The antigen presenting potential of Vγ9Vδ2 T-cells during *Plasmodium falciparum* blood-stage infection.

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Summary: Vγ9Vδ2 T-cells with APC-like phenotype can be found *in vivo* in *P. falciparum*-infected patients. Vγ9Vδ2 T-cells stimulated *in vitro* with *P. falciparum*-infected red blood cell acquire an APC-like phenotype, induce naïve αβ T-cell activation and perform antigen cross-presentation.

Running title: γδ T-APC POTENTIAL IN MALARIA

ABSTRACT

During *Plasmodium falciparum* infections, erythrocyte-stage parasites inhibit dendritic cell maturation and function; compromising development of effective anti-malarial adaptive immunity. Human Vγ9Vδ2 T-cells can act *in vitro* as APCs and induce αβ T-cell activation. However, the relevance of this activity in pathophysiological contexts *in vivo* has remained elusive. Since Vγ9Vδ2 T-cells are activated during the early immune response against *P.falciparum* infection, we investigated whether they could contribute to the instruction of adaptive immune responses toward malaria parasites. In *P.falciparum*-infected patients, Vγ9Vδ2 T-cells presented an increased surface expression of APC-associated markers HLA-DR and CD86. In response to infected red blood cells *in vitro*, Vγ9Vδ2 T-cells readily up-regulated surface expression of HLA-DR, HLA-ABC, CD40, CD80, CD83 and CD86, induced naive αβ T-cell responses, and cross-presented soluble prototypical protein to antigen-specific $CD8^+$ T-cells. Our findings indicate that *P. falciparum* parasites induce genuine APC properties in Vγ9Vδ2 T-cells and qualify this subset as an alternative professional APC in malaria patients, which could be harnessed for therapeutic interventions and vaccine design.

Key words: γδ T-cells, malaria, antigen presentation, *P. falciparum* infection

INTRODUCTION

Accumulating evidence has shown that a non-conventional T-cell, the γδ T-cell, is involved in the early immune response to erythrocyte stage infection of *Plasmodium spp.* malaria parasites, including *P.falciparum* and *P.vivax*. A subset unique to primates and expressing the Vγ9Vδ2 T-cell receptor (TCR) is activated in a major histocompatibility complex (MHC)-independent manner by small non-peptidic phosphorylated molecules commonly referred to as phosphoantigens. The most potent phosphoantigen is HMBPP (4-hydroxy-3-methyl-but-2-enyl diphosphate), produced by the non-mevalonate isoprenoid pathway used by numerous microorganisms, including *Apicomplexan* parasites such as *Plasmodium spp.* (reviewed[1]). *In vivo,* human blood Vγ9Vδ2 T-cells are activated and expand during primary *P.falciparum* infection [2, 3]. *In vitro,* it has been shown that Vγ9Vδ2 T-cells inhibit erythrocyte stage proliferation [4] by granulysin release targeting the extracellular invasive parasite form (merozoites) [2, 3]. Granulysin levels are elevated in malaria patients and correlate with the percentage of CD3-positive T-cells expressing Vδ2 [2] consistent with substantial degranulation of Vγ9Vδ2 T-cells during *P.falciparum* infection.

In addition to a cytotoxic role, it has been proposed that Vγ9Vδ2 T-cells can mimic in part dendritic cell (DC) function and license primary αβ T-cell responses. Vγ9Vδ2 T-cells stimulated *in vitro* by synthetic phosphoantigens, express antigen presenting cell (APC) phenotype and display a DC-like morphology. They are able to present both viral and bacterial antigens to $CD4^+$ αβ T-cells and can even cross-present antigens to $CD8^+$ αβ T-cells [5-10]. However, their APC potential in response to an infectious agent *in vivo* has yet to be demonstrated. It is possible that Vγ9Vδ2 T-cells represent an alternative route of antigen presentation during the immune response

that follows *P.falciparum* infection. This question is particularly relevant as there is both *in vitro* and *ex vivo* evidence that *Plasmodium* erythrocyte stage parasites inhibit effective DC maturation and function [11-15].

We therefore investigated the antigen presenting ability of Vγ9Vδ2 T-cells after exposure to erythrocyte stage *P.falciparum* parasites (iRBC), looking at both their phenotype and function. We found that *in vivo* there is an increase in surface expression of APC-associated makers on blood Vγ9Vδ2 T-cells from *P. falciparum*-infected patients. *In vitro*, Vγ9Vδ2 T-cells up-regulated surface expression of a range of APC-markers in response to iRBC, and exhibited functions of professional APCs in mixed lymphocyte reactions and a cross-presentation assay. To our knowledge, this is the first demonstration of an APC activity of Vγ9Vδ2 T-cells in an infectious context, and has implications for therapeutic interventions and improved vaccine design.

MATERIALS AND METHODS

Clinical samples

Clinical blood samples were collected from *P.falciparum*-infected patients admitted to the University Hospital of Bordeaux on admission prior to treatment (day 0) and, for those who came back for a medical follow-up, 7 days after treatment (day 7). Non-malaria febrile control samples were collected as were malaria-samples from patients presenting with high-grade fever and a record of travel to malaria endemic zones, but for whom malaria attack was excluded. Healthy donors from the blood bank (Etablissement Français du Sang, EFS-Aquitaine) were processed as healthy controls. Malaria patients were classified as described in the text according to [16, 17]. Severe malaria was defined as described [18]. All patients gave informed

consent. The ethical agreement obtained for this study was restricted to leftovers from the blood samples used for diagnosis, corresponding to approximately 0.5-1 ml of blood.

APC phenotyping by flow cytometry

For *ex vivo* analysis, 300 µl whole blood was labelled with anti-CD3-BV510, CD45RA-PECF5940, CD27-Alexafluor 700 (all from BD Biosciences); anti-Vδ2-PC7, CD80-FITC, CD86-PE mAbs (all from Beckman Coulter) and anti-CD40-APC mAb (Miltenyi-Biotech). For *in vitro* analysis cells were labelled with anti-CD3-BV510, HLA-DR-Alexafluor 700, HLA-ABC-V450 mAbs (all from BD Biosciences); anti-Vδ2-FITC, CD80-PC7, CD83-PE, CD86-APC Alexafluor 750 mAbs (all from Beckman Coulter) and anti-CD40-APC mAbs (Miltenyi-Biotech). FACS data were acquired using a LSR Fortessa 2-Blue 6-Violet 3-Red 5-Yelgr laser configuration, a CantoII 4-Blue 2-Violet 2-Red laser configuration and a Canto 4-Blue 2-Red laser configuration (all BD Biosciences). Analysis was performed using Diva 8 (BD Biosciences) and FlowJo 9.3.2 (Tristar) softwares. Total lymphocyte counts from patient blood samples were carried out by the immunology laboratory (Bordeaux University Hospital) using FC500 Beckman Coulter flow cytometers.

Parasite Culture

FCR3 *P.falciparum* parasites were cultured in type O^+ red blood cells (RBCs) (EFS-Aquitaine), in RPMI-1640 supplemented with 10% human serum, gentamicin, glutamine and hypoxanthine, as previously described [2, 19]. Infected red blood cell (iRBC) cultures were regularly tested for absence of *Mycoplasma* contamination. Parasitemia was assessed using hydroethidine staining, as described [20] or examination of Giemsa stained smears. Parasite cultures were synchronized by sorbitol and/or heparin treatment as described [21, 22]. Mid-stage to late schizonts

around 36 hours post infection (hpi), were purified by gel floatation on gelofusin [20]. Parasitemia after purification was >80%. Supernatant was produced as previously described [21]. For Alkaline Phosphatase treatment, supernatants were treated with 0.015 U/µl shrimp alkaline phosphatase for 30 min at 37°C, as described [23].

In vitro stimulation of Vγ9Vδ2 T-cells

PBMCs from malaria-naive healthy donors (EFS-Aquitaine) were isolated from blood by Ficoll density gradient and cultured in complete medium (RPMI-1640, supplemented with glutamine, antibiotics, non-essential amino acids, sodium pyruvate (all from Gibco Life Technologies), and 10% of heat-inactivated fetal calf serum (PAA Laboratories)) supplemented with 100 IU/ml interleukin (IL)-2 (Peprotech) in 24 well plates (Thermo Scientific). PBMCs ($1.5x10^6$ cells/ml) were stimulated with $5x10^6$/ml iRBC or 10 nM HMBPP (Echelon Biosciences). uiRBCs ($5x10^6$/ml) or CM alone were used as respective negative controls for phenotyping assays. 100 IU/ml IL-2 and 25 ng/ml IL-15 (Peprotech) were added after 72 h of culture. After 12 days in culture, Vγ9Vδ2 T-cell purity was assessed by flow cytometry. If total $Vδ2^+$ T-cell purity was <90% cells were sorted as described below.

Mixed lymphocyte reaction

Monocytes from malaria-naive healthy donors' PBMCs were depleted using CD14-beads (Miltenyi-Biotech). The CD14-negative fraction was then labelled with either anti-Vδ2-FITC or anti-Vγ9-PC5 antibody (Beckman Coulter) and anti-FITC or anti-PE beads (Miltenyi-Biotech). Labelled cells were filtrated through LS then MS columns (Miltenyi-Biotech) and used when purity was >95%. Freshly sorted $Vγ9^+$ T-cells (10^5) were cultured with 10^4 autologous irradiated (50 Gy) monocytes as feeders and stimulated with either 10^5-$2x10^5$ iRBCs, 0.2-1 nM HMBPP or 50% v/v iRBC-

supernatant in complete medium supplemented with 100 IU/ml IL-2. After 6 days, Vδ2 purity and phenotype were checked and the cells were used as effectors in MLR. Total allogenic $CD4^+$ and $CD8^+$ T-cells were sorted based on expression of CD4/CD8. Naive $CD4^+$ and $CD8^+$ T-cells were sorted as $CD45RA^+$, and $CCR7^+$ to purities of >99.2% from PBMCs of healthy donors. Sorted cells were labelled with 5 µM CFSE (Invitrogen) in PBS-5% FCS for 5 min, washed thoroughly and starved 1 h in complete medium at 37°C before being used as responder cells in MLR. $CD4^+$ and $CD8^+$ cells ($2x10^5$) were mixed with decreasing ratios of irradiated (12 Gy) $Vγ9^+$ T-cells cells, namely -1:10, 10:1, 100:1, 1000:1, in 96 well round bottom plates. CD4/CD8 T-cells alone were incubated with 4 µg/ml Staphylococcus Enterotoxin B (SEB, Sigma-Aldrich) and 40 IU/ml IL-2 or in complete medium alone. After 5 days, cells were labelled with DAPI, anti-CD3-BV510, anti-CD4-PC7 and anti-CD8-APC mAbs. The percentages of $CFSE^{low}$ $CD4^+$ and $CD8^+$ T-cells were assessed by flow cytometry. For measurement of intracellular cytokine expression, MLR cultures were stimulated with PMA/Ionomycin (10 ng/ml and 1 µM, both from Sigma). Brefeldin A (10 µg/ml, BioLegend) was added 1 h later. Following 5 h of stimulation, cells were processed for extracellular and intracellular staining using the eBioscience Fix/Perm Kit. Cultures were stained with anti-CD4-APC H7, CD8-PCy7, CD3-FITC, Vγ9-PCy5 and IFNγ-BV421 mAbs.

M1 antigen presentation assay

CD8 T-cells specific for M1p58-66 presented in an HLA-A2 background were generated using the influenza matrix M1 protein-derived M1p58-66 peptide (GILGFVFTL) as described [6, 24]. $HLA\text{-}A2^+$ or $HLA\text{-}A2^-$ *in vitro*-stimulated Vγ9Vδ2 T-cells ($3x10^5$) were incubated overnight with 0.01-1 µM M1 protein in a 96 well round bottom plate (Falcon) for loading. M1p58-66 (0.01µM) was identically loaded

for 1 h immediately prior to co-culture. Effector cells were washed and co-cultured with M1p58-66-specific CD8 T-cell responders ($0.3x10^6$) for 4.5 h in complete medium plus 1 µg/ml brefeldin A. PMA/Ionomycin were used as positive polyclonal control for CD8 T-cell activation. Cells were labelled with anti-CD3-APC, CD8-PC7 mAbs (both from BD Biosciences), and anti-Vδ2-FITC mAb (Beckman Coulter), fixed with FACs lysis buffer and permeabilized using Perm2 (both from BD Biosciences), then labelled with anti-IFNγ-PE mAb (BD Biosciences).

Statistical analysis

Statistical analysis were performed using Graph-Pad Prism as follows: non-parametric Mann Whitney U-test for comparisons of unpaired populations; Wilcoxon signed rank test for pairwise day0 vs day7 comparisons; Friedman test with Dunn's multi-comparison adjustment for pairwise comparisons; 2-way ANOVA test with Bonferroni correction for time-course comparisons. Differences were considered significant at $P<0.05$.

RESULTS

Vγ9Vδ2 T-cells in *P.falciparum* malaria patients show increased expression of APC markers compared to healthy and non-malaria febrile controls.

We first compared the surface expression of APC-markers Vγ9Vδ2 T-cells in blood obtained from *P.falciparum*-infected adult patients, healthy controls and patients presenting with fever for reasons other than malaria (Figure 1 and Supplementary Figure 1). Overall, we observed higher levels of HLA-DR and CD86 expression on $Vδ2^+$ T-cells from malaria patients at admission compared to healthy and non-malaria febrile controls. Both non-malaria febrile controls and malaria patients had a

decreased CD40 expression on day 0 compared to healthy controls, although this was less pronounced in malaria patients. There were no significant differences in percentage of $V\delta2^+/CD3^+$ T-cells between the three groups (Figure 1A) and no CD80 expression was observed. With regard to memory phenotype, malaria patients presented less T_{naive} and more T_{emra} $V\delta2^+$ T-cells compared to both healthy and non-malaria febrile controls (Figure 1B). Interestingly there was strong homogeneity between the febrile controls free of malaria infection, despite presenting with heterogeneous infectious events (Table 1). Despite limited statistical power due to the low number of patients for whom both D0 and D7 blood samples were available, there appears to be a trend for an increase in percentage of Vδ2 T-cells (as previously documented), and a decrease in HLA-DR and CD86 expression, though not significant (Figure 1C), concomitant with parasite clearance. There was also an increase in the T_{cm} and a decrease in the T_{emra} $V\delta2^+$ T-cells subsets (Figure 1C).

To assess the effect of different previous exposures to malaria, we stratified the malaria patients between those of sub-Saharan African origin who were migrant to France and visiting friends and relatives in their country of origin (VFR), travelers -Caucasian- of European origin (TEO), and travelers -Caucasian- of European origin with a history of frequent travel to endemic regions (FTEO). The majority of the cohort patients were VFR (n=22) (Table 1). While there were no significant differences in APC- or memory-phenotype between the groups, the VFR group was the only group consistently significantly different from the healthy and febrile controls (Supplementary Figure 1B). There was no correlation between parasitemia and APC phenotype. However, our cohort contained one highly severe case with 19% RBC parasitemia upon admission (Table 1, Patient 11), who showed high expression of HLA-DR and CD86 on $V\delta2^+$ T-cells and a predominant T_{em} phenotype, which all diminished post-parasite clearance (Figure 1C,D). Altogether, these data

demonstrate that Vγ9Vδ2 T-cells presenting phenotypic features of APCs can be found *in vivo* in the blood of malaria patients.

Vγ9Vδ2 T lymphocytes acquire an APC-like phenotype after *in vitro* stimulation by blood-stage *P.falciparum* parasites.

To investigate whether *P.falciparum* can induce APC maturation of Vγ9Vδ2 T-cells, PBMCs from malaria naive, afebrile healthy donors were stimulated with intact $5x10^6$/ml iRBC. The synthetic phosphoantigen HMBPP was used as a positive control, $5x10^6$/ml uninfected RBC (uiRBC) and complete medium were used as negative controls respectively (Figure 2). We found that iRBC stimulation significantly increased expression of all APC-associated markers tested over a 12-day culture period, though with different kinetics (Figure 2A, Supplementary Figure 2). iRBC induced a lower magnitude of expression than HMBPP. Expression of all APC markers was induced in the negative controls, but to lower/marginal levels, and with delayed kinetics. This was likely due to the presence of IL-2 and IL-15 in the culture medium, indicating that APC-like phenotype acquisition is not solely dependent on TCR signaling.

These results indicate that *P.falciparum* can promote *in vitro* the expression of an APC phenotype by Vγ9Vδ2 T-cells.

Vγ9Vδ2 T lymphocytes acquire APC-like functions after *in vitro* stimulation by blood-stage *P.falciparum* parasites.

One of the hallmarks of APCs is the ability to stimulate allogenic αβ T-cells. We thus tested the capacity of peripheral-blood isolated Vγ9Vδ2 T-cells stimulated *in vitro* with *P.falciparum* to activate $CD4^+$ and $CD8^+$ αβ T-cells in a mixed lymphocyte

reaction (MLR). Vγ9Vδ2 T-cells stimulated by intact iRBCs induced proliferation of total allogenic $CD4^+$ and $CD8^+$ T-cells (Figure 3A). Moreover, Vγ9Vδ2 T-cells stimulated with iRBC culture supernatant induced both proliferation (Figure 3B) and cytokine production (Figure 3C) by allogenic naive $CD4^+$ and $CD8^+$ T-cells, demonstrating genuine professional APC activity. Indeed, naive αβ T-cell activation was not observed with unstimulated Vγ9Vδ2 T-cells (Figure 3B). Much lower activity was observed when using the uiRBC culture supernatant and alkaline phosphatase-treated iRBC culture supernatant. This is in agreement with the susceptibility of parasite-derived phosphoantigens to dephosphorylation, and rules out the requirement of unrelated parasite or RBC molecules for the induction of APC features in Vγ9Vδ2 T-cells.

It has been documented that isopentenylpyrophosphate- or HMBPP-stimulated Vγ9Vδ2 T-cells can efficiently cross-present exogenous antigens to $CD8^+$ T-cells [6, 24]. To assess whether *P.falciparum*-stimulated Vγ9Vδ2 T-cells have the same ability, we used an antigen presentation assay based upon the processing and presentation of the Influenza virus M1 antigen to M1p58-66-specific $CD8^+$ T-cells in a HLA-A2 background (Figure 4). We found that *P.falciparum*-stimulated, M1 protein-loaded, Vγ9Vδ2 T-cells activated the M1p58-66-specific $CD8^+$ T-cell responder line as efficiently as HMBPP-stimulated Vγ9Vδ2 T-cells (Figure 4B). This activation was dependent on both M1 protein loading and HLA-A2 expression by the Vγ9Vδ2 T-cells, demonstrating that activation was due to M1 protein up-take, processing and cross-presentation of the M1p58-66 epitope.

Taken together, our results show that *P.falciparum*-stimulated Vγ9Vδ2 T-cells readily acquire the potential to act as professional APCs and induce responses by $CD4^+$ and $CD8^+$ T-cells.

DISCUSSION

Protective immunity to malaria infection in endemic areas is acquired slowly over many years of exposure and is not stable. There are many explanations for this including *P.falciparum* antigenic diversity, T-cell inhibition and B-cell modulation in infected individuals (reviewed [25]). In addition, parasites have evolved mechanisms to inhibit the antigen presenting machinery. There is both *in vitro* and *ex vivo* evidence that iRBC inhibit effective DC maturation and function [11-15]. Our current results indicate that Vγ9Vδ2 T-cells, which are efficiently activated *in vivo* during *P.falciparum* infection, could thus represent an alternative route for antigen presentation to αβ T-cells in the context of malaria. We show that *in vivo*-stimulated Vγ9Vδ2 T-cells from malaria patients display a membrane phenotype associated with conventional APCs, which is not observed in the febrile non-malaria infected controls. The size increase and stability of the peripheral-blood memory subpopulations indicate that, although the patients were lymphopenic, malaria didn't damage the blood central memory compartment as reported for HIV infections [26]. From our malaria patient sub-grouping, it appears that originating from a malaria endemic area correlates with a higher *ex-vivo* Vγ9Vδ2 T-cell APC-phenotype upon infection. Previous studies have shown that $Vδ1^+$, not $Vδ2^+$ T-cells are expanded in the peripheral blood of malaria patients from endemic regions [27]. Our data may possibly reflect a situation where $Vδ2^+$ T-cells take on an APC phenotype once their inflammatory functions are downregulated. Vγ9Vδ2 T-cells express the chemokine CCR7 after phosphoantigen stimulation *in vitro* [28], thus their absence from peripheral blood could be due to their migration to lymphoid organs. However, as the sample size of the TEO and FTEO groups was small (n=4 in each group), this

interpretation needs to be further substantiated. Further, we cannot rule out a genetic effect in patients of different ethnic backgrounds in the VFR and TEO/FTEO groups. Systematic information on previous malaria infection(s), the duration of residence of VFR in both their native country or in France, and the frequency of visits to endemic regions in the VFR and FTEO groups were not available. In this study, we focused on samples taken pre-treatment, resulting in mainly recruiting non- and mildly-severe malaria cases. This may have led to underestimating the average level of Vγ9Vδ2 T-cells displaying an APC phenotype in malaria patients, given that the strongest APC phenotype was seen in our unique extremely severe malaria patient. From this severe hyperparasitemic case, it is clear that high HLA-DR and CD86 expression can occur in Vγ9Vδ2 T-cells during acute malarial infection. The dramatic decrease of both HLA-DR and CD86 expression by day 7, after patient recovery, further indicates that this APC-like phenotype was transient in blood and associated with acute infection.

Little *in vivo* evidence of APC-like phenotype and functions of γδ T-cells have been documented. In mice, where a Vγ9Vδ2 T-cell equivalent population has yet to be found, Frossard *et al.* described the APC-like phenotype of γδ T-cells induced *in vivo* by cholera toxin, and their role in the modulation of oral tolerance in food allergy pathogenesis [29]. More recently, Wan *et al.* described γδ T-cells with APC-like capabilities in zebrafish [30]. Thus, this work presents the first evidence to our knowledge of Vγ9Vδ2 T-cell APC-like differentiation occurring physiologically in humans, in a pathological situation.

The presence of Vγ9Vδ2 T-cells with an APC-like phenotype in malaria patients is supported by our demonstration that *in vitro* iRBC stimulated Vγ9Vδ2 T-cells readily express both presenting and co-stimulatory molecules associated with APCs. As a confirmation of the functional relevance of these findings, we showed that iRBC-

stimulated Vγ9Vδ2 T-cells can take up, process and cross-present exogenous M1 protein to M1p58-66-specific CD8 T-cells as effectively as *in vitro* HMBPP-stimulated Vγ9Vδ2 T-cells. *P.falciparum*-stimulated Vγ9Vδ2 T-cells were also capable of activating naive allogenic αβ T-cells, as shown by our MLR experiments. In this model, we observed some activation of αβ T-cells by Vγ9Vδ2 T-cells cultured with uiRBC supernatant, consistent with the induction of a small proportion of APC Vγ9Vδ2 T-cells in this condition (Figure 2) that is probably due to the presence of IL-2 and IL-15 in the culture medium. HMBPP stimulation consistently induced increased expansion and APC-marker expression in Vγ9Vδ2 T-cells compared to iRBC stimulation. This could be due to lower phosphoantigen levels with iRBC stimulation ($5x10^6$/ml iRBC equates to ~1 nM HMBPP [31]), but we cannot rule out the presence of some iRBC-derived inhibitor mitigating the activation. iRBC released molecules such as hemozoin have been shown to inhibit DC maturation [13]. However, if similar inhibition occurs for the Vγ9Vδ2 T-cells, it does not overtake their activation by phosphoantigens. CD36-dependent iRBC adhesion has also been proposed to mediate DC inhibition [15]. The FCR3 parasite strain used in our assays was non-adherent, therefore CD36-mediated inhibition would not occur in our experimental system. As CD36 expression of Vγ9Vδ2 T-cells post zoledronate stimulation has been documented [32], it will be important to assess Vγ9Vδ2 T APC-like maturation after co-culture with CD36-binding iRBCs. Recent data on *Mycobacteria* immunity [33] showed that APC functions of Vγ9Vδ2 T-cells do not require soluble mediators and involve cell-to-cell contact. In our hands, both intact iRBC and soluble products from *P.falciparum* were able to stimulate Vγ9Vδ2 T-cells. Moreover, the abrogation of such stimulation after phosphatase treatment of the supernatants indicates that soluble phosphorylated mediators are necessary to

induce APC-like functions. Thus, it is likely *M. tuberculosis* and *P.falciparum* differ in their mechanisms of APC induction in Vγ9Vδ2 T-cells.

Magnitudes and kinetics of APC-marker stimulation differ in the publications mentioned above [29, 30, 32, 33], suggesting that *in vitro* culture conditions can modulate Vγ9Vδ2 T-APC maturation kinetics. Under the stimulatory conditions we used, there appears to be two patterns of expression at least within the timeframe of our observation, with first transient expression of CD40 and CD83 alongside increased HLA class I expression and later on stable expression of HLA-DR, alongside expression of CD80 and CD86. Though peak expression points didn't coincide for the two types of phenotype, expression of all markers was above base-line between days 6 and 9 when Vγ9Vδ2 T-cells were stimulated among the PBMCs and between days 3 and 6 when they were purified before stimulation. In the latter situation, the pattern was sufficient to induce allogenic αβ T-cell stimulation. From our kinetics data, one can surmise that *in vivo* the Vγ9Vδ2 T-APCs have matured sufficiently after 3-5 days of iRBC exposure. Based on their parasitemia at admission, the patients have experienced on average 3-5 replication cycles, *i.e* ~6-10 days of blood-stage infection. Thus, the reduced CD40 and high HLA-DR and CD86 levels in patients Vγ9Vδ2 T-cells correlate with the kinetics of expression observed in our cultured lines. It will be important to assess the functional ability of *in vivo* iRBC-induced Vγ9Vδ2 T-APC-like cells. Our study design did not allow investigating this point, which requires to collect from patients much larger volumes of blood.

Taken together, our present study proposes a novel role for human Vγ9Vδ2 T-cells in malaria-infected patients, promoting initiation of the adaptive response despite a possible impairment of conventional APCs.

NOTES

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Conflict of interest

The authors have no financial conflicts of interest.

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REFERENCES

1. Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev **2007**; 215:59-76.
2. Costa G, Loizon S, Guenot M, et al. Control of Plasmodium falciparum erythrocytic cycle: gammadelta T cells target the red blood cell-invasive merozoites. Blood **2011**; 118:6952-62.
3. Farouk SE, Mincheva-Nilsson L, Krensky AM, Dieli F, Troye-Blomberg M. Gamma delta T cells inhibit in vitro growth of the asexual blood stages of Plasmodium falciparum by a granule exocytosis-dependent cytotoxic pathway that requires granulysin. Eur J Immunol **2004**; 34:2248-56.
4. Troye-Blomberg M, Perlmann P, Mincheva Nilsson L, Perlmann H. Immune regulation of protection and pathogenesis in Plasmodium falciparum malaria. Parassitologia **1999**; 41:131-8.
5. Altvater B, Pscherer S, Landmeier S, et al. Activated human gammadelta T cells induce peptide-specific CD8+ T-cell responses to tumor-associated self-antigens. Cancer Immunol Immunother **2012**; 61:385-96.
6. Brandes M, Willimann K, Bioley G, et al. Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. Proc Natl Acad Sci U S A **2009**; 106:2307-12.
7. Brandes M, Willimann K, Moser B. Professional antigen-presentation function by human gammadelta T Cells. Science **2005**; 309:264-8.
8. Khan MW, Curbishley SM, Chen HC, et al. Expanded Human Blood-Derived gammadeltaT Cells Display Potent Antigen-Presentation Functions. Front Immunol **2014**; 5:344.
9. Landmeier S, Altvater B, Pscherer S, et al. Activated human gammadelta T cells as stimulators of specific CD8+ T-cell responses to subdominant Epstein Barr virus epitopes: potential for immunotherapy of cancer. J Immunother **2009**; 32:310-21.
10. Tyler CJ, McCarthy NE, Lindsay JO, Stagg AJ, Moser B, Eberl M. Antigen-presenting human γδ T-cells promote intestinal CD4+ T-cell expression of IL-22 and mucosal release of calprotectin. J Immunol **2017**; In press.
11. Bettiol E, Carapau D, Galan-Rodriguez C, Ocana-Morgner C, Rodriguez A. Dual effect of Plasmodium-infected erythrocytes on dendritic cell maturation. Malar J **2010**; 9:64.

12. Elliott SR, Spurck TP, Dodin JM, et al. Inhibition of dendritic cell maturation by malaria is dose dependent and does not require Plasmodium falciparum erythrocyte membrane protein 1. Infect Immun **2007**; 75:3621-32.

13. Millington OR, Di Lorenzo C, Phillips RS, Garside P, Brewer JM. Suppression of adaptive immunity to heterologous antigens during Plasmodium infection through hemozoin-induced failure of dendritic cell function. J Biol **2006**; 5:5.

14. Pinzon-Charry A, Woodberry T, Kienzle V, et al. Apoptosis and dysfunction of blood dendritic cells in patients with falciparum and vivax malaria. J Exp Med **2013**; 210:1635-46.

15. Urban BC, Ferguson DJ, Pain A, et al. Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. Nature **1999**; 400:73-7.

16. Pistone T, Diallo A, Mechain M, Receveur MC, Malvy D. Epidemiology of imported malaria give support to the hypothesis of 'long-term' semi-immunity to malaria in sub-Saharan African migrants living in France. Travel Med Infect Dis **2014**; 12:48-53.

17. Pistone T, Guibert P, Gay F, et al. Malaria risk perception, knowledge and prophylaxis practices among travellers of African ethnicity living in Paris and visiting their country of origin in sub-Saharan Africa. Trans R Soc Trop Med Hyg **2007**; 101:990-5.

18. Societe de Pathologie Infectieuse de Langue F, College des Universitaires de Maladies Infectieuses et T, Societe Francaise de Medecine des A, et al. Recommendations for clinical practice. Management and prevention of imported Plasmodium falciparum malaria. (Revision 2007 of the 1999 Consensus conference). Short text. Med Mal Infect **2008**; 38:54-67, 39-53.

19. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science **1976**; 193:673-5.

20. van der Heyde HC, Elloso MM, vande Waa J, Schell K, Weidanz WP. Use of hydroethidine and flow cytometry to assess the effects of leukocytes on the malarial parasite Plasmodium falciparum. Clin Diagn Lab Immunol **1995**; 2:417-25.

21. Boyle MJ, Richards JS, Gilson PR, Chai W, Beeson JG. Interactions with heparin-like molecules during erythrocyte invasion by Plasmodium falciparum merozoites. Blood **2010**; 115:4559-68.

22. Lambros C, Vanderberg JP. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol **1979**; 65:418-20.

23. Davey MS, Lin CY, Roberts GW, et al. Human neutrophil clearance of bacterial pathogens triggers anti-microbial gammadelta T cell responses in early infection. PLoS Pathog **2011**; 7:e1002040.

24. Meuter S, Eberl M, Moser B. Prolonged antigen survival and cytosolic export in cross-presenting human gammadelta T cells. Proc Natl Acad Sci U S A **2010**; 107:8730-5.

25. Stanisic DI, Barry AE, Good MF. Escaping the immune system: How the malaria parasite makes vaccine development a challenge. Trends Parasitol **2013**; 29:612-22.

26. Okoye AA, Picker LJ. CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure. Immunol Rev **2013**; 254:54-64.

27. Hviid L, Kurtzhals JA, Adabayeri V, et al. Perturbation and proinflammatory type activation of V delta 1(+) gamma delta T cells in African children with Plasmodium falciparum malaria. Infect Immun **2001**; 69:3190-6.

28. Brandes M, Willimann K, Lang AB, et al. Flexible migration program regulates gamma delta T-cell involvement in humoral immunity. Blood **2003**; 102:3693-701.

29. Frossard CP, Asigbetse KE, Burger D, Eigenmann PA. Gut T cell receptor-gammadelta(+) intraepithelial lymphocytes are activated selectively by cholera toxin to break oral tolerance in mice. Clin Exp Immunol **2015**; 180:118-30.

30. Wan F, Hu C-b, Ma J-x, Gao K, Xiang L-x, Shao J-z. Characterization of γδ T Cells from Zebrafish Provides Insights into Their Important Role in Adaptive Humoral Immunity. Frontiers in Immunology **2017**; 7.

31. Guenot M, Loizon S, Howard J, et al. Phosphoantigen Burst upon Plasmodium falciparum Schizont Rupture Can Distantly Activate Vgamma9Vdelta2 T Cells. Infect Immun **2015**; 83:3816-24.

32. Muto M, Baghdadi M, Maekawa R, Wada H, Seino K. Myeloid molecular characteristics of human gammadelta T cells support their acquisition of tumor antigen-presenting capacity. Cancer Immunol Immunother **2015**; 64:941-9.

33. Abate G, Spencer CT, Hamzabegovic F, Blazevic A, Xia M, Hoft DF. Mycobacterium-Specific gamma9delta2 T Cells Mediate Both Pathogen-Inhibitory and CD40 Ligand-Dependent Antigen Presentation Effects Important for Tuberculosis Immunity. Infect Immun **2015**; 84:580-9.

LEGENDS TO THE FIGURES

Figure 1. Vγ9Vδ2 T lymphocytes from *P.falciparum* malaria patients show increased expression of presenting and co-stimulatory molecules compared to healthy and non-malaria febrile infected controls. Vγ9Vδ2 T lymphocytes from *P.falciparum*-infected patients collected at admission pre-treatment were assessed for both APC and memory phenotypes. Patients with non-malaria febrile symptoms, and non-malaria exposed healthy donors were used as controls. % of **(A)** HLA-DR, CD40 and $CD86^+/V\delta2^+$ T-cells and **(B)** T_{naive}, T_{cm}, T_{em} and T_{emra} $V\delta2^+$ T-cells in whole blood from healthy controls (n=11), febrile controls (n=6) and malaria patients at the time of hospital admission (n=27). Each point represents an individual patient. The mean is indicated by the horizontal bar. Data were analysed using Mann Whitney U test for unpaired samples (**P<0.01, *P<0.05). **(C)** % of HLA-DR, CD40, $CD86^+/V\delta2^+$ T-cells (left panel) and % T_{naive}, T_{cm}, T_{em} and T_{emra} $V\delta2^+$ T-cells (right panel) in the 5 patients from whom paired blood samples on the day of admission (D0) and on day 7 post-treatment (D7) were obtained. The trends observed were non significant as calculated by a Wilcoxon signed rank tests for matched pairs. **(D)** Histograms (Unstained control in grey) at D0 and % of APC and memory phenotype of $V\delta2^+$ T-cells on D0 and D7 in whole blood of a severe malaria case (patient#11) presenting with 19% parasitemia at admission.

Figure 2. Vγ9Vδ2 T lymphocytes acquire an APC-like phenotype after *in vitro* stimulation by *P.falciparum* blood-stage parasites. Kinetics of $V\delta2^+$ T-cell expansion and HLA-DR, CD80, CD86, CD83 and CD40 expression on $V\delta2^+$ T-cells stimulated 12 days with 5 $x10^6$/ml iRBC (red), $5x10^6$/ml uiRBC (blue), 10nM HMBPP (green) or complete medium (black). **(A)** Representative donor and **(B)** Mean of 5 donors. Expressed % positive $V\delta2^+$ T-cells. The mean is indicated by the horizontal

bar. Statistical significance was calculated using 2-way ANOVA with Bonferroni correction, with comparing at each time point the values for % of marker expression in (uiRBC – iRBC) (***P<0.001, **P<0.01, *P<0.05).

Figure 3. *P.falciparum*-stimulated Vγ9Vδ2 T-cells are able to induce αβ T lymphocyte allogenic stimulation. Freshly isolated Vγ9Vδ2 T-cells were stimulated for 6 days and mixed with allogenic $CD4^+$ (left) and $CD8^+$ (right) T-cells. Proliferation was assessed at day 5 as % of CFSE low in **(A)** total αβ $CD4^+$ and $CD8^+$ T-cells co-cultured with Vγ9Vδ2 T-cells stimulated with 0,2-1 nM HMBPP or 1-2x10^6 intact iRBC/ml or **(B)** naive αβ $CD4^+$ and $CD8^+$ T-cells co-cultured with (black circles) either fresh unstimulated Vγ9Vδ2 T-cells (unstim) or Vγ9Vδ2 T-cells stimulated with 50% v/v supernatants (SN) of iRBC or uiRBC, or cultured with phosphatase alkaline-treated iRBC supernatants (iRBC-SN+AP). **(C)** Intracellular IFNγ expression by αβ T-cells was measured after the first 5 h of co-culture shown in B. αβ T-cells alone non-stimulated (ns) or stimulated with Staphylococcus Enterotoxine B (SEB) were used as negative and positive controls respectively (grey circles). Mean ±SD of three independent experiments. Data were analyzed using a Friedman test with Dunn's multi-comparison correction. In **A,** global analysis was performed excluding SEB condition used as positive control for αβ T-cell proliferation (***P<0.001, **P<0.01, *P<0.05).

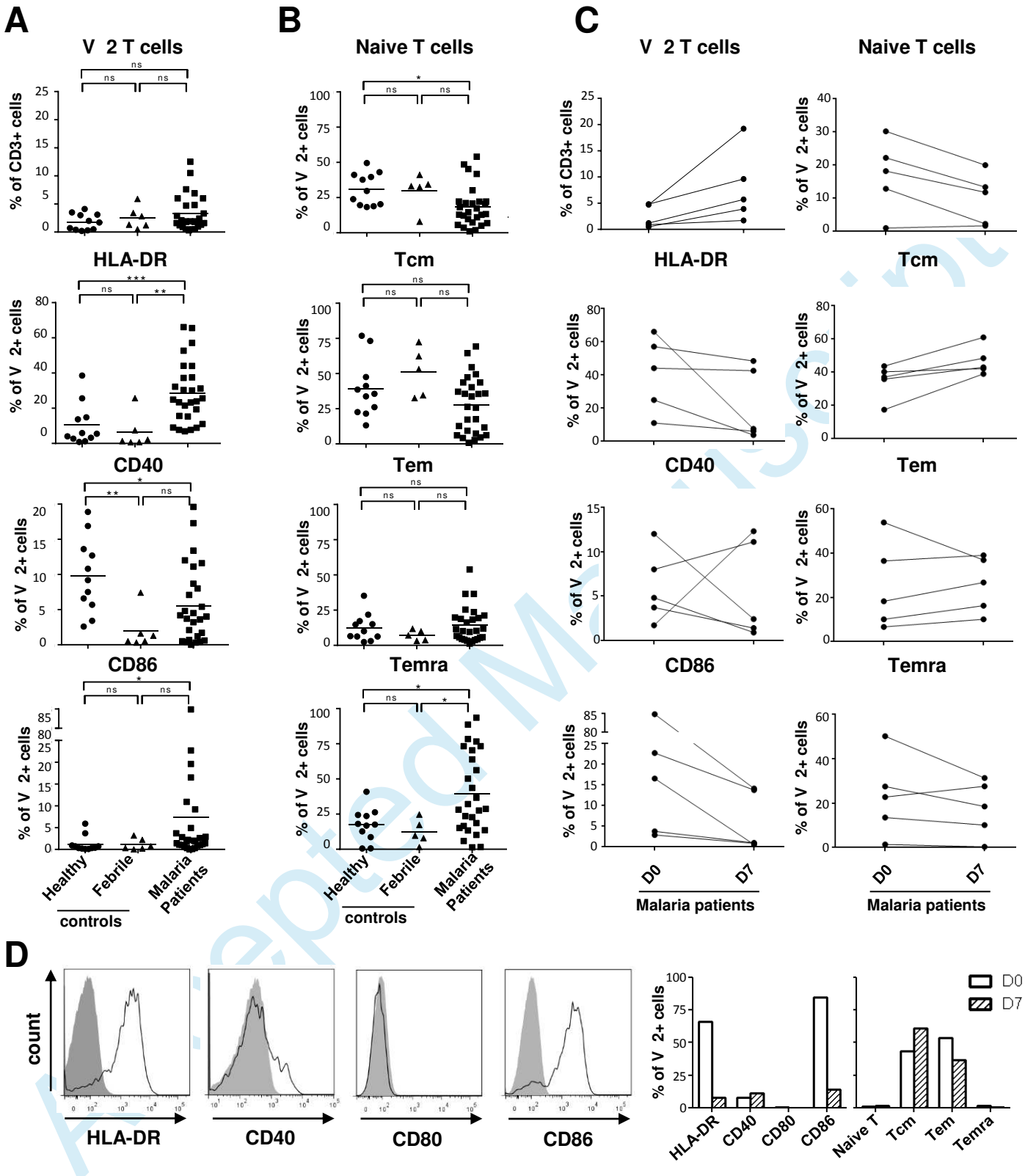
Figure 4. iRBC-stimulated Vγ9Vδ2 T-cells are able to cross present an M1 derived antigen to specific $CD8^+$ αβ T lymphocytes. HLA-A2-positive and -negative Vγ9Vδ2 T-cell lines generated by stimulation for 12 days with 5x10^6 iRBC/ml (black bars) or 10 nM HMBPP (white bars), sorted by positive selection and loaded overnight with M1 protein, or loaded 1 h with 0.01 µM M1pp58-66. Vγ9Vδ2 T-

cells were then washed thoroughly and co-cultured with M1pp58-66 specific CD8 T-cells and the IFNγ production by the CD8 T-cells measured. **(A)** Gating strategy for $IFN\gamma^+$ $CD8^+$ T-cell identification. Unstimulated control in grey histogram. **(B)** Intracellular IFNγ expression by $CD8^+$ αβ T-cells. $CD8^+$ αβ T-cells alone, non-stimulated (ns) or stimulated with PMA/ionomycin (P/I) were used as negative and positive controls, respectively (grey bars). Mean ±SEM of two independent experiments with n=3 for $HLA\text{-}A2^+$ and n=2 for $HLA\text{-}A2^-$ Vγ9Vδ2 T-cells.

Table 1: Patient cohort information.

Patient	Febrile Infection	Parasite-mia	Details	Duration of stay in malaria endemic zone	Group	Disease attributes	Anti-infection specific treatment after sampling
P1	Febrile, non-malarial	NA		ND	FC		
P2	Dengue fever	NA	Non-migrant	ND	FC	Non-complicated	Unknown
P3	E Coli urinary infection (compatible with pyelonephritis)	NA	Non-migrant	ND	FC	Non-complicated / RAS	Ceftriaxone
P4	Prostatitis - Klebsiella pneumonia BLSE + Lambliasis (fortuitous discovery)	NA	Migrant, from the Congo	ND	FC	Acute kidney dysfunction with urinary retention.	Ceftriaxone, then ertapenem then sulfamethoxazole and trimethoprim (bactrim)+albendazole
P5	EBV infection (compatible with infectious mononucleosis)	NA	Migrant, regularly travel to Tchad.	ND	FC	Non-complicated / RAS	Unknown
P6	Undocumented viral infection	NA	Migrant, from Cameroon	3 weeks	FC		Sampled before treatment
P7	P. falciparum	0.5	Non migrant	2 weeks humanitarian mission.	FTEO	Non-complicated	DHA-PQP + metronidazole (blastocytis hominis in feces)
P8	P. falciparum	0.6	Non-migrant, previous malaria episodes.	2 months in Ivory Coast	FTEO	Non-complicated / high platelets	DHA-PQP
P9	P. falciparum	3	Military.	5 months military mission	FTEO	Non-complicated	DHA-PQP
P10	P. falciparum	4.4	Non-migrant, previous malaria cases.	7 months humanitarian mission	FTEO	Non-complicated	DHA-PQP
P11	P. falciparum	19	Non-migrant, frequent trips to the Ivory Coast.	2 weeks	FTEO	Severe: hyperparasitemia, prostration, instability, hemodynamic.	Artesunate then DHA-PQP + ceftriaxone
P12	P. falciparum	<0,01	Not specified	ND	TEO		
P13	P. falciparum	0.05	Not specified	2 weeks	TEO	Non-complicated	Atovaquone/proguanil
P14	P. falciparum	0.5	Non-migrant		TEO	Non-complicated	DHA-PQP
P15	P. falciparum	2.23	Non-migrant	1 month	TEO	Non-complicated	DHA-PQP
P16	P. spp	0 (PCR+)	Migrant, born in the Ivory Coast.	Internship	VFR	Non-complicated	Atovaquone/proguanil
P17	P. falciparum	<0,01	Migrant, from the Ivory Coast.	5 weeks	VFR	Non-complicated	DHA-PQP then atovaquone/proguanil
P18	P. falciparum	0.02	Migrant, of many years.	Expatriate	VFR	Non-complicated	DHA-PQP
P19	P. falciparum	0.02	Migrant, resident many years in Equatorial Guinea and Cameroon.	2 weeks	VFR	Non-complicated	DHA-PQP
P20	P. falciparum	0.06	Migrant, resident many years in Gabon.	2 weeks	VFR	Non-complicated	DHA-PQP
P21	P. falicparum	0.14	Migrant, from Cameroon.	1 month	VFR	Non-complicated	DHA-PQP. Already treated quinine three days in Cameroon. Possible resistant parasite.
P22	P. falciparum	0.2	Migrant, from Cameroon.	3 months	VFR	Non-complicated	DHA-PQP
P23*	P. falciparum	0.3	Migrant, originally from the Ivory Coast.		VFR	Non-complicated	DHA-PQP
P24	P. falciparum	0.5	Migrant, from the Congo.	3 months	VFR	Non-complicated / supplementary conjunctivitis	DHA-PQP
P25*	P. falciparum	0.6	Migrant, from Congo.	ND	VFR	Non-complicated	DHA-PQP
P26	P. falciparum	1.1	Migrant, from Cameroon.	15 days	VFR	Non-complicated	DHA-PQP
P27	P. falciparum	1.44	Migrant, from Ivory Coast	2 months	VFR	Non-complicated	Atovaquone/proguanil (anomaly ecg - no DHA-PQP)
P28	P. falciparum	1.6	Migrant, born in Cameroon, multiple malaria cases in childhood.	10 days	VFR	Non-complicated	DHA-PQP
P29	P. falciparum	1.6	Migrant, lived long time in Ivory coast.	1 week	VFR	Non-complicated	DHA-PQP
P30	P. falciparum	3	Resident Cameroon, in France for work/tourism.	Resident	VFR	Sever: prostration	DHA-PQP
P31	P. falciparum	4.5	Migrant, from Cameroon, resident 7 years.	Expatriate (resident 2 years Douala)	VFR	Sever: jaundice	DHA-PQP
P32	P. falciparum	5	Migrant, from Angola	2 month	VFR	Sever: parasitemia 5%	DHA-PQP
P33	P. falciparum	5.5	Migrant, from Cameroon	1 month	VFR	Sever: parasitemia 5,5%	DHA-PQP
P34*	P. falciparum	6	Migrant, from Cameroon.	1.5 months Cameroon	VFR	Severe: parasitemia 6%	DHA-PQP
P35	P. falciparum	2	Migrant, from Senegal.	6 months, Senegal	VFR	Non-complicated	DHA-PQP
P36	P. falciparum + P. malariae (PCR only, not seen on blood smear)	0.85	Migrant	4 months Senegal	VFR	Non-complicated	DHA-PQP
P37	P. falciparum	0.04	Migrant, from Ivory Coast	4 weeks, Ivory Coast	VFR	Non-complicated	DHA-PQP

Abbreviations: ND: unknown; NA : non-applicable; P: Plasmodium ; EBV: Epstein-Barr virus; E: Escherichia ; DHA-PQP : dihydroartemisinin-piperaquine; FC : febrile controls; VFR : malaria patients migrants to France born in malaria endemic countries and visiting friends and relatives in malaria endemic areas; TEO : malaria patients, travellers of European (Caucasian) origin; FTEO : malaria patient, travellers of European (Caucasian) origin with a history of frequent visits to endemic areas; Migrant: migrant to France; Non-migrant: born and resident in France. *Insufficient (<200) Vγ9Vδ2 T-cell events recorded therefore patient not included in Figure 1 analysis.



NEW Figure 1.

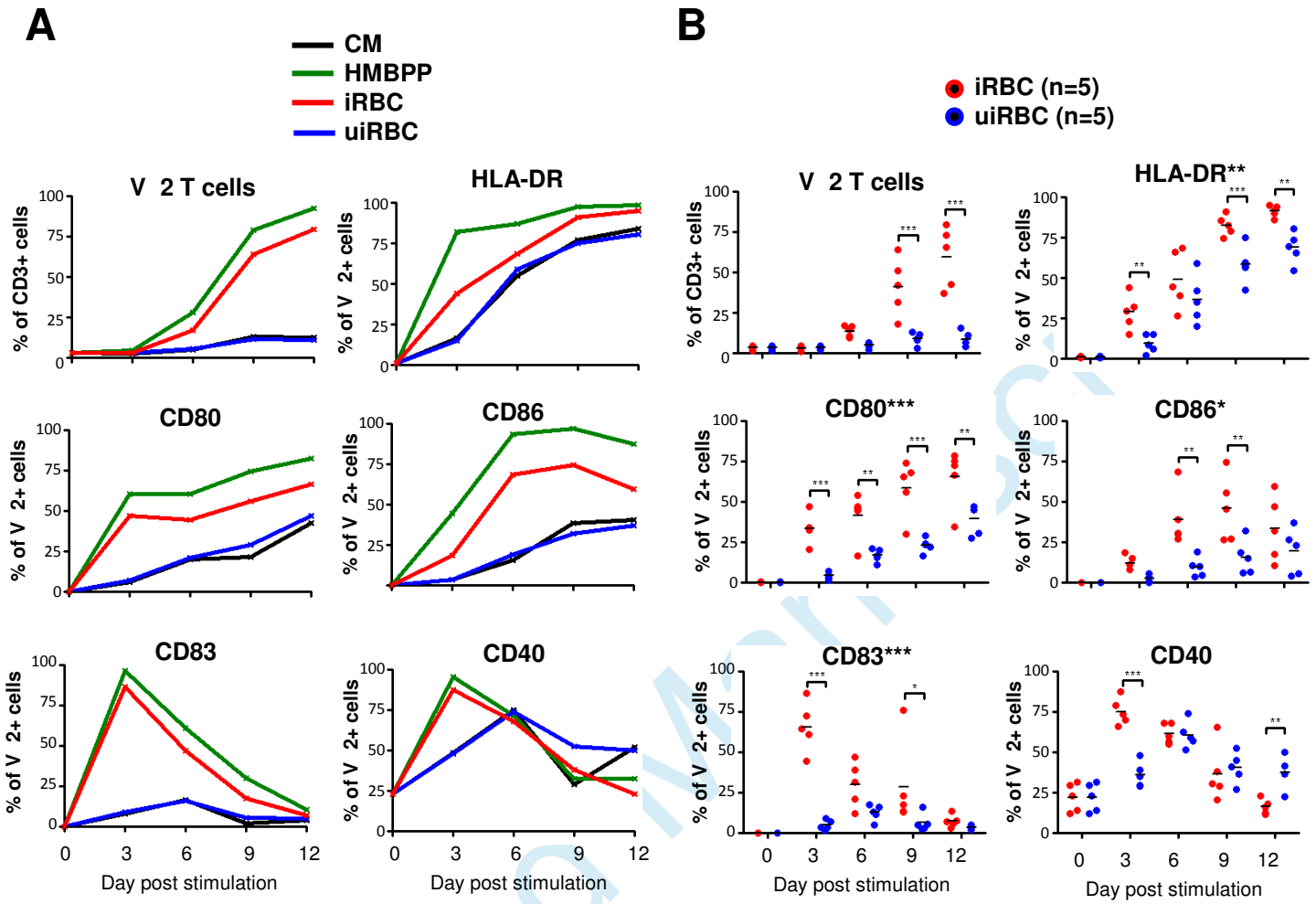
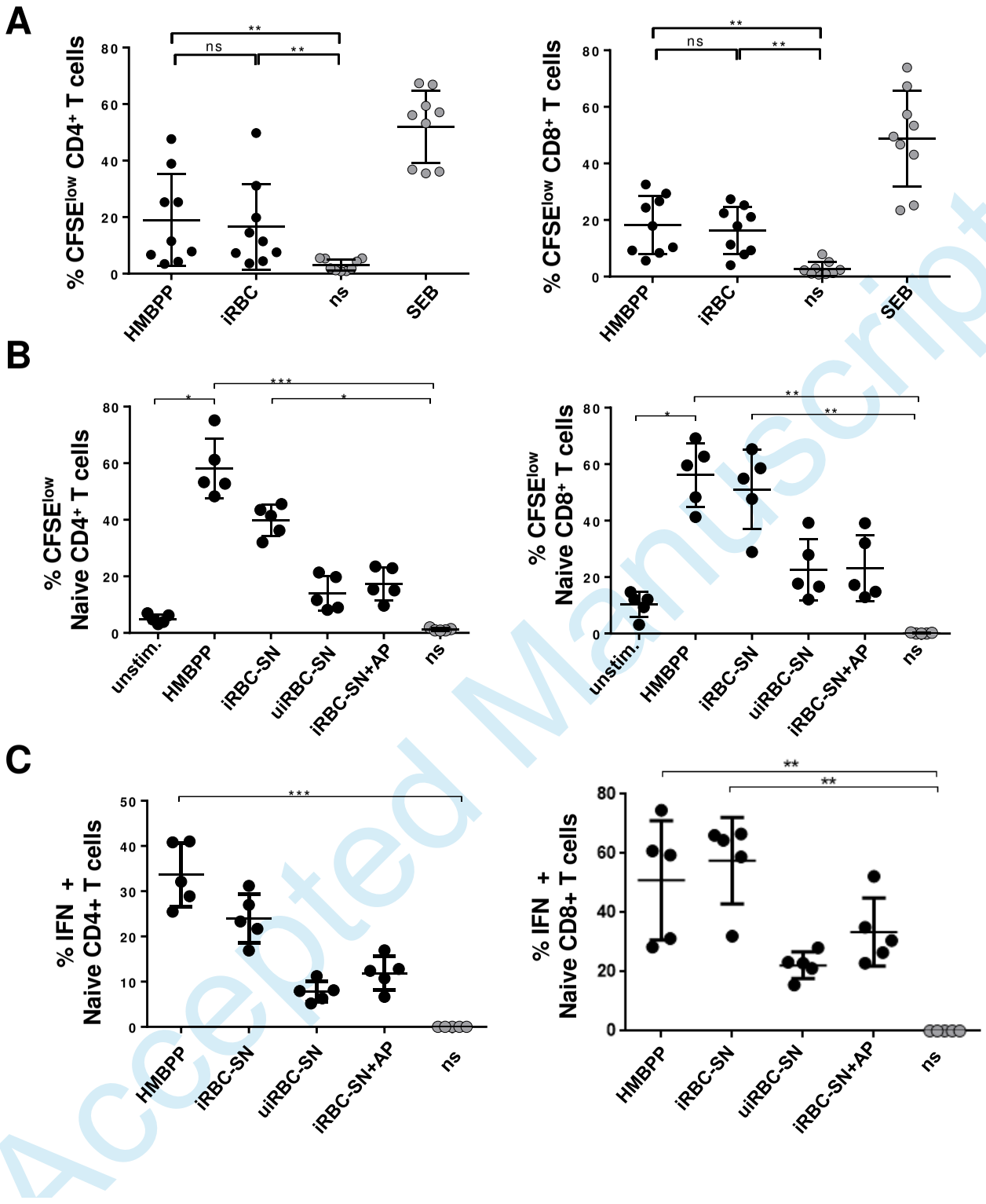


Figure 2.



NEW Figure 3.

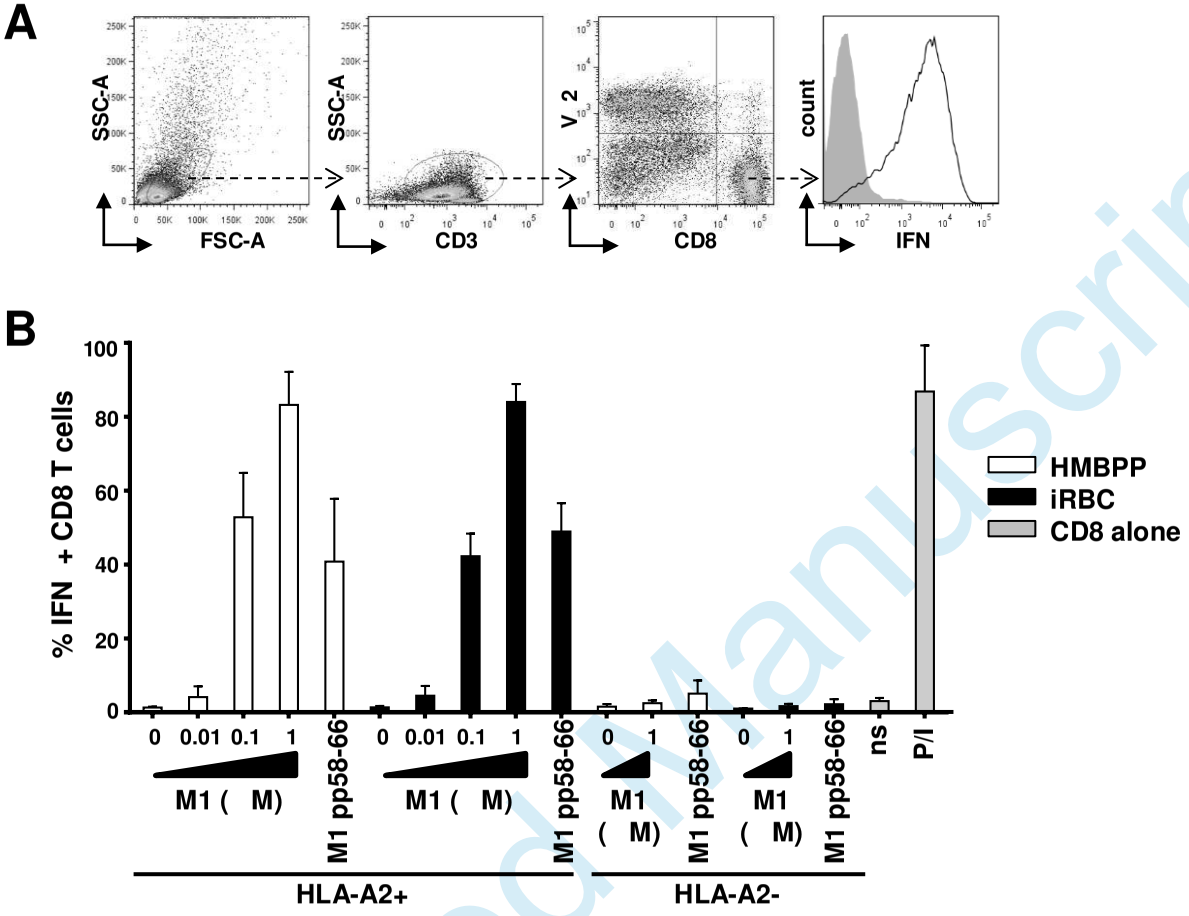


Figure 4.