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OMIP-064: A 27-Color Flow Cytometry Panel to Detect and Characterize Human NK Cells and Other Innate Lymphoid Cell Subsets, MAIT Cells, and γδ T Cells

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Abstract

This 27-color flow cytometry panel was developed in order to assess immunological changes over the course of an immunization and challenge regimen in two experimental malaria vaccine trials. The aim of the study was to find correlates of vaccine-induced protection. Several studies have indicated that protection against malaria appears to involve immune responses at various immunological sites, with liver-resident responses playing an essential role. As it is not feasible to monitor the immune responses within the liver in humans, this panel is developed with the aim to thoroughly characterize the immune responses over time in blood in addition to detecting changes that might reflect what happens in other immunological sites like the liver. The focus of this panel is to detect several innate lymphoid cell populations, including NK cells and their activation status. Moreover, unconventional T cells like mucosal associated invariant T cells and γδ T cells are assessed in the panel.

Keywords

flow cytometry; human PBMC; γδ T cells; NK cells; T cells; MAIT cells; innate lymphoid cells

BACKGROUND

Malaria is still a major health threat, with 200 million cases and approximately 400,000 deaths annually, mostly among children under 5 (1–3). The disease is caused by mosquito-transmitted *Plasmodium* parasites that have a complicated lifecycle which occurs in multiple sites of the body, including the liver and the blood. Although efforts to design a potent anti-malaria vaccine have been ongoing for nearly a century, there is still no vaccine

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

that confers adequate durable immunity to infection. Furthermore, naturally occurring sterilizing immunity to malaria is rare, despite repeated infections (4,5). Immunization with radiation-attenuated sporozoites (RAS), which are parasites that have retained their ability to infect the liver, has been shown to confer sterilizing protection. However, the mechanism behind this protection is incompletely understood (1,6–9). Remarkably, the occurrence of natural infection seems to inhibit the development of protective sterilizing immunity, as clinical trials in nonendemic regions consistently report higher vaccine efficacies than those in malaria-endemic regions (10). It has been suggested that tolerogenic responses, immune exhaustion, and senescence play a role (11). Identifying the underlying immune mechanisms of anti-malarial immunity or the lack thereof will aid the development of a protective vaccine that is suitable for mass distribution. High parameter multicolor flow cytometry enables thorough characterization of immune responses against pathogens. Here we describe a 27-color panel that aims to detect immune responses that correlate with protection in experimental malaria vaccine trials, in addition to comparing responses in individuals from malaria endemic regions with those from nonendemic regions. This panel was developed for use with three other panels, in order to extensively phenotype the immune responses triggered by RAS-immunizations. The other panels we developed focus on T and B cells and we used a dendritic/monocyte panel published as an optimized multicolor immunofluorescence panel (OMIP) (Table 1) (12).

The panel described here is developed with a focus on innate lymphoid cells (ILCs), conventional NK cells and their activation status, γδ T cells, mucosal associated invariant T (MAIT) cells in addition to the major lineages including T cells, B cells and monocytes, both for exclusion of other lineages and to characterize potential expression of NK-relevant markers in these other cell types. All the reagents are listed in Table 2. The panel includes lineage markers CD14 and CD33 to gate out monocytes, dendritic cells, and granulocytes and CD19 for the exclusion of B-cells. For the gating of T cells, CD3, CD4, and CD8 are used to identify the conventional T cell subsets, although these markers can be expressed on several other cell types detected by this panel, such as NK cells. CD161 and TCRvα7.2 are included to gate on MAIT cells. These cells are detected in peripheral blood, although they are more abundant at mucosal sites and in the liver. Their role in the protection against malaria infection is unknown, although MAIT cells were observed to contract and then expand in a controlled human malaria-infection(CHMI) study (13).

Another unconventional T cell subset that has generated attention in the malaria immunology field is the subset of T cells expressing the γδ T cell receptor (TCR). These γδ T cells have been shown to be expanded in acute malaria infection, and a recent study showed that the Vδ2 subset was found to correlate with protection in a large cohort of healthy, malaria-exposed individuals that were immunized with a RAS-vaccine (14). In addition, their relevance to malaria has also been demonstrated in animal models, for example, γδ T cells are required for the induction of sterile immunity in a rodent model (14). We have therefore included antibodies detecting the γδ TCR and Vδ2 to identify γδ T cells and the Vδ2 subset.

Animal models have helped elucidate some of the mechanisms needed for protective immune responses in the liver, and NK cells have previously been shown to be important

in the immune responses during the early phases of liver stage infection. NK cells likely contribute through the production of IFNγ and potentially through direct cytolysis of infected hepatocytes (15,16).

In humans, NK cells have been shown to play a role in malaria disease, both as having protective effects against the pathogen in addition to contributing to pathology in cerebral malaria (17). We have included several markers to extensively characterize NK phenotypes, in which maturation status, differentiation, and activation markers are included. To phenotype NK cells, we used CD56 in combination with CD16 to delineate different NK subsets. An extensive set of NK cells markers were included to monitor maturation and differentiation, which can also indirectly indicate the functionality of these NK subsets. Recently, so-called adaptive NK cells that lack FCεRI-γ were associated with protective effects in a large cohort of seasonal malaria transmission monitoring (18). This marker is combined with NKG2C, CD57 and a lack of NKG2A expression to identify these adaptive NK cells (19). CD27 has been implicated as another maturation or memory-like marker, and has therefore been included (20). CD27 on NK cells mark mature NK cells with low cytotoxic potential (21). CD27-expressing NK cells were also indicated as being memory-like NK cells in a murine tuberculosis model (22). NKp30, NKp46, and CD38 are included to monitor activation of NK cells. CD38 was recently described to be a key regulator in NK cells that are enhanced in their cytotoxic abilities and cytokine producing potential (23,24). The activating receptor NKG2D was shown to be highly expressed in liver-resident NK cells in a rodent model, and implicated in humans (20,25,26). NKG2D ligands are upregulated in response to type I interferons, which have been shown to be induced in plasmodium-infected hepatocytes in mice, and these cells could therefore be of interest to monitor (15,27). Several of the NK markers in this panel have been shown to be expressed on the conventional and unconventional T cells detected by this panel. For instance, NKG2A and CD27 can be detected on γδ T cells as indirect markers for IFNγ-producing γδ T cells (28), and CD57 expression on T cells can be used as an exhaustion marker, previously shown to be upregulated in Plasmodium falciparum infection (11).

The markers CD16, CD161, CD127 c-kit, and CRTH2 can together be used to differentiate the ILC subsets, which are found in low abundance in the blood, although they are more prevalent in tissues (29). The role of ILCs in malaria vaccine responses has not been identified yet, although sparse data indicate that ILCs may have a role in infection. For instance, blood stage infection led to a rapid loss of group 1 ILCs in the blood of subjects participating in a CHMI study (30). Another study suggested that group 2 ILCs were involved in protection against cerebral malaria (31). As an additional marker to phenotype cellular responses, we included Ki67 as a marker for proliferation and recent in vivo activation. Figure 1 shows an example of how these markers can be used to gate on different cell subsets. This panel can be used for preipheral blood mononuclear cells (PBMC), and possibly for other sample types.

SIMILARITY TO PUBLISHED OMIPs

This panel is unique, although there is some overlap with OMIP-056 (32), which also looks at MAIT, γδ T cells, and NK cells (Table 1). However, the focus of that panel is more on

functional responses in the context of HIV infection, and does not deeply phenotype subsets of NK cells and unconventional T cells, as in this panel. The 21-marker panel described in OMIP-055 (33) has nine markers in common with this panel, although four of those are lineage markers and all lineage markers are assigned to the same fluorochrome, which limits the depth of the analysis to ILC subsets. OMIP-039 (34) and OMIP-029 (35) describe panels that phenotype NK cells that partly overlap with this panel, although both panels are less elaborate, as they include 14 and 13 colors, respectively, and can therefore not combine both NK cell markers with MAIT and γδ T cells. OMIP-058 also has several markers that overlap with our panel, although the emphasis is on γδ T cells and iNKT cells and the depth of NK subset phenotyping is more limited than the panel we describe here. Overall, our panel is unique because it enables a deep assessment of NK subsets in addition to conventional and unconventional T cells, combined with an extensive set of activation and maturation markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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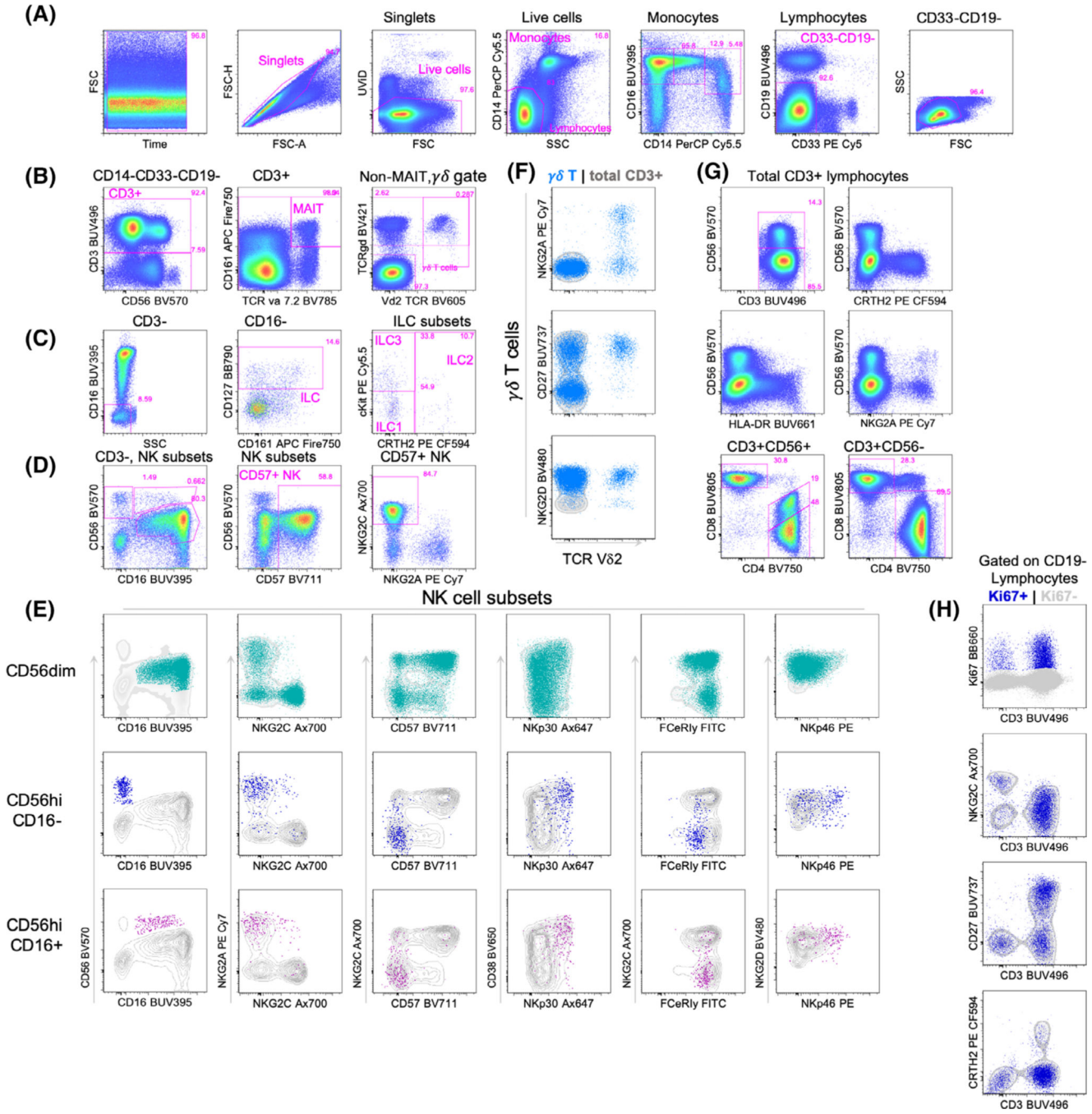

FIGURE 1.

Example of the gating strategy of the 27-color measurement of cryopreserved PBMCs. (**A**) The cells from a healthy donor were stained and the data were subsequently acquired on a BD FACSymphony instrument. The plot depicting forward scatter (FSC) vs. time is to verify that there are no pressure fluctuations that might affect fluorescent signals. The FSC-Area (A) vs. FSC-Height (H) is to gate on single cells and to exclude doublets. Next, the UViD-negative events are gated to exclude dead or damaged cells. CD14 vs. Side scatter (SSC)-A is used to gate monocytes (CD14+ and SSChi) vs. lymphocytes (CD14− and

SSClo), and CD14 and CD16 are used to gate on the different monocyte subsets. In parallel, CD19 and CD33 are used as exclusion channels, to gate out B cells and granulocytes. Another clean-up gate for lymphocytes is included based on FSC and SSC. (**B**) The negative fraction is then plotted for CD3 to gate on T cell subsets. First, MAIT cells are gated as CD3+ TCRγδ− CD161+ and TCRva7.2+. The non-MAIT cells are then used to gate γδ T cells, as CD3+ and TCRγδ+. The vδ2 T cells are double positive for TCRγδ+ and the vδ2TCR. The negative fraction is then used to gate on conventional T cells that are either CD4+ or CD8+. (**C**) The CD3-gate is use for further delineation of ILCs. The ILC subsets are gated as CD3-CD16-CD127 + CD161+, although ILC1 and LTi have been shown to lack CD161 expression, so ILCs can be gated on without this marker as well. Further differentiation between ILC1, ILC2, and ILC3 can be done with CRTH2 and cKit, with CRTh2-CKit- cells gated as ILC1, CRTh2+ cells gated on as ILC2 and ILC3 are cKit+. (**D**) The CD3-fraction is alternatively gated as CD56 vs. CD16 for NK subsets. The second plot in this row shows that CD57 is mainly expressed on CD56dim NK cells. These cells are then plotted as NKG2A vs. NKG2C, the latter of which are adaptive NK cells. (**E**) The 3 NK subsets as shown in the left plot in each row are plotted separately for the remaining NK subset markers (CD57, NKG2A, NKG2C, NKG2D, NKp30, and NKp46), overlaid on the total CD3− population in gray. Also the expression of FCεRIγ is measured, “adaptive NK cells” have a low expression of this marker (36). (**F**) Depiction of the γδ T cells (blue overlay) and NKG2A, NKG2D and CD27 against vδ2.The background depicts total CD3+ cells, in gray. (**G**) A subset of CD3+ cells is positive for CD56 and these will include NK T cells. The expression of CRTH2, HLA-DR, and NKG2A is plotted vs. CD56 to demonstrate several phenotypic differences between the CD56+ and CD56− populations. The two plots in the third row show the CD4 versus CD8 pattern and how the CD56+ CD3+ cells have a CD4+ population that dimly expressed CD8. (**H**) Ki67+ is used as a proliferation marker and plotted against CD3+ on all CD14-CD33-CD19− lymphocytes, and shown as overlay combined with several markers that are different between the CD3+ and CD3− populations. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1

Summary table for application of OMIP-064

Purpose	Extensive phenotyping
Species	Human
Celltype	PBMC
Cross-references	OMIP-029, OMIP-039, OMIP-044, OMIP-055, OMIP-056, and OMIP-058

Table 2

Reagents used for OMIP-0

MARKER	ANTIBODY CLONE	FLUOROCHROME	PURPOSE
CD16	3G8	BUV395	NK, monocyte, ILC gating
Viability	NA	Fixable blue	Dead cell dump
CD3	UCHT1	BUV496	T cells
CD19	SJ25C1	BUV563	B cells
HLA-DR	G46–6	BUV661	Activation
CD27	L128	BUV737	NK phenotyping
CD8	SK1	BUV805	CD8+ T cells
TCRγδ	11F2	BV421	Pan-γδ T cells
NKG2D	1D11	BV480	NK phenotyping
CD56	HCD56	BV570	NK phenotyping
Vδ2 TCR	B6	BV605	γδ T cell subset vδ2
CD38	HB-7	BV650	Activation
CD57	HNK-1	BV711	NK phenotyping
CD4	SK3	BV750	CD4+ T cells
TCRvα7.2	3C10	BV786	MAIT cells
FCεRIγ	Poly	FITC	NK phenotyping
Ki-67	Ki67	BB660	Proliferation
CD14	M®P9	PerCP-Cy5.5	Monocytes
CD127	HIL-7R-M21	BB790	ILC phenotyping
NKp46	9E2/NKp46	PE	NK phenotyping
CRTh2	BM16	PE-CF594	ILC phenotyping
CD33	WM53	PE-Cy5	Granulocyte/monocyte exclusion
c-kit	104D2	PE-Cy5.5	ILC phenotyping
NKG2A	Z199	PE-Cy7	NK phenotyping
CD337/Nkp30	AF29–4D12	APC	NK phenotyping
NKG2C	134,591	Ax700	NK phenotyping
CD161	HP-3G10	APC-Fire750	MAIT cells/ILC phenotyping