-Delta2-CD3+ cells) were quadranted to 4 populations by anti-CD27 and anti-CD45RA. Two distinct populations between CD45RA- CD27-and CD45RA+ CD27- in non-Vγ9Vδ2 T cells but not in Vγ9Vδ2 T cells B, Comparison spreads of background fluorescence between unstained sample and FMO sample (minus Gamma9). Spread of background fluorescence in Gamma9 axis of unstained sample.
sample is less than the background of FMO sample. The quadrant should be higher than it in unstained control according to FMO control.

Proliferation assay

The formula used for acquiring the absolute number of Vγ9Vδ2 T cells in each sample: number of total cells × percentage of Vγ9Vδ2 T cells in total cells × volume of cell suspension of sample. Fold increase is introduced to investigate whether Vγ9Vδ2 T cells proliferate under various conditions by comparison of absolute numbers of Vγ9Vδ2 T cells before and after stimulation. Some studies assess if the Vγ9Vδ2 T cells proliferate by comparison of the percentages of Vγ9Vδ2 T cells in total cells or lymphocytes [22, 138]. Our method is further compared with the absolute number of Vγ9Vδ2 T cells before and after growing. Some proliferation assays such as thymidine incorporation assay cannot determine proliferation of subpopulation from PBMCs. Carboxyfluorescein succinimidyl ester (CFSE) proliferation assay can be used to estimate proliferation of Vγ9Vδ2 T cells [139]. Since CSFE is a fluorescence dye stable bond to cytoplasm, the intensity of CSFE demonstrates division times of cells. This way is convenient but indirect. In addition, CSFE effects development and proliferation of lymphocytes [140](Table 3).

Table 3. Comparison different proliferation assays

<table>
<thead>
<tr>
<th>Name</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACS by comparison of percentages of target cells in total cells</td>
<td>This method illustrates the proliferation by FACS dot plots.</td>
<td>This method does not take into account the difference in the number of cells between samples.</td>
</tr>
<tr>
<td>Combination of FACS and absolute number estimation</td>
<td>This method directly shows the number of cells from subpopulation increases or decreases after culture.</td>
<td>Time consuming. There are many steps in the method.</td>
</tr>
<tr>
<td>CSFE assay</td>
<td>It is convenient and an effective method.</td>
<td>It indirectly assesses the proliferation by cell division. It effects development and proliferation of lymphocytes.</td>
</tr>
</tbody>
</table>
Results

Paper I

Antigenic molecules to Vγ9Vδ2 T cells are released from *P. falciparum*-infected erythrocytes at the ring stage

To demonstrate that the *Anopheles gambiae*’s immunity system can encounter the phosphoantigens from infected erythrocyte blood, examining whether the blood possibly contains phosphoantigens is crucial. Very few parasites at the schizont or trophozoite stage are found in peripheral blood [141] since most of them accumulate in vesicular tissues of some organs such as adipose tissue, skeletal muscle and the heart [142]. If the phosphoantigens are released due to schizont rupture only [63-65], there are still questions. For example, are there many schizonts around the *Anopheles* biting site? To bypass the questions, we reported the parasites at the ring stage released molecules antigenic to Vγ9Vδ2 T cells in this paper. In detail, media were collected from infected cells synchronized to the ring stage and the media were added into PBMCs for stimulation. After incubation of 12 days with assistance of IL-2, the data were acquired with flow cytometry and cell counting.

Small isoprenoid pyrophosphates stimulate proliferation of Vγ9Vδ2 T cells but HMBPP has much higher antigenic bioactivity than other isoprenoid pyrophosphates [143, 144] in Vγ9Vδ2 T cell lines. In addition, the antigenic molecules are pyrophosphates according to the findings of our paper II. As a result, HMBPP should have crucial function in the proliferation. The concentration of HMBPP can be estimated by “HMBPP unit based on the EC50” method. EC50 is defined as half maximal proliferation of Vγ9Vδ2 T cells in PBMCs at the certain concentration of IL-2 [145]. One unit of HMBPP is defined as the concentration of HMBPP at EC50. As a result, the lower concentration at EC50, the higher the antigenic bioactivity.

By comparison of dilution series and the HMBPP dose-response curve (Paper II, figure 1), the concentration HMBPP in media can be estimated roughly. Using 20× diluted media, there was less proliferation than it from the 4× diluted media. It suggests the concentration of HMBPP in the 4 × dilution is
lower or close to 80pM. So the concentration in the media should be 320pM or lower.

HMBPP increases phosphorylation of the MAPK/Erk activated proteins p38, JNK and the transcription factor FOXO in an *Anopheles gambiae* immune cell line.

MAPK/ERKand PI-3K pathways are activated by HMBPP in the absence of IL-2 in Vγ9Vδ2 T cells. The response is T cell receptor related [124]. In this paper, we find the homologous response in *Anopheles gambiae*. HMBPP stimulation induces an increase of the phosphorylation of MAPK/Erk activated proteins JNK (c-Jun N-terminal kinases), p38 and transcription factor FOXO (Forkhead box class O) in insect immune competent hemocyte-like cell line – 4a3B but does not upregulate phosphorylation of Erk.

JNK is involved in the regulation of hydrogen peroxide detoxifying enzymes of *Anopheles gambiae* [146]. In addition, the transcription factor FOXO is involved in oxidative stress [147] and p38 is phosphorylated when hydrogen peroxide challenges [148]. Based on the findings, HMBPP plausibly upregulates the resistance to oxidative stress. This is further supported by our result. Our result shows the HBMPP-pretreated 4a3B cells give tolerance to 1mL hydrogen peroxide.

HMBPP induces changes in the transcription of genes related to the immune response in *Anopheles gambiae*

To test if the HMBPP stimulates the immune response in *Anopheles gambiae*, 6 genes related to the immune response were selected to investigate if their transcription changes after adding HMBPP to a blood meal in *Anopheles gambiae*. Transcription of five of the six genes changed significantly compared with control feed. The five genes were 3 AMP (antimicrobial peptide) genes and two genes implicated in H2O2/NO generation- dual oxidase (DUOX) and nitric oxide synthase (NOS). DUOX has an important function in the midgut epithelial immunity of *Anopheles gambiae* [149]. Moreover, HMBPP stimulation regulates the abundance of bacteria in the midgut. The number of bacteria in the midgut is related the parasite infection rate [150-152]. It has been reported that peptidoglycan (PGN) regulates transcription of AMP genes in *Anopheles gambiae* by PGN Recognition Protein LC [151]. Our paper also shows the PGN regulates transcription of two of the three AMP genes significantly but only HMBPP regulates DUOX and NOS.
Paper II

Vγ9Vδ2 T cells response to HMBPP

To find out the concentration of HMBPP that induces near maximum proliferation of Vγ9Vδ2 T cells, various concentrations of HMBPP were tested for expansion of Vγ9Vδ2 T cells in present of IL-2. Concentrations from 800fM to 8nM of HMBPP induce proliferation and the peak is from 8pM to 8nM. 80pM HMBPP was chosen as a positive control for subsequent experiments.

Simultaneous acquisition of synchronized cultures at ring, trophozoite, schizont and merozoite stages

A new protocol has been developed for simultaneous acquisition of cultures of P. falciparum at all asexual blood stages by using Plasmion and sorbitol. Briefly, two subcultures are separated from a mother culture. Merozoite and trophozoite are synchronized from one subculture. Schizont and ring stage parasite are acquired from the other. The synchronized cultures are incubated in new media for several hours. The time of incubation cannot exceed the transformation time to the next stage. That means, after incubation, the synchronized cultures still were in the same stage had not developed to next stage. The media was collected by filtering out the erythrocytes and parasites.

Media from parasite-infected erythrocytes at all asexual blood stages induce proliferation of Vγ9Vδ2 T cells

Two phosphoantigens HMBPP and IPP are produced by apicoplast of P. falciparum. In Paper I, we demonstrate the antigenic molecules that were released to media collected from ring stage culture support Vγ9Vδ2 T cells proliferation. In Paper II, media collected from all blood cycle stages were tested if they support proliferation. Several batches of cultures and PBMCs from different donors were applied for the experiment. The data were gathered and analysed by generalized linear mixed model (GLMM) model. The model has been used for many medical studies [153]. We found a significant difference between our experimental groups and negative control group. There was no difference among the experimental groups. This result challenges the traditional view that phosphoantigens are released to media by schizont rupture and the burst of erythrocytes.

To investigate if parasites at ring, trophozoit and schizont stages release antigens to stimulate Vγ9Vδ2 T cells; some other possibilities must be considered. In Paper II, we figured out 3 possibilities: How pure were the synchronized cultures? Investigate if the antigens were leaked from lysed infected
erythrocytes? And do phosphoantigens released from uninfected erythrocytes support proliferation?

Purity of the synchronized cultures

More than 90% purity of schizonts should be acquired from Plasmion that is a commercial gelatin solution [113] and the purity of ring stage parasites in infected erythrocytes should be 95% after sorbitol lysis [154]. In our study, we checked the purity by microscopy and found there was around 90% purity in the ring culture and the remaining parasites were at the trophozoite stage. Purity in the trophozoite culture was around 90% and the remaining parasites were schizonts. There was 100% purity in schizont culture. In merozoit/schizont rupture culture, we find around 80% of parasites were at merozoite/ruptured schizont stage. Importantly, the result shows almost no parasite at schizont rupture was mixed into the other 3 cultures (ring, trophozoite and schizont).

The phosphoantigens in culture media are not due to broken infected erythrocytes

It is possible that infected erythrocytes are lysed during culture and release antigens to the culture’s media. To investigate this possibility, we measured the iron released from broken erythrocytes in some samples. By comparison between lysed erythrocytes control and samples, we found average level of rupture in total erythrocytes is 0.3-0.4 % while around 5 % erythrocyte were infected in the parasite cultures. The lysis rate in infected samples was not higher than the uninfected samples. These results strongly suggest the phosphoantigens in culture media are not due to infected erythrocytes.

The level of iron in media of the culture with the parasites that had undergone schizont and erythrocyte rupture was not higher than media from other cultures. It suggests the parasites used almost all hemoglobin. In the infected cells, the parasites consume hemoglobin to construct their proteins and non-protein part, heme (with iron), is released from hemoglobin. The heme is toxic to parasite and the parasite converts it to the insoluble hemozoin (with iron) [155]. At the late trophozoite stage, 61% iron of the host erythrocyte was found in hemozoin [156]. The hemozoin crystals were removed when we filtered the cultures to get the cell-free media.

Media collected from uninfected cultures cannot cause the proliferation

Erythrocytes produce IPP and other pyrophosphates by the mevalonate pathway though the amount of pyrophosphates seems unlikely to support the
proliferation because the much higher bioactivity of HMBPP. The result shows the Vγ9Vδ2 T cells cannot proliferate with stimulation by the media collected from uninfected erythrocyte cultures. Furthermore, Vγ9Vδ2 T cells do not proliferate with lysed uninfected erythrocyte but proliferate with the media from infected cultures.

**Phosphatase treatment stops the proliferation**

Though no paper report (based on my knowledge) the Vγ9Vδ2 T cells recognize peptidic antigens, other cells such as CD4+αβ T cell may release cytokines like IL-2 to induce proliferation of Vγ9Vδ2 T cells. These cells are likely to be stimulated by peptidic antigens from parasite. As a result, we tested if some proteins stimulated the Vγ9Vδ2 T cells indirectly. Heating (95°C, 10min) the media collected from the infected cultures could not stop the proliferation of Vγ9Vδ2 T cells. This suggests the antigenitic molecules are not proteins.

After the media collected from infected cultures were treated by phosphatase (apyrase), Vγ9Vδ2 T cells did not proliferate. Apyrase catalyzes the pyrophosphate hydrolysed to alcohols or monophosphates and lowers the antigenic bioactivity (Paper III). This result strongly suggests the proliferation was induced by pyrophosphate-containing phosphoantigens.

**Paper III**

In this study, we investigated if Vγ9Vδ2 T cells in PBMCs responded to various isoprenoid phosphates and addressed their response ability at different conditions. To address if the phosphoantigens strengthens cytotoxicity of Vγ9Vδ2 T cells to adherent cells, we developed a new FACS based assay.

**Cryopreservation of PBMCs for proliferation of Vγ9Vδ2 T cells**

We are successful to expand Vγ9Vδ2 T cells from cryopreserved PBMCs with stimulation of HMBPP. This procedure can be used to preserve large numbers of PBMCs to save PBMCs preparation time and experimental material. Based on statistics, proliferation from cryopreserved Vγ9Vδ2 T cells is better than it of fresh samples from the same donors. In the cryopreservation procedure, much DMSO (dimethyl sulfoxide) was used to protect cells from lysis due to crystals of water. DMSO affects permeability of the cell membrane and other function of cells [157, 158]. To investigate if DMSO affects proliferation, 0,1% DMSO was administrated to the PBMCs. There was no significant change in the phosphoantigens-mediated proliferation.
DMSO has a negative effect in 3 of 4 freshly isolated samples but the positive effect in 3 of 5 cryopreserved samples in HMBPP-responded proliferation. Moreover, cryopreserving may lower percentage of regulatory cells in PBMCs that suppress the proliferation in some samples of PBMCs [159, 160]. Another explanation for the high proliferation in cryopreserved cells is that the healthiest cells survived the cryopreservation and that these cells proliferate fast.

Isoprenoid pyrophosphates length C5-C20 and monophosphate DMAP stimulate Vγ9Vδ2 T cells

Except IPP and HMBPP, other length C5-C20 isoprenoid pyrophosphates stimulate proliferation of Vγ9Vδ2 T cell lines. Moreover, it has been reported that IPP has higher biological activity than DMAPP (5 carbons, GPP (10 carbons), FPP (15 carbons) and GGPP (20 carbons) [144, 161]. In this paper, we show DMAPP, GPP, FPP and GGPP can stimulate proliferation of Vγ9Vδ2 T cells in PBMCs collected from different donors.

Cells can take up isoprenoid alcohols – DMA, G, F and incorporate them to prenylated proteins [162]. This finding suggests the alcohols can be phosphorylated in cells. The rephosphorylated alcohols bind can to B30.2 intracellular domain of BTN3A1 to induce activation of Vγ9Vδ2 T cell. None of isoprenoid alcohols stimulated Vγ9Vδ2 T cells in PBMCs in our experiments. Among the isoprenoid monophosphates, only DMAP shows immunogenicity.

HMBPP strengthens the cytotoxicity of Vγ9Vδ2 T cells towards colon cancer cells

FACS based cytotoxicity assay shows the absolute number of live cells and injured cells after cell killing. To address it is only suitable for suspension cells, a flow-cytometry based method for assessing cytotoxicity towards adherent cells was developed. By microscopy, we demonstrated all the adherent target cells could be resuspended and collected after incubation. It has been reported that HMBPP increases the cytotoxicity of Vγ9Vδ2 T cell towards suspension target cells [163]. By using our newly developed assay, we find the HMBPP improves cytotoxicity to adherent colon cancer cell lines (HT29 and SW620) at different ratios.
Paper IV

Immunotherapy for colon cancer using patient themselves Vγ9Vδ2 T cells is a potential therapy. To acquire status and more information of Vγ9Vδ2 T cells in the patients, we characterised the frequency, proliferation capacity, differentiation and homing potential of Vγ9Vδ2 T cells from colon cancer patients.

The frequency of Vγ9Vδ2 T cells in colon cancer patients

Frequency or percentage of circulating γδ T cells or/and Vγ9Vδ2 T cells is found to be lower than healthy donors in some cancers [164-166]. First, we investigated if the impairment occurs in colon cancer. We compare the percentages of Vγ9Vδ2 T cells in lymphocytes between age-matched donors and colon cancer patients at stage I, II, III and IV (Table 4). The average The mean percentage of age-matched control group (3%) was higher than it of the four groups of patient samples (1.5%-2.5%).

The capability of Vγ9Vδ2 T cell proliferation in colon cancer patients

Except the lower frequency, it has been reported that the capability of proliferation is impaired in cutaneous primary melanoma [164]. In this study, we investigated if the capability of proliferation in colon cancer patients was lower than the control. We found IL-2 and HMBPP induce proliferation of Vγ9Vδ2 T cells in all groups but some samples did not respond to HMBPP. As a result, we grouped the samples to non-responder, low responder, medium responder and high responder. The number of high responders in the control group was higher than it in the colon cancer stage I, III and IV groups. The number of non-responders in control group is lower than all patient sample groups.

Based on these results, it is suggested that the Vγ9Vδ2 T cell proliferation is impaired in colon cancer patients. But the impairment does not appear to be related to the stage of colon cancer.

CXCR3 and CCR5 expression in Vγ9Vδ2 T cells in colon cancer patients

The inflammatory chemokine receptors, CXCR3 and CCR5 are related to direct the infiltrated T cells to tumors [167-169]. Vγ9Vδ2 T cells express the two chemokine receptors and upregulate them after differentiation. To investigate the infiltration potential of Vγ9Vδ2 T cells, we tested the expression of the two receptors in cancer samples compared with age-matched samples.
After proliferation with stimulation of HMBPP with help of IL-2, there was a significant increase in CCR5 expression of Vγ9Vδ2 T cells and 90% the cells expressed CCR5 in all groups. Interestingly, the expression of CXCR3 in all patient sample groups except stage IV was significantly higher than control group after proliferation. Moreover, expression of CCR5 before proliferation in control group is lower than it in the four cancer groups. These results show the Vγ9Vδ2 T cells in colon cancer patients was potential to infiltrate tumors and that this capacity increases when they are stimulated by phosphoantigens.

Vγ9Vδ2 T cells differentiation in colon cancer patients

We characterised differentiation status of the Vγ9Vδ2 T cells in patient samples. We found a significant increase in the percentages of T_{EM} after proliferation while a decrease in the percentages of T_{N} and T_{EMRA} in all sample but one exception. T_{EM} cells and T_{EMRA} cells play main role in cytotoxicity. We find the combined T_{EM} cells and T_{EMRA} cells population significantly increased after proliferation in all samples. In summary, the Vγ9Vδ2 T cells of patients can differentiate to cytotoxic cells to cancer.
Summary and conclusion

Paper I
We address how Vγ9Vδ2 T cells, as well as Anopheles gambiae immunity cells, respond to phosphoantigens released from malaria parasites. In detail, we found that the phosphoantigen HMBPP increases phosphorylation of the MAPK/Erk proteins causing their activation. The HMBPP increases the resistance of oxidative stress by upregulation of the transcription of dual oxidase and nitric oxide synthase. We also have shown that Vγ9Vδ2 T cells were substantially stimulated by media collected from synchronized parasite-infected erythrocytes at the ring stage.

Paper II
We found that Vγ9Vδ2 T cells were stimulated and proliferated by media collected from infected erythrocytes at all blood stages, i.e. both asexual and sexual stages. Moreover, the stimulation was independent of schizont rupture. Our result suggests that the parasites used all hemoglobin during their development from the ring to the schizont stage. The molecules stimulating Vγ9Vδ2 T cell were determined to be phosphoantigens. Phosphoantigens did not appear in the media due to the lysis of erythrocytes assessed by measuring the iron level in the media. These findings demonstrate phosphoantigens are released from parasites during all stages of blood cell cycle and suggest that phosphoantigens are transported through several membranes when they are released.

Paper III
A protocol for proliferation of Vγ9Vδ2 T cells from cryopreserved samples was designed and we find the cryopreservation does not abrogate the proliferation response. Actually, it was higher than the proliferation from freshly isolated samples. Although many cells were lost during cryopreservation, no significant change in percentages of δ2+ cells CD3+ cells was found. DMSO
was tested if it enforces the proliferation but the DMSO did not show a significant effect. We also show that fresh media should be used for proliferation. Most small natural pyrophosphate intermediates of isoprenoid synthesis induced proliferation but most corresponding monophosphates did not with the exception is the monophosphate DMAP. Vγ9Vδ2 T cells respond to stimulation of phosphoantigens and phosphoantigens enforce cytotoxicity to cancer cells. An assay was developed for testing the cytotoxicity of HMBPP-stimulated Vγ9Vδ2 T cells against adherent colon cancer cell lines.

**Paper IV**

In this study, we characterised the frequency, proliferation capacity, and inflammatory homing potential of Vγ9Vδ2 T cells from colon cancer patients. The patients were classified to 4 groups (stage I-IV) according to TNM systems. We found the impairment of capability of Vγ9Vδ2 T cells proliferation in all patient sample groups. In addition, the percentage of Vγ9Vδ2 T cells in total lymphocytes in all patient groups was lower than control group. The expression of CXCR3 and CCR5 in patient samples is not lower and even higher than it of control group. These results suggest the Vγ9Vδ2 T cells of patients have potential to infiltrate tumor. After proliferation, the percentage and number of T_EM cells increase in both control and patient groups, the result suggests the Vγ9Vδ2 T cells have potential to be used for adoptive immunotherapy.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)