



Research paper

Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation

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ABSTRACT

Polychromatic flow cytometry allows the capture of multidimensional data, providing the technical tool to assess complex immune responses. Interrogation of the adaptive T cell response to infection or vaccination already has benefited greatly from standardized protocols for polychromatic flow cytometric analysis. The innate immune system plays an important role in health and disease, and presents potentially important therapeutic and diagnostic modalities. We describe here a high-throughput polychromatic flow cytometry-based platform that enables the rapid interrogation and large scale screening of human blood antigen presenting cell responses to Toll-like receptor (TLR) ligands and other innate immune modulators. Using this assay, we found that for certain stimuli (e.g., TLR9 and TLR3 ligands), the general protocol for intracellular cytokine cytometry had to be significantly modified to allow response detection. Furthermore, high concentrations of TLR7/8 and TLR4 stimuli caused substantial changes in lineage markers, potentially confounding analysis if one were to use a conventional “lineage-negative” cocktail. The assay we developed is reproducible and has been used to show that a given individual's TLR response pattern is relatively stable over at least several months. This protocol is in strict compliance with published guidelines for polychromatic flow cytometry, provides a common platform for scientists to compare their results directly, and may be applicable to the diagnostic evaluation of Toll-like receptor function and the rapid screening of promising therapeutic innate immune modulators.

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1. Introduction

The innate immune system provides the first contact between potentially invading microbes and the host's defense response. It represents a highly sophisticated system for recognition of specific pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), of which Toll-

like receptors (TLRs) are the best studied example. In the course of a normal host response to infection, antigen presenting cells (APCs) take up the invading microbe, process and present parts of it to the adaptive immune system (T and B cells) in the context of costimulatory surface molecules and cytokines, which in turn leads to a pathogen-specific adaptive immune response efficient at eliminating the offending invader. The magnitude and quality of APC responses is largely directed by PAMPs binding to PRRs on these cells. Stimulation with purified TLR ligands has been shown to change antigen-processing and to induce the maturation, migration and the production of cytokines and costimulatory markers on professional APCs. The APCs integrate information from multiple PRRs, resulting in a fine-tuned signal

Abbreviations: ICC, Intracellular Cytokine Cytometry; PBMC, peripheral blood mononuclear cells; WB, whole blood; TLR, Toll-like receptors.

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Table 1

TLR ligand panel used to stimulate innate immune cells

TLR	Ligand	Source	Catalog #	Concentration range
TLR 2/1	PAM3CSK4	EMC microcollections	L2000	0.01–10 µg/mL
TLR 2/6	R-FSL-1	EMC microcollections	L7022	0.01–10 µg/mL
TLR 3	poly I:C	Amersham	27-4729-01	6.25–100 µg/mL
TLR 4	0111:B4 LPS	InVivogen	tlr1-pelps	0.1–1000 ng/mL
TLR 7	3M-013	3M	MTA	0.01–100 µM
TLR 7/8	3M-003	3M	MTA	0.01–100 µM
TLR 8	3M-002	3M	MTA	0.01–100 µM
TLR 9	CpG A	Coley	N/A contact Coley	6.25–100 µg/mL
TLR9	ODN 2336			
TLR9	CpG B	Coley	N/A contact Coley	6.25–100 µg/mL
TLR 9	ODN 10103			
TLR 9	CpG C	Coley	N/A contact Coley	6.25–100 µg/mL
	ODN 2395			

Reagents 3M-013, 3M-003, and 3M-002 were a generous gift from 3M Pharmaceuticals. All other reagents are commercially available as indicated.

that instructs the subsequent adaptive immune response (Akira et al., 2006; Steinman and Hemmi, 2006).

While this highly complex system appears to work well in most individuals most of the time, changes in any of the essential components of the PRR signaling pathways increase the risk for several disease processes. For example, alteration of essential signaling PRR components leads to a state of heightened susceptibility to infection in both mice and humans. Even subtle single nucleotide changes in the sequence of some of the molecules involved in this orchestrated response to PAMPs have potentially lethal consequences if the individual is exposed to the right microbe at the wrong time (Turvey and Hawn, 2006). The response of APCs to TLR stimulation also appears to be developmentally regulated, with important clinical implications for pre-term labor and heightened risk for infection early in life (Levy, 2007). Several TLRs have been identified as potentially major contributors to autoimmune disease (Marshak-Rothstein, 2006), chronic inflammation and cancer (Karin et al., 2006). TLR activity has also been proposed to change the function of T regulatory cells (Kabelitz et al., 2006) and with that allograft rejection (Tesar and Goldstein, 2007). All of the above emphasizes the centrality of the innate immune system to our health and well-being. Yet, our understanding of the cause–effect relationships is very rudimentary. A reliable yet versatile assay of innate immune function would allow scientists to unravel the mechanisms at work, providing the foundation for rational therapeutic interventions.

Despite this lack of knowledge, the realization that innate immune responses can be altered through TLR stimulation has propelled TLR ligands to the stage of clinical trials as immune modulators (Amati et al., 2006). For example, TLR agonists are under consideration as therapies for asthma and allergy (Goldman, 2007). Most advanced is the concept of coupling TLR ligands as adjuvants to vaccine antigens (Pulendran and Ahmed, 2006). A high-throughput assay allowing the rapid screening of hundreds of potential innate immune modulators and adjuvants would speed up the discovery phase of these promising applications (Kanzler et al., 2007).

Previously, assays for innate immune analysis were limited in scope and speed, analyzing either only innate

immune cell numbers (Vuckovic et al., 2004), or cytokines secreted into culture supernatants (Deering and Orange, 2006). While this global assessment of innate immune function has its advantages, it does not provide the necessary precise single-cell-specific information to accurately assess an innate response to TLR ligation. One previously described flow cytometric assay allowed assessment of specific innate cell types using four-color flow cytometry, with only two colors as a response read out (Ida et al., 2006). The complexity of the innate response to TLR stimulation far exceeds the capacity of this assay. We reasoned that recent developments of polychromatic (>5 colors) flow cytometry (Perfetto et al., 2004, 2006) should allow the inherent complexity of the innate immune response to be captured more fully. We strived to design an automated flow cytometry-based assay to provide a

Table 2

Staining panel used for polychromatic flow cytometry

	Characteristic being measured	Antibody name Clone name	Vendor cat# Dilution used
A			
<i>Violet</i>			
AmCyan	Cell surface protein	CD123 AmCyan (9F5)	BD#custom 1/50
Pacific Blue	Intracellular protein	IL12p40/70 (C8.6)	eBio#57-7129 1/100
<i>Red</i>			
APC	Cell surface protein	CD11c (5HCL3)	BD#340714 1/50
APC-Cy7	Intracellular protein	IL6 (MQ2-13A5)	BD#custom 1/100
Alexa 700	Intracellular protein	TNFα (Mab11)	BD#557996 1/100
<i>Blue</i>			
FITC/OG	Intracellular protein	IFNα (A11)	Antigenix#MC100133 1/100
PerCPCy5.5	Cell surface protein	MHCII TU36	BD#custom 1/100
PE-Cy7	Cell surface protein	CD14 (61D3)	eBio #25-0149 1/50
B			
<i>Violet</i>			
Pacific Blue	Cell surface protein	CD123 (9F5)	BD#custom 1/100
<i>Red</i>			
APC	Cell surface protein	CD11c (5HCL3)	BD#340714 1/50
Alexa 700	Cell surface protein	CD14 (M5E2)	BD#557923 1/50
<i>Blue</i>			
FITC/OG	Cell surface protein	CD40 (5C3)	eBio #11-0409 1/100
PE	Cell surface protein	CD86 (IT2.2)	eBio #12-0869 1/100
PerCPCy5.5	Cell surface protein	MHCII TU36*	BD#custom 1/100
PE-TEXRed	Cell surface protein	CD19-biotin (HIB19)	BD#555411
		streptavidin PE-TR	BD#551487 1/50
PE-Cy7	Cell surface protein	CCR7 (3D12)	BD#557648 1/100

A) Panel used for intracellular cytokine staining.

B) Panel used for cell surface staining.

widely usable innate immune analysis platform. Bulk cytokine information as done previously can easily be gathered in parallel with the more detailed cell-specific information from this flow cytometry platform to create a detailed picture of innate immune function. Here we outline the optimization and validation of these multiplex assays to analyze complex innate immune responses to TLR stimulation.

2. Methods

2.1. TLR stimulation plates

Deep-96-well (VWR) source plates containing 1.3 mL of various TLR ligands at 10× the desired concentration were prepared using sterile procedures under a laminar air-flow

hood. Ten TLR ligands were used, each diluted in RPMI-1640 medium (Invitrogen) containing Glutamax (RPMI) over a 5 log₁₀ concentration range for a total of 50 different stimuli (Table 1). Two unstimulated wells were included as a negative control, using the same medium. Brefeldin A (BFA, Sigma) was added at a concentration of 100 µg/mL (10× the desired final concentration of 10 µg/mL) to all wells except those wells containing TLR3 and TLR9 ligands (see below). Compounds containing the TLR ligands PAM₃CSK₄, R-FSL-1 or LPS were sonicated at least 10 min and vortexed thoroughly before each initial dilution and between serial dilutions, while the other ligands were not sonicated but vortexed initially and between serial dilutions. Source plates were sealed with sterile plate sealers (USA Scientific), frozen at -80 °C and thawed prior to use. 20 µl from each well of the source plate was dispensed into each well

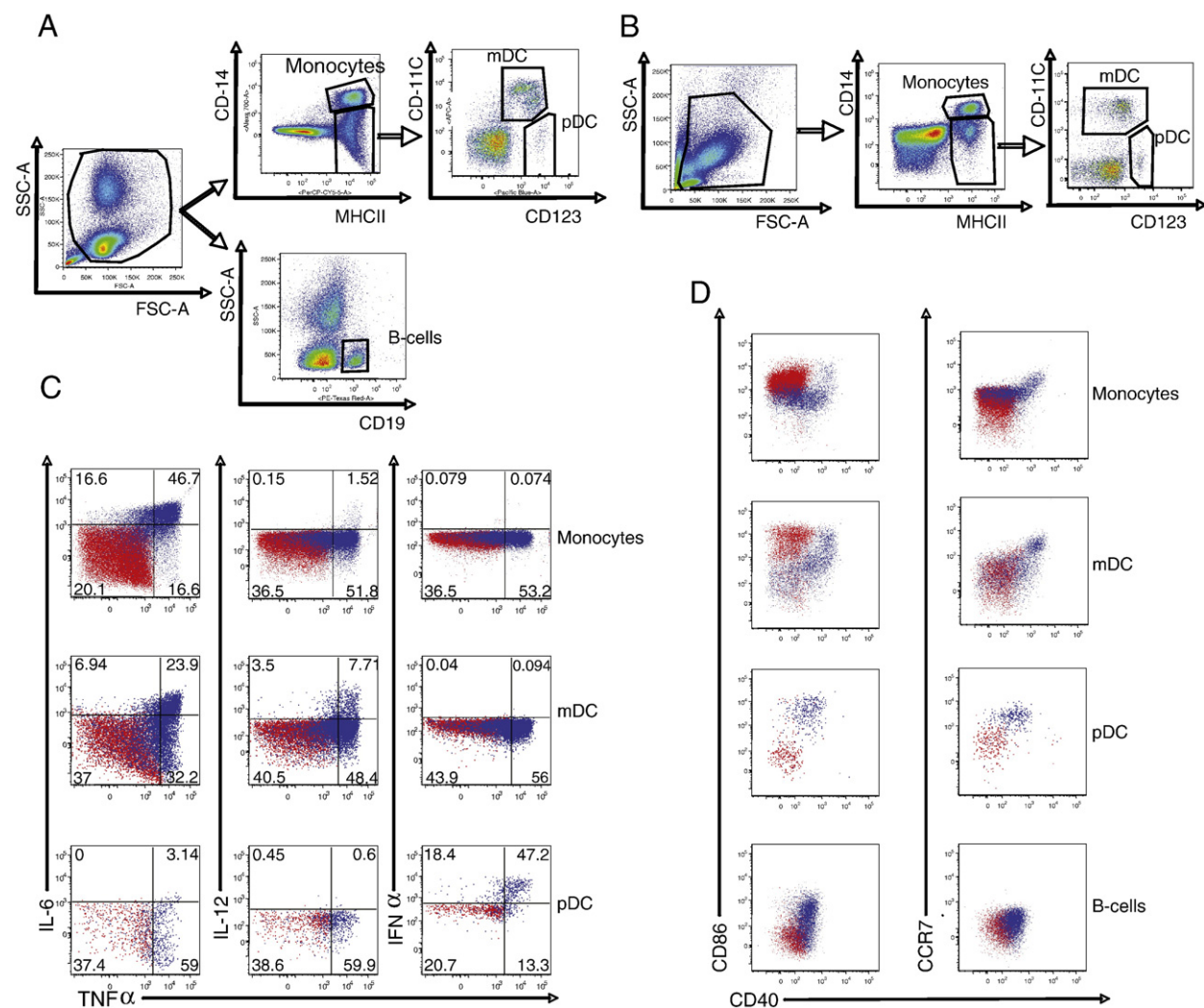


Fig. 1. Gating strategy and staining profile for 8-color flow cytometry. Shown is a PBMC sample in A, C, and D, and a whole blood sample in B. Shown in A and B on an unstimulated sample is the gating strategy to identify various innate immune cell subsets: monocytes (MHCIIpos, CD14pos), B cells (MHCIIpos, CD19pos), myeloid DCs (MHCIIpos, CD14neg/low, CD123neg, CD11cpos) and plasmacytoid DCs (MHCIIpos, CD14neg/low, CD11cneg, CD123pos). C. Shown is a PBMC sample from the same adult subject, either unstimulated (red) or stimulated with 10 µM of the TLR7/8 ligand 3M-003 (blue) for 6 h in the presence of BFA. Innate immune cell subsets were identified as indicated above, then analyzed for expression of IL-6, IL-12, and IFN-α with TNF-α. D. Shown is a PBMC sample from the same adult subject, either unstimulated (red) or stimulated with TLR7/8 ligand 3M-003 (blue) for 18 h without any Golgi-blocker. Innate immune cell subsets were identified as indicated above, then analyzed for expression of CD86, CCR7 and CD40.

of recipient 96-well round bottom polystyrene plates (Corning) using the Evolution™ P3 Precision Pipetting Platform (Perkin Elmer), under a laminar air-flow hood using sterile procedures. Recipient plates were sealed with sterile aluminum sealers (USA Scientific) and frozen at -80°C until use.

2.2. Blood sample processing and *in vitro* stimulation

All studies were approved by the Institutional Ethics Review Board at both the University of Washington and the University of British Columbia. We obtained blood from healthy adult individuals via sterile venipuncture into vacutainers containing Na-Heparin (Becton Dickinson). Blood and all reagents were kept at room temperature. PBMC were isolated by density gradient centrifugation; whole blood (WB) was mixed 1:1 with sterile RPMI. Peripheral blood mononuclear cells (PBMC) were cultured in RPMI supplemented with 100 units penicillin/mL, 100 μg streptomycin/mL (Invitrogen) and 10% human AB serum (Gemini Bio-Products). 200 μl /well of cell suspension (either PBMC or WB mixed 1:1 with RPMI) was added to premade 96 well plates containing the specific TLR ligands at the desired concentrations. Cells were incubated for 6 h at 37°C in 5% CO_2 . For TLR3 and TLR9 ligands BFA was added for the final 3 h at a final concentration of 10 $\mu\text{g}/\text{mL}$. An identical set of plates was incubated in parallel for 18 h without BFA; at 18 h, these plates were spun and 100 μl of supernatant was removed and frozen at -80°C for later Luminex analysis. Then EDTA was added to a final of 2 mM, and plates were incubated for 10 min at 37°C to detach adherent cells. Plates containing PBMC were spun and resuspended in 100 μl FACSlyse (Becton Dickinson); for plates containing WB, the entire mixture was added to 1400 μl FACSlyse in deep 96-well plates (VWR). Both plates were then frozen at -80°C until staining.

2.3. Staining, acquisition and analysis

Antibodies (clones and source) for either intracellular cytokine cytometry at 6 h or activation-induced cell surface molecule expression at 18 h are shown in Table 2A and B, respectively. The concentrations of all antibodies were

titrated prior to use, and the optimal dilutions for each are shown in Table 2. Frozen plates were thawed, either in a 37°C incubator for 15 min for PBMC (100 μl per well in microwell plates) or for 1.5 h in a 37°C water bath for WB (1.6 ml/mL per well in deep well plates) and spun, then pellets were resuspended in 200 μl FACS Permeabilizing Solution (BD) and incubated at room temperature for 10 min; permeabilizing solution was not added to 18 h plates. After one wash (or two washes for WB) in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide (PBSAN), cells were stained in a final volume 100 μl PBSAN for 30–60 min at room temperature. After two further washes with PBSAN, cells were resuspended in PBS containing 1% paraformaldehyde and immediately analyzed on an LSRII Flow Cytometer (Becton Dickinson) set up according to published guidelines (Perfetto et al., 2004, 2006). Compensation beads (CompBeads, Becton Dickinson) were used to standardize voltage settings and were used as single stain controls as described (Maecker and Trotter, 2006; Lamoreaux et al., 2006). 3 μl of anti-mouse Ig CompBeads and 3 μl of anti-FBS negative control CompBeads were added to 94 μl of PBSAN in the same plate as the cells, and stained with 3 μl of antibody for each single stain control at the same time as cells were stained. For the rest of the protocol, CompBeads were treated in the same way as the cells. For PBMC at least 200,000 and for whole blood at least 1,000,000 events were acquired uncompensated through a high-throughput sampler (set to mix at medium and to acquire at no more than 2 $\mu\text{l}/\text{s}$). Extra volume of at least 50 μl beyond what the 96-well sampler uses was added to the wells to prevent introduction of air into the system. Compensation was set in FlowJo (TreeStar, OR) or Labkey Flow (Labkey, WA), and samples were analyzed compensated. Cytometer optimization and calibration were performed according to published guidelines (Perfetto et al., 2004, 2006).

2.4. Statistical analysis

Graphs were prepared using Excel software (Microsoft, WA). For statistical analysis, the Prism (GraphPad Software) software analysis program was used.

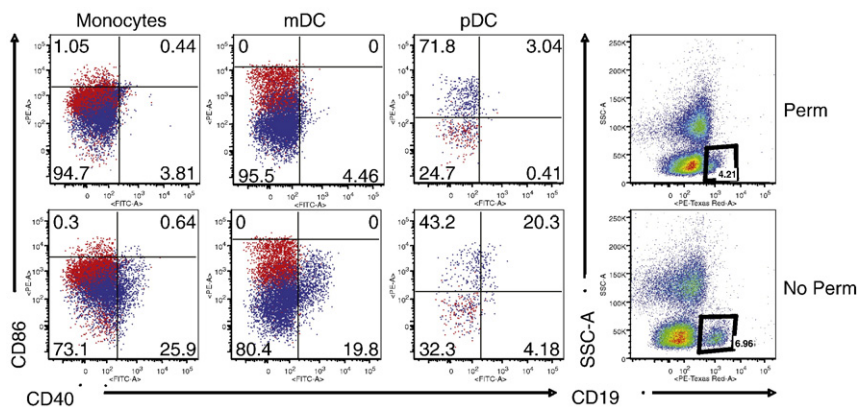


Fig. 2. Permeabilization impacts surface marker detection. Adult PBMC were either left unstimulated (red) or stimulated with 10 μM of the TLR7/8 ligand 3M-003 (blue) for 18 h without any Golgi-blocker. Subsequently, samples were either processed as described for intracellular cytokine cytometry (i.e., permeabilized) or left untreated. Both samples were then stained to identify innate immune cell subsets as indicated in Fig. 1A. The impact of permeabilization on expression of CD86 and CD40 by monocytes, mDCs and pDCs is shown in the left panels and the impact of permeabilization on CD19 expression is shown on the far-right panel.

3. Results

3.1. Optimization of reagents and equipment

Through initial pilot experiments we realized that all materials, including plastic hardware (96 well plates, pipettes etc.), blood-draw equipment (e.g. vacutainer tubes, heparin, etc.), that came into contact with the innate immune cells, had to be tested and shown to be free of innate immune stimulating activity in order to avoid potential artifacts resulting from unintended activation of the cells. This testing was performed not only via commercially available systems (e.g., Limulus amoebocyte lysate test), which only screen for one specific contaminant, but more effectively through exposure of fresh PBMC from healthy adult subjects to the materials to be tested, followed by analysis for activation of innate immune cells. For example, we plated unstimulated PBMC with different batches of human AB serum to identify the source

and batch that provides the highest signal-to-noise ratio (lowest background activation, yet the highest TNF α production in mDC and macrophages if stimulated with 10 ng/mL of LPS). Once optimal reagents were identified, we procured sufficient quantities to complete the ongoing study. Of note, there was no difference between fetal calf serum (FCS) or human AB serum as a culture additive when responses were assessed by flow cytometry (data not shown). TLR ligands were tested in reporter cell lines to confirm that each of those used in our assays was specific for the appropriate TLR and lacked activity for other TLRs (Hajjar et al., 2006). The source of each TLR ligand that satisfied this stringent assessment is shown in Table 1. The optimal antibody and fluorochrome combination used for the polychromatic innate immune evaluation was chosen according to published guidelines (Maecker et al., 2004, 2005). Neither positive interference (antibodies added to our cocktail inhibiting each other's binding) nor negative interference (reagents contained in

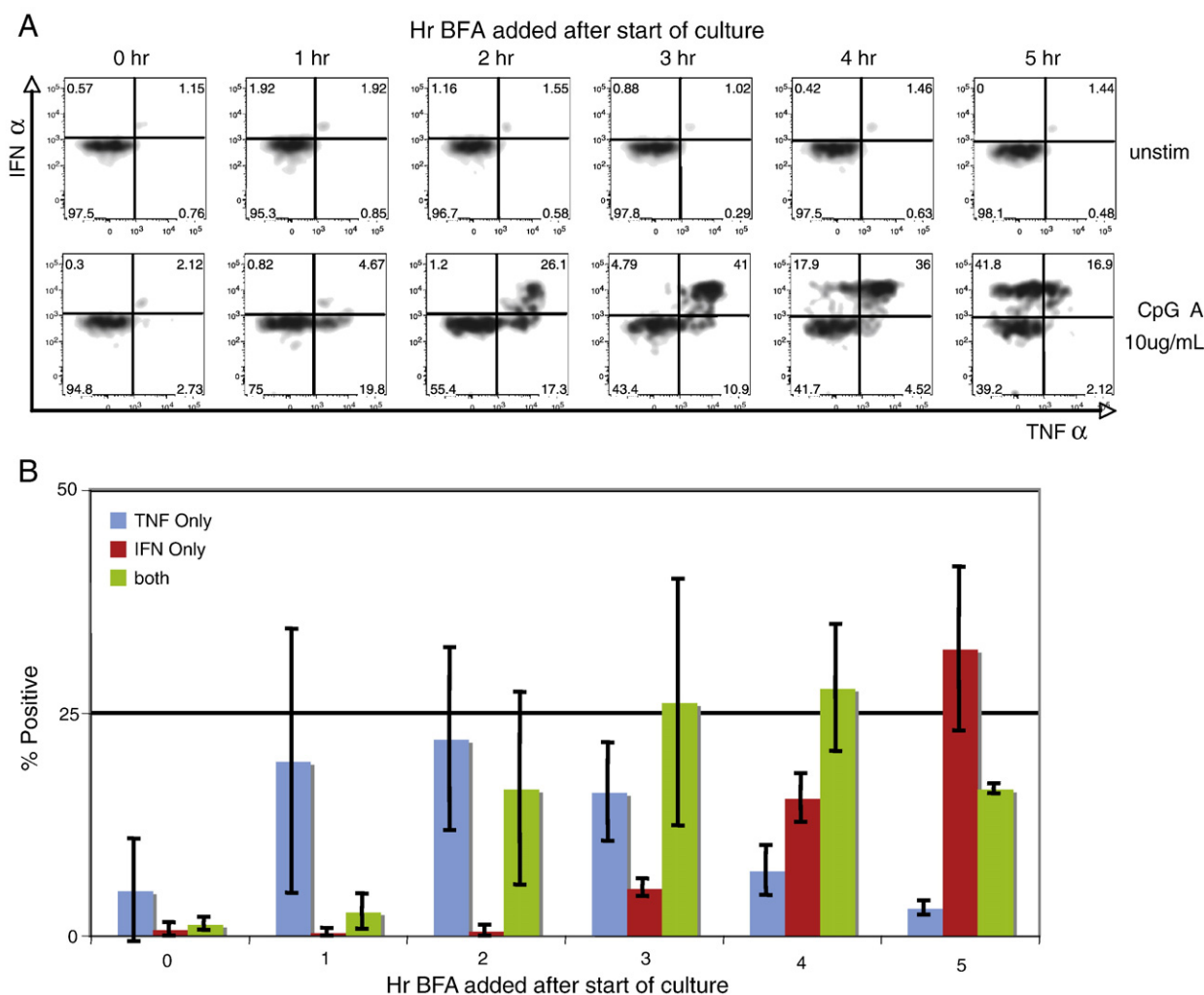


Fig. 3. Timing of Brefeldin A addition has significant impact on responses to CpGs. A. Adult PBMC were either left unstimulated (top row), or stimulated with 10 μ g/mL of the TLR9 ligand CpG A (lower row) for 6 h. The Golgi-blocker Brefeldin A (BFA) was added hourly right from the start (0 h) or up to 5 h later. All cells were incubated for the same total time of 6 h, and processed further for intracellular expression of cytokines in the various innate immune cell subsets. Shown is the co-expression of TNF- α and IFN- α in plasmacytoid DCs. B. Graph summarizing co-expression of TNF- α and IFN- α in plasmacytoid DCs in three different adult subjects with the bars indicating the standard deviation.

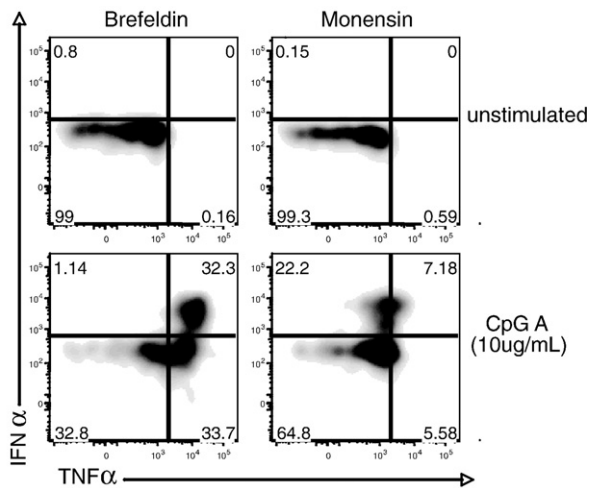


Fig. 4. Brefeldin A is the better Golgi blocking reagent for intracellular cytokine analysis in innate immune cells. Adult PBMC were either left unstimulated (top row) or were stimulated with 10 μ g/mL of the TLR9 ligand CpGA (lower row) for 6 h. The Golgi-blockers Brefeldin A (BFA) or Monensin were added 3 h into the incubation period. Cells were incubated for another 3 h, then processed further for intracellular expression of cytokines. Shown is the co-expression of TNF- α and IFN- α in plasmacytoid DCs.

culture medium or its additives inhibiting the binding of antibodies present in our cocktail) was detected for the final clones chosen (Phillips et al., 2006). This required testing of over 70 clone/fluorochrome combinations to assemble the satisfactory panel of antibodies shown in Table 2, with Table 2A listing the optimal panel for ICC, and Table 2B listing those optimal for staining of surface markers of activation in the innate target cells.

3.2. Optimization of stimulation and analysis

3.2.1. Gating strategy

The gating strategy for PBMC and whole blood (WB) from a representative healthy adult subject that were stimulated with the TLR7/8 ligand 3 M-003 is shown in Fig. 1A and B respectively. Our assay allows the simultaneous analysis of monocytes (identified based on FSC/SSC, MHCII^{hi}, CD14^{hi}), myeloid DC (mDC; identified based on FSC/SSC, MHCII^{hi}, CD14-/low, CD123-, CD11c^{hi}), plasmacytoid DC (pDC; identified based on FSC/SSC, MHCII^{hi}, CD14-/low, CD11c-, CD123^{hi}), and B cells (identified based on FSC/SSC, MHCII^{hi}, CD14-, CD19^{hi}). This approach allows specific identification of the target cell populations (gate 'in'), and stands in contrast to other widely used gating strategies that rely on stable expression of certain lineage markers to exclude (i.e. gate 'out') the "undesired" target cells (Ida et al., 2006). The Golgi-secretion inhibitor BFA was added to the culture to permit detection of intracellular cytokines by flow cytometry (ICC) on permeabilized cells (Dinter and Berger, 1998; Maecker et al., 2004, 2005) as shown in Fig. 1C. Cultures stimulated for 18 h in the absence of BFA can be used to concomitantly assess changes of cell surface (e.g. costimulatory and homing receptors) markers (Fig. 1D) and to detect cytokines secreted into the supernatant (not shown) as parallel functional read outs. It is important to recognize that certain surface molecules such as costimulatory molecule CD40 as well as the

surface marker CD19 were sensitive to membrane permeabilization (Fig. 2), while all other surface antibodies listed in Table 2B were not affected by permeabilization.

3.2.2. Choice of Golgi-secretion inhibitors

The addition of the Golgi-inhibitors BFA or Monensin concomitantly with TLR stimulation yielded optimal results for all TLR ligands except for CpG and pl:C. For these two ligands, a measurable response was obtained only if BFA (or Monensin) addition was delayed, as illustrated by the pDC response to CpG (Fig. 3); for these ligands, addition of BFA at 3–4 h after addition of the CpG or pl:C appeared to be optimal for detection of intracellular cytokines. We directly compared Monensin and BFA and found no substantial difference between them except for TNF- α and to a lesser extent IL-6 (data not shown), for which BFA performed better than Monensin after CpG stimulation (Fig. 4).

3.2.3. Dose response titration and kinetics

Performing similar analysis for each of the TLR ligands listed in Table 1, across a wide range of concentrations, we found that different APC subsets have different concentration optima for different cytokines. Furthermore, differences in dose–response curves also exist between PBMC and WB responses, as illustrated for the TLR7/8 ligand 3M-003 and the TLR4 ligand LPS (Fig. 5). Importantly, very high concentrations of TLR7/8 (>10 μ M) or TLR4 (>100 ng/mL) ligands altered the expression of the lineage markers CD14 and CD11c (Fig. 6). We were able to solve this problem by reducing the concentrations of these ligands and by sequentially gating, first on MHCII⁺ and CD14^{high} for monocytes, then on MHCII⁺ and CD11c^{high}/CD123 negative for mDCs (see Fig. 1), rather than trying to use CD14 vs. CD11c to differentiate between monocytes and mDCs as shown in Fig. 6. For detection of intracellular cytokines by flow cytometry, a total incubation period of ~5–8 h appeared optimal (Fig. 7), with incubation periods of less than 5 h resulting in substantial reduction of the response, and incubation periods of over 8 h producing a steady increase in dead cells (data not shown).

3.2.4. Precision

Precision refers to the ability of the assay to generate reproducible results as operational parameters change (Horton et al., 2007). We looked at replicate variability between same plate vs. separate plate experimental set-ups. Well-to-well variability was assessed by comparing triplicate wells stimulated within the same plate, while plate-to-plate variability was assessed for the same individuals by comparing triplicates across 3 separate plates. As expected, we found the standard deviation to be somewhat higher across separate plates (Fig. 8), but the mean of the 'same plate' triplicates still fell within the standard deviation of the triplicates measured across three different plates.

3.2.5. SOP outline

The amount of hands-on work necessary to complete the innate immune response to TLR stimulation according to our protocol is shown in Fig. 9.

4. Discussion

The innate immune system plays an important role in health and disease, and presents potentially important therapeutic

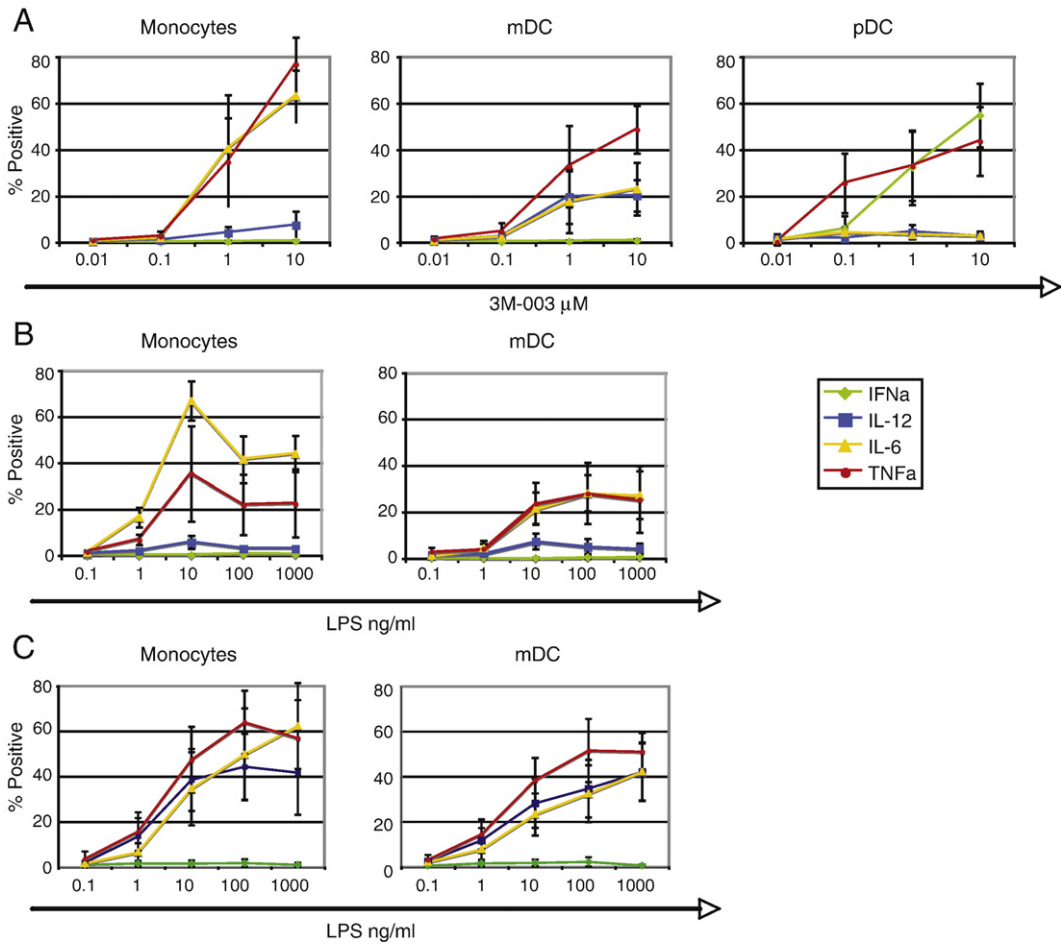


Fig. 5. Dose response profiles for 3M-003 and LPS. Adult PBMC were either left unstimulated or stimulated with different concentrations of the TLR7/8 ligand 3M-003 (A) or the TLR4 ligand LPS (B). C. Adult whole blood samples were either left unstimulated or stimulated with different concentrations of the TLR4 ligand LPS. (C) Shown in all graphs is the result of intracellular cytokine detection in samples stimulated for 6 h in the presence of BFA. These graphs depict the % cytokine positive cells for each of the cytokines analyzed from 5 different adult subjects ($n=5$). Error bars indicate standard deviation.

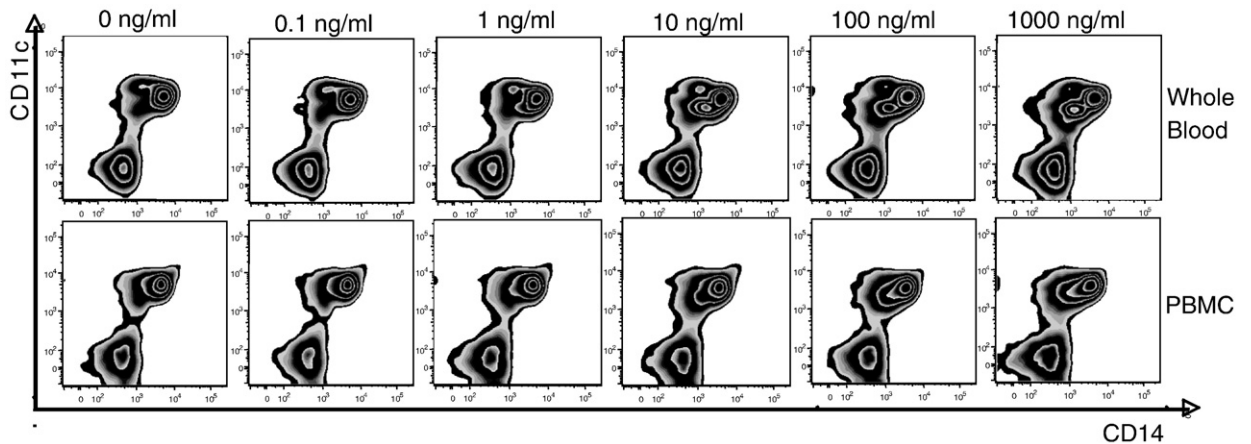


Fig. 6. Lineage marker expression is altered with TLR stimulation. Adult whole blood (top row) or PBMC (bottom row) were either left unstimulated or stimulated for 6 h with the indicated concentrations of the TLR4 ligand LPS in the presence of the Golgi-blocker BFA. Samples were processed as described for intracellular cytokine cytometry and analyzed for the surface expression of CD11c and CD14.

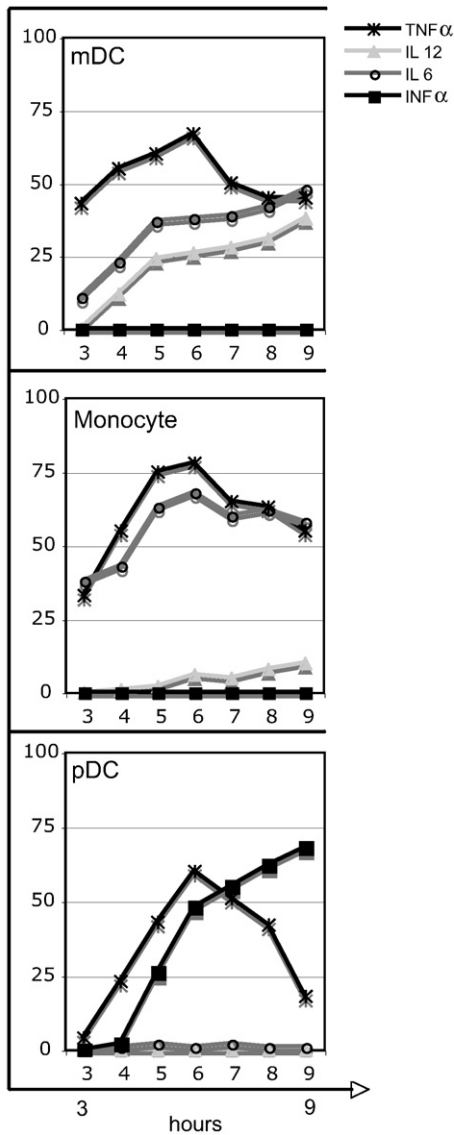


Fig. 7. Optimal time point of total incubation length. Adult PBMC were either left unstimulated or stimulated with 10 μ M of the TLR7/8 ligand 3M-003. Shown is the result of intracellular cytokine detection in myeloid DC, monocytes, or plasmacytoid DC stimulated for 3 to 9 h in the presence of BFA. The graphs depict the % cytokine positive cells for each of the cytokines analyzed. This graph is representative of results obtained with 3 different adult subjects.

and diagnostic targets. We describe here a high-throughput polychromatic flow cytometry-based platform that produces reliable and reproducible data that can easily be used in parallel with a multiplexed bulk cytokine assay. Given that this platform is in strict compliance with published guidelines for polychromatic flow cytometry, we anticipate this protocol will provide a common platform for innate immune evaluation.

Previous innate immune assays lack the speed and scope provided by our assay. For example, Deering and Orange describe a clinical assay to evaluate TLR function, but limit their analysis to cytokines present in culture supernatants of PBMC stimulated with TLR ligands (Deering and Orange,

2006). Ida et al. (2006) describe a flow cytometric method to assess DC function in response to TLR stimulation, but only employ 4 colors, 2 of which are used to identify the DC subsets, leaving only 2 for a functional read out (Ida et al., 2006). Neither of these assays provides the detail necessary to adequately evaluate the complex innate immune response, and neither has been developed into a rigorously standardized, high-throughput platform.

Based on our data, there are several interesting points to emphasize:

1. The sensitivity of CD40 to permeabilization procedures precluded the simultaneous detection of the CD40 surface marker alongside intracellular cytokines. Detection of intracellular cytokines in B cells requires the use of other B cell surface markers than CD19 for subset identification, as CD19 detection is reduced in permeabilized cells.
2. The lack of response to TLR3 and TLR9 ligands if Golgi blockers were added at the start of culture (Fig. 3) might be

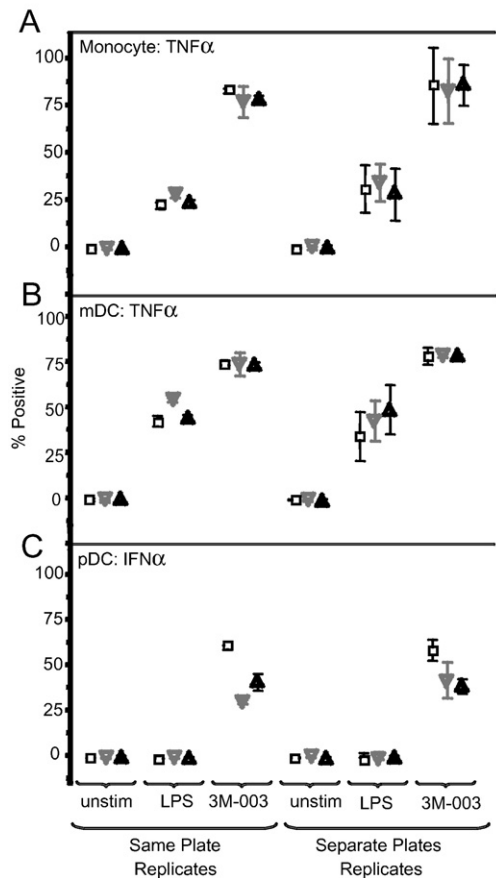


Fig. 8. Precision of the assay. PBMC from 3 different adults were either left unstimulated or stimulated with 10 μ M of the TLR7/8 ligand 3M-003 or 10 ng/mL of the TLR4 ligand LPS for 6 h in the presence of BFA. Triplicate wells were assayed either within the same plate (same plate) or across three different plates (separate plates). Furthermore, samples from these 3 adult subjects were taken three months apart between the assay using the same plate (time=0 months) and those taken to assay triplicates across 3 different plates (time=3 months), to estimate changes in a given subject over time. Shown is the result of intracellular TNF- α detection in monocytes (A) and mDCs (B), and for INF- α in pDCs (C); error bars indicate standard deviation.

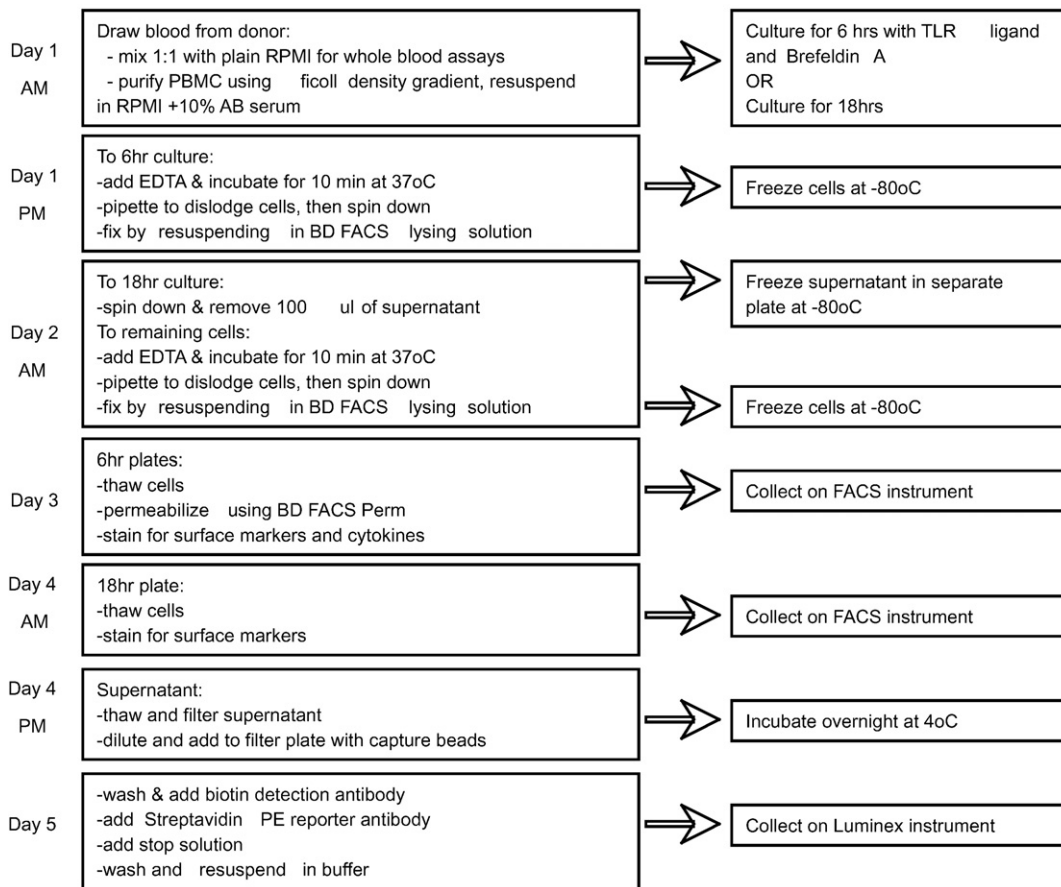


Fig. 9. Summary of the protocol.

related to transport requirements for these ligands or their receptors from the endoplasmic reticulum to and from the endosome (Latz et al., 2004; Ivanov et al., 2007; Kim et al., 2008). This effect was observed with both BFA and Monensin. The optimal delay in the addition of BFA to cells stimulated with TLR3 or TLR9 ligands was 3–4 h, as this delay allowed assessment of the maximal direct TLR stimulation response (TNF- α and INF- α double positive cells) (Theofilopoulos et al., 2005).

- While optimal time and dose response kinetics varied between TLR ligand and cytokine read out (Figs. 5 and 7), we chose the 6 h time point for total length of stimulation for ICC, as it captured most of the cytokines within their maximum response. We chose the range of concentrations listed in Table 1 based on providing a range of low to high response for most cytokines in most APC subsets in WB as well as PBMC (Fig. 5). As indicated in Fig. 5, we did note potential differences between WB and PBMC in some TLR response patterns. This has been described previously (Levy, 2007). We are currently in the process of identifying the precise mechanisms underlying this phenomenon.
- The down-regulation of CD14 and CD11c after stimulation with a high concentration of TLR7/8 and TLR4 ligand (Fig. 6) was surprising, but similar observations in response to histamine (Takahashi et al., 2003) and LPS (Lin et al., 2004) have been made before. Whereas the mechanisms respon-

sible for this phenomenon are not clear, it is important to note that flow cytometric strategies relying on 'dump-gates' (e.g. excluding T-, B-, NK-cells and CD14+ monocytes all in one channel), as described in the one previously published flow cytometric assessment of innate immune function (Ida et al., 2006), would fail to detect the loss of these TLR ligand-responsive cells, producing an artifact that would likely underestimate the response to this ligand in the affected cell populations. Our assay allows one to capture these cells, since we specifically include ('gate-in') our target population through the use of the polychromatic antibody cocktails described above. To avoid this problem, one can also choose concentrations of TLR7/8 and TLR4 ligands at which this phenomenon is minimal such that the relevant cell populations can still be discriminated from each other.

- Fig. 8 suggests that our assay delivers precise measurements of an individual's response to TLR stimulation. Two different aspects are presented in Fig. 8 simultaneously: A. The standard deviation (SDEV) of blood samples from 3 individuals stimulated on the same plate vs. those stimulated on different plates were compared. The SDEV was clearly larger if samples were stimulated on different plates. While this is not surprising, it is important to keep in mind when serial samples of an individual are compared over time (e.g., the length of a given study) since by definition they would be stimulated on different plates. B.

To test whether an individual's innate immune response changes over time, one needs to control for potential artifacts due to the use of different stimulation plates. Our data indicate that a given subject's response appears relatively stable over at least 3 months as indicated by the fact that the mean of the first time point, which was derived from three replicates stimulated on the same plate, still falls within the SDEV of three replicates of PBMC obtained 3 months later, even though these 3 replicates were each stimulated in a different plate. This stability has important practical ramifications, as population-wide searches for changes in TLR response might otherwise be handicapped by variation over time. To adequately evaluate if potentially significant differences in a subject's response over time occur, we are currently in the process of investigating longer time periods with many more subjects.

Since cryopreservation of innate cells is known to alter their response profile (Deering and Orange, 2006), and we thus had to use fresh samples for stimulation. However, it is important to point out that our assay is compatible with cryopreservation and shipment on dry ice after stimulation and fixation in FACS lysing solution of cells, and thus can easily be employed in multi-center studies with only one central analysis facility. Furthermore, the assay described here has been optimized for whole blood (WB) and peripheral blood mononuclear cells (PBMC), allowing one to compare responses in the presence or absence of soluble factors contained in an individual's plasma and thus to assess the potential impact of such factors on the innate immune response (Levy, 2007). This platform is easily coupled to other multiplex assays, such as cytokine bead arrays, without increasing the necessary sample volume. Our polychromatic flow cytometric assay platform described here is clearly amenable to study not only TLR ligands, but of any innate immune modulator, and thus should be of general applicability to studies of human innate immune responses.

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