Cytometry PART A
Journal of the International Society for Advancement of Cytometry

MIFlowCyt

OMIP-038: Innate Immune Assessment with a 14 Color Flow Cytometry Panel

Kinga K. Smolen,[1,2] Bing Cai,[3] Tobias R. Kollmann[1,3]*

[1]Department of Experimental Medicine, University of British Columbia, CFRI A5-147, 950 W28th Ave, Vancouver, British Columbia, V5Z 4H4, Canada

[2]Institute for Medical Immunology, Université Libre de Bruxelles, Rue Adrienne Bolland 8, Gosselies, B-6041, Belgium

[3]Department of Pediatrics, University of British Columbia, CFRI A5-147, 950 W28th Ave, Vancouver, British Columbia, V5Z 4H4, Canada

Received 15 September 2016; Revised 5 February 2017; Accepted 22 March 2017

Additional supporting information may be found in the online version of this article.

*Correspondence to: Tobias R. Kollmann, Department of Pediatrics, University of British Columbia, CFRI A5-147, 950 W28th Ave, Vancouver, British Columbia, V5Z 4H4, Canada. E-mail: tkollm@mac.com

Published online 17 April 2017 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23109

• **Key terms**
innate immunity; intracellular cytokine staining; innate immune profiling; whole blood; multiparametric flow cytometry

Purpose and Appropriate Sample Types

Profiling the innate immune system of young children in a state of health and disease, is an important but arduous task, plagued with restrictions and limitations. Once blood samples are obtained, no matter how limited in volume, they are extremely precious. Therefore great care and consideration must be taken in how these samples are assayed.

The purpose of the panel presented here is to assess the innate immune response in whole blood after Toll-like receptor (TLR) ligand or cytokine stimulation, at the single-cell level. In addition to the identification of many of the innate and innate-adaptive interface immune cell subsets, this panel also enables the detection of key intracellular cytokines involved in the immune response.

This panel was optimized for the evaluation of TLR-ligand and cytokine stimulated whole blood, which has been fixed and frozen, prior to flow cytometric analysis to allow batched analysis to reduce technical artifacts. The panel presented here is an expansion of a panel previously described in Refs. (1–4), but expanded here to capture most of the major innate immune subsets found in peripheral blood and their key functional cytokines. Although the specific application of this panel was to identify the baseline difference between groups of healthy children, shown in Ref. (5), this panel is readily applied to a wide range of normal and diseased-states in humans.

Background

The innate immune system instructs the adaptive immune response. Thus, an understanding of the innate immune response is essential in vaccine development and prediction of protective host immune responses following infection.

Often human blood samples are limited in volume; this is especially true for pediatric samples. These precious samples require an efficient multi-color analysis to extract the maximum information out of the smallest of samples. The focus of this panel is to precisely characterize the innate immune responses on the single cell level in a highly standardized way, allowing for the identification of specific cell subtypes, the exclusion of potential contaminating cells, and the incorporation of innate-adaptive interface cells and their function. Our immune profiling approach allows for exactly that; by the determining of cell phenotype and frequencies, differentiating between various cell subtypes, and quantifying functional cytokine response (Table 1).

ISAC
International Society for Advancement of Cytometry

Table 1. Summary table for application of panel

PURPOSE	THE CHARACTERIZATION OF THE INNATE IMMUNE RESPONSE
Species	Human
Cell Type	Whole Blood
Cross-references	none

Surface Phenotype

Our panel assesses the frequency of monocytes, dendritic cells (DC), neutrophils, $\gamma\delta$ T cells, T cells and B cells, as well as cytokines of interest. First, fixable viability dye (FVD) was used to stain the cells prior to incubation in FACSLyse, followed by freezing and storage of the samples prior to intracellular staining and flow cytometric analysis. The FVD specifically is able to withstand freezing and permeabilization of the cells contained in whole blood.

After the exclusion of dead cells, cell subset specific anchor markers were employed either alone or in combination to delineate many of the innate immune cell subsets in peripheral blood. CD66 allowed for the identification of neutrophils. CD14 versus HLA-DR (MHC class II) allowed for the initial separation of antigen presenting cells (APC), specifically monocytes and DC (all HLADR+), from the other leukocytes (HLADR−). The CD123 and CD11c markers provided further resolution between plasmacytoid DC (pDC) (HLADR+ CD11c− CD123+), conventional DC (cDC) (HLADR+ CD11c+ CD123−), and B cells (HLADR+ CD11c− CD123−) (3). Unlike the other cell types, B cells were identified using a previously described exclusion gating strategy (3). Next, CD16 allowed for the investigation of the three monocyte subsets; CD14+ CD16− (classical), CD14+ CD16+ (inflammatory), and CD14dimCD16+ (patrolling). These various monocytes subsets have been shown to have different functional profiles under certain stimulatory conditions or disease states (6).

T cells were identified by CD3. In combination with other markers, CD3 can be used to identify innate and adaptive interface cells. In conjunction with $\gamma\delta$ TCR, CD3 was used to identify $\gamma\delta$ T cells, while when combined with CD16 and CD56, it can be used to identify Natural Killer (NK).

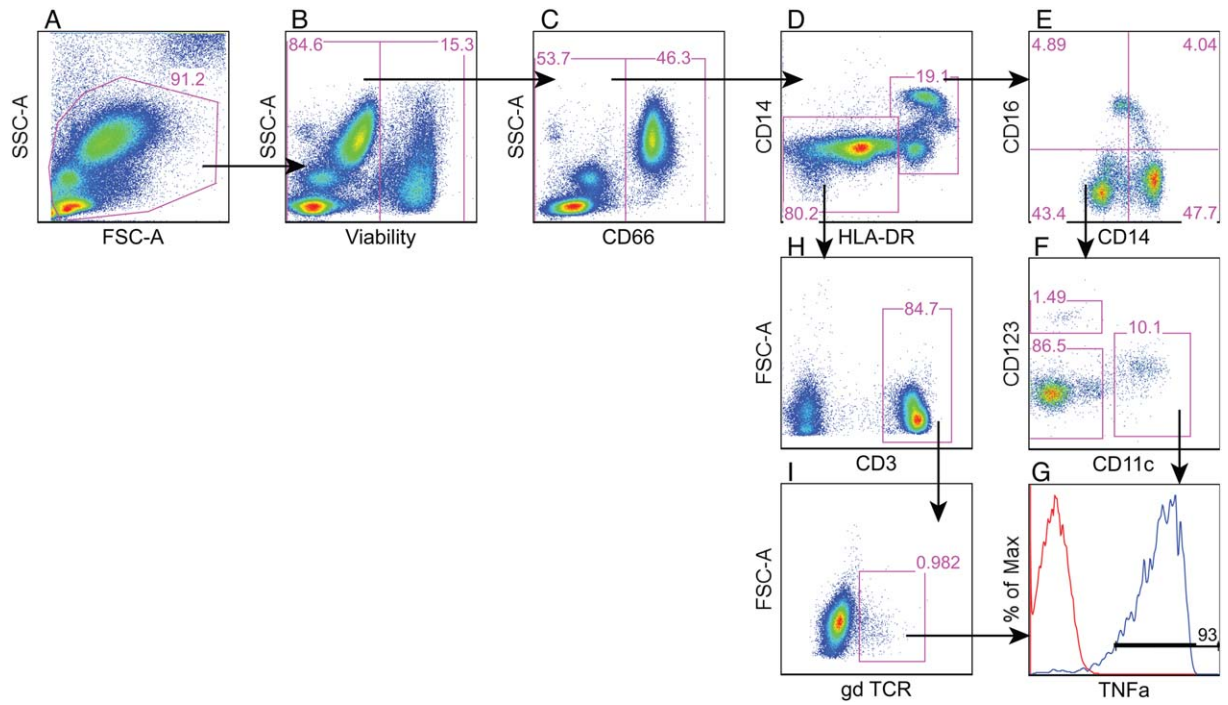

Figure 1. Gating strategy. After excluding doublet cells, we can select cells based on SSC-A versus FSC-A (A) followed by the viability discriminator (B). The CD66 marker identifies the neutrophil population allowing for their exclusion (C). The CD14 versus HLA-DR gate allows for the distinction of total monocytes (HLA-DR+ CD14+) and DCs (HLA-DR+ CD14−) from the total leukocyte population (HLA-DR− CD14−) (D). The HLA-DR+ population in gate D can be plotted using CD14 vs.CD16 to reveal the three-monocyte subsets; CD14+ CD16− (classical), CD14+ CD16+ (inflammatory), and CD14dimCD16+ (patrolling), as well as CD14− CD16− (DCs populations) in gate E. The CD14− CD16− population can be further divided into the DCs subset using CD123 versus CD11c providing further resolution between pDC (CD123+ CD11c−), cDC (CD123− CD11c+), and B cells (CD123− CD11c−) as seen in gate F. The production of relevant cytokines can readily be assessed for each cell subset (G). The leukocyte population can be identified as the CD14− HLA-DR− gate (D), from which CD3+ and CD3− populations can be identified (H). The CD3+ population can be subcategorized as $\gamma\delta$ T cells and T cells with the $\gamma\delta$ TCR marker (I). The production of relevant cytokines can readily be assessed for each cell subset (G). The histogram in G shows TNFα production, the blue line shows the unstimulated population and the red line show the R848 stimulated population. [Color figure can be viewed at wileyonlinelibrary.com]

Table 2. Reagents used for panel

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
Viability Dye		eFluor 780	Live/Dead
TNFα	MAb11	Alexa 700	Function
CD11c	S-HCL-3	APC	cDC
CD123	6H6	PE-Cy7	pDC
CD3	UCHTI	PE-CF594	T lineages
IFNa	7N4-1	PE	Function
IL-6	MQZ13A5	PerCP-eFluor 710	Function
gdTCR	B1.1	FITC	gd T cells
CD66	ASL-32	Biotin	Neutrophils
Streptavidin		BV786	–
IFNg	4S.B3	BV711	Function
CD16	3G8	BV650	NK cells, NK T cells, Monocyte
HLA-DR	LN3	eFluor 605	B cells, Monocytes
CD14	M5E2	V500	Monocytes
IL-12	C8.6	eFluor 450	Function

Intracellular Staining

In addition to the phenotypic description of the cells involved, the panel investigates the major cytokines of interest for each cell type described using intracellular staining, for a functional analysis of the cell-subsets. FACSLysed whole blood (WB) samples were permeabilized and stained to provide a cell-specific cytokine response. The pro-inflammatory cytokines TNFα, IL-12, and IL-6 are common cytokines produced by the APC within this panel. The magnitude of response is dependent on the stimulation used. IFNα is mainly produced by the pDC in response to TLR 7/8 and 9 stimulation. Lastly, IFNγ is also widely produced by T cells and γδ T cells.

The gating strategy for evaluating all these subsets using the developed panel is shown in Figure 1. Figure 1 depicts a fully stained WB sample, which has been stimulated with the TLR7/8 ligand R848, FACSLysed, and frozen prior to antibody staining. The reagents used within this panel may be found in Table 2. Additional information about the development of this panel and its gating strategy are provided in the supplementary data.

Similarity to Published OMIPS

While this current work is based on previous work found in Refs. (1–5), there are several published OMIP panels that are related. These include; OMIP 019, 020, 021, and 024 for the identification of γδ TCR, OMIP 023 that identify monocytes and NK cells, and OMIP 027 for NK cell function.

Acknowledgments

We would like to thank all the previous members of our lab that have inspired this panel.

Literature Cited

1. Jansen K, Blimkie D, Furlong J, Hajjar A, Rein-Weston A, Crabtree J, Reikie B, Wilson C, Kollmann T. Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation. J Immunol Methods 2008;336:183–192.
2. Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, Lavoie PM, Furlong J, Fortuno ES, Hajjar AM, et al. Neonatal innate TLR-mediated responses are distinct from those of adults. J Immunol 2009;183:7150–7160.
3. Blimkie D, Fortuno 3rd ES, Thommai F, Xu L, Fernandes E, Crabtree J, Rein-Weston A, Jansen K, Brinkman RR, Kollmann TR. Identification of B cells through negative gating-An example of the MIFlowCyt standard applied. Cytometry A 2010;77A:546–551.
4. Blimkie D, Fortuno 3rd ES, Yan H, Cho P, Ho K, Turvey SE, Marchant A, Goriely S, Kollmann TR. Variables to be controlled in the assessment of blood innate immune responses to Toll-like receptor stimulation. J Immunol Methods 2011;366:89–99.
5. Smolen KK, Cai B, Gelinas L, Fortuno ES, Larsen M, Speert DP, Chamekh M, Cooper PJ, Esser M, Marchant A, et al. Single-cell analysis of innate cytokine responses to pattern recognition receptor stimulation in children across four continents. J Immunol 2014;193:3003–3012.
6. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J Leukocyte Biol 2006;81:584–592.