Published in final edited form as:
J Immunol Methods. 2008 July 31; 336(2): 183–192. doi:10.1016/j.jim.2008.04.013.

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

Kirstin Jansen[1], **Darren Blimkie**[2], **Jeff Furlong**[1], **Adeline Hajjar**[1], **Annie Rein-Weston**[1], **Juliet Crabtree**[1], **Brian Reikie**[2], **Christopher Wilson**[1], and **Tobias Kollmann**[23]

1*Department of Immunology, University of Washington, Seattle, WA*

2*Department of Pediatrics, Division of Infectious and Immunological Diseases, Vaccine Evaluation Center, BC Children's Hospital, University of British Columbia, Vancouver Canada*

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.

Keywords

Flow cytometry; Intracellular cytokine staining; Cytokine bead array; Innate immunity; Toll-like receptors; Assay validation

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

3Address correspondence to: Tobias Kollmann MDPhD, CFRI 304, 950 W 28th Ave, Vancouver, BC V5Z 4H4, Canada; tkollmann@cw.bc.ca.

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

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). 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). Several TLRs have been identified as potentially major contributors to autoimmune disease (Marshak-Rothstein), chronic inflammation and cancer (Karin et al.). TLR activity has also been proposed to change the function of T regulatory cells (Kabelitz et al.) and with that allograft rejection (Tesar and Goldstein). 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 onto the stage of clinical trials as immune modulators (Amati et al.). For example, TLR agonists are under consideration as therapies for asthma and allergy (Goldman). Most advanced is the concept of coupling TLR ligands as adjuvants to vaccine antigens (Pulendran and Ahmed). 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.).

Previously assays for innate immune analysis were limited in scope and speed, analyzing either only innate immune cell numbers (Vuckovic et al.), or cytokines secreted into culture supernatants (Deering and Orange). 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.). 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.) 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 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 10x 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 $\log_{10}$ 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 (10x 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 PAM_3CSK_4, 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. 20μl from each well of the source plate was dispensed into each well 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, 100μg 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 hours at 37 °C in 5% CO2. For TLR3 andTLR9 ligands BFA was added for the final 3 hours at a final concentration of 10μg/mL. An identical set of plates was incubated in parallel for 18hrs without BFA; at 18hrs, these plates were spun and 100μl of supernatant was removed and frozen at −80°C for later Luminex analysis. Then EDTA was added to a final of 2mM, 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 6hr or activation-induced cell surface molecule expression at 18 hr are shown in Table 2A and 2B, 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 minutes for PBMC (100μl per well in microwell plates) or for 1.5 hours in a 37°C water bath for WB (1.6ml 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 hr plates. After one wash 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 minutes 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.).

Compensation beads (CompBeads, Becton Dickinson) were used to standardize voltage settings and were used as single stain controls as described (Maecker and Trotter; Lamoreaux et al.) 3 μl of anti-mouse Ig CompBeads and 3 μl of anti-FBS negative control CompBeads were added to 94μl of PBSAN in the same plate as the cells, and stained with 3μl 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 2μl/sec). Extra volume of at least 50μl 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.).

2.4. Statistical analysis

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

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 amebocyte 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 TNFα production in mDC and macrophages if stimulated with 10ng/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 were specific for the appropriate TLR and lacked activity for other TLRs (Hajjar et al.). 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 were chosen according to published guidelines (Maecker et al.). Neither positive interference (antibodies added to our cocktail inhibiting each other's binding) nor negative interference (reagents contained in culture medium or its additives inhibiting the binding of antibodies present in our cocktail) was detected for the final clones chosen (Phillips et al.). 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 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 3M-003 is shown in Figure 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.). 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; Maecker et al.) as shown in Figure 1C. Cultures stimulated for 18 hrs in the absence of BFA can be used to concomitantly assess changes of cell surface (e.g. costimulatory and homing receptors) markers (Figure 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 (Figure 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 pI: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 (Figure 3); for these ligands, addition of BFA at 3–4 hrs after addition of the CpG or pI: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 (Figure 4).

3.2.3. Dose-Response Titration & 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 optimums for different cytokines. Furthermore, difference 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 (Figure 5). Importantly, very high concentrations of TLR7/8 (>10 μM) or TLR4 (>100 ng/ml) ligands altered the expression of the lineage markers CD14 and CD11c (Figure 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 Figure 1), rather than trying to use CD14 vs. CD11c to differentiate between monocytes and mDCs as shown in Figure 6. For detection of intracellular cytokines by flow cytometry, a total incubation period of ~5–8 hours appeared optimal (Figure 7), with incubation periods of less than 5 hours resulting in substantial reduction of the response, and incubation periods of over 8 hours 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.). 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 (Figure 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 Figure 9.

4. DISCUSSION

The innate immune system plays an important role in health and disease, and presents potentially important therapeutic 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). Ida et al. 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..) 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 (Figure 3) might be related to a transport requirements for these ligands or their receptors from the endoplasmic reticulum to and from the endosome (Latz et al.;Ivanov et al.;Kim et al.). 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 hours, as this delay allowed assessment of the maximal direct TLR stimulation response (TNF-α and INF-α double positive cells) (Theofilopoulos et al..)
3. While optimal time and dose response kinetics varied between TLR ligand and cytokine read-out (Figure 5 and Figure 7), we chose the 6 hour 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 (Figure 5). As indicated in Figure 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.
4. The down-regulation of CD14 and CD11c after stimulation with a high concentration of TLR7/8 and TLR4 ligand (Figure 6) was surprising, but similar observations in response to Histamine (Takahashi et al.) and LPS (Lin et al.) have been made before. Whereas the mechanisms responsible 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.), 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 chose 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.

5. Figure 8 suggests that our assay delivers precise measurements of an individual's response to TLR stimulation. Two different aspects are presented in Figure 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.

While cryopreservation of innate cells is known to alter their response profile (Deering and Orange), and we thus had to use fresh samples for stimulation, 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). 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.

Abbreviations

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

Acknowledgements

This work was supported by N01-AI-50023 from the National Institute of Allergy and Infectious Diseases. The research of TK is supported in part by a Carreer Award in the Biomedical Sciences from the Burroughs Wellcome Fund, and by a Carreer Development award from the Canadian Child Health Clinician Scientist Program. We would like to thank Isidro Zarraga of 3M Pharmaceuticals for providing TLR7 and TLR8 ligands.

REFERNCES

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801. [PubMed: 16497588]

Amati L, Pepe M, Passeri ME, Mastronardi ML, Jirillo E, Covelli V. Toll-like receptor signaling mechanisms involved in dendritic cell activation: potential therapeutic control of T cell polarization. Curr Pharm Des 2006;12:4247–4254. [PubMed: 17100626]

Deering RP, Orange JS. Development of a clinical assay to evaluate toll-like receptor function. Clin Vaccine Immunol 2006;13:68–76. [PubMed: 16426002]

Dinter A, Berger EG. Golgi-disturbing agents. Histochem Cell Biol 1998;109:571–590. [PubMed: 9681636]

Goldman M. Translational mini-review series on Toll-like receptors: Toll-like receptor ligands as novel pharmaceuticals for allergic disorders. Clin Exp Immunol 2007;147:208–216. [PubMed: 17223960]

Hajjar AM, Harvey MD, Shaffer SA, Goodlett DR, Sjostedt A, Edebro H, Forsman M, Bystrom M, Pelletier M, Wilson CB, Miller SI, Skerrett SJ, Ernst RK. Lack of in vitro and in vivo recognition of Francisella tularensis subspecies lipopolysaccharide by Toll-like receptors. Infect Immun 2006;74:6730–6738. [PubMed: 16982824]

Horton H, Thomas EP, Stucky JA, Frank I, Moodie Z, Huang Y, Chiu YL, McElrath MJ, De Rosa SC. Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. J Immunol Methods 2007;323:39–54. [PubMed: 17451739]

Ida JA, Shrestha N, Desai S, Pahwa S, Hanekom WA, Haslett PA. A whole blood assay to assess peripheral blood dendritic cell function in response to Toll-like receptor stimulation. J Immunol Methods 2006;310:86–99. [PubMed: 16455104]

Ivanov S, Dragoi AM, Wang X, Dallacosta C, Louten J, Musco G, Sitia G, Yap GS, Wan Y, Biron CA, Bianchi ME, Wang H, Chu WM. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. Blood. 2007

Kabelitz D, Wesch D, Oberg HH. Regulation of regulatory T cells: role of dendritic cells and toll-like receptors. Crit Rev Immunol 2006;26:291–306. [PubMed: 17073555]

Kanzler H, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med 2007;13:552–559. [PubMed: 17479101]

Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell 2006;124:823–835. [PubMed: 16497591]

Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature 2008;452:234–239. [PubMed: 18305481]

Lamoreaux L, Roederer M, Koup R. Intracellular cytokine optimization and standard operating procedure. Nat Protoc 2006;1:1507–1516. [PubMed: 17406442]

Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol 2004;5:190–198. [PubMed: 14716310]

Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol 2007;7:379–390. [PubMed: 17457344]

Lin SM, Frevert CW, Kajikawa O, Wurfel MM, Ballman K, Mongovin S, Wong VA, Selk A, Martin TR. Differential regulation of membrane CD14 expression and endotoxin-tolerance in alveolar macrophages. Am J Respir Cell Mol Biol 2004;31:162–170. [PubMed: 15059784]

Maecker HT, Frey T, Nomura LE, Trotter J. Selecting fluorochrome conjugates for maximum sensitivity. Cytometry A 2004;62:169–173. [PubMed: 15536642]

Maecker HT, Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. Cytometry A 2006;69:1037–1042. [PubMed: 16888771]

Maecker HT, Rinfret A, D'Souza P, Darden J, Roig E, Landry C, Hayes P, Birungi J, Anzala O, Garcia M, Harari A, Frank I, Baydo R, Baker M, Holbrook J, Ottinger J, Lamoreaux L, Epling CL, Sinclair E, Suni MA, Punt K, Calarota S, El-Bahi S, Alter G, Maila H, Kuta E, Cox J, Gray C, Altfeld M, Nougarede N, Boyer J, Tussey L, Tobery T, Bredt B, Roederer M, Koup R, Maino VC, Weinhold K, Pantaleo G, Gilmour J, Horton H, Sekaly RP. Standardization of cytokine flow cytometry assays. BMC Immunol 2005;6:13. [PubMed: 15978127]

Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. Nat Rev Immunol 2006;6:823–835. [PubMed: 17063184]

Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. Quality assurance for polychromatic flow cytometry. 2006

Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. Nat Rev Immunol 2004;4:648–655. [PubMed: 15286731]

Phillips DJ, League SC, Weinstein P, Hooper WC. Interference in microsphere flow cytometric multiplexed immunoassays for human cytokine estimation. Cytokine 2006;36:180–188. [PubMed: 17306558]

Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. Cell 2006;124:849–863. [PubMed: 16497593]

Steinman RM, Hemmi H. Dendritic cells: translating innate to adaptive immunity. Curr Top Microbiol Immunol 2006;311:17–58. [PubMed: 17048704]

Takahashi HK, Morichika T, Iwagaki H, Tamura R, Kubo S, Yoshino T, Mori S, Akagi T, Tanaka N, Nishibori M. Histamine downregulates CD14 expression via H2 receptors on human monocytes. Clin Immunol 2003;108:274–281. [PubMed: 14499251]

Tesar BM, Goldstein DR. Toll-like receptors and their role in transplantation. Front Biosci 2007;12:4221–4238. [PubMed: 17485369]

Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol 2005;23:307–336. [PubMed: 15771573]

Turvey SE, Hawn TR. Towards subtlety: understanding the role of Toll-like receptor signaling in susceptibility to human infections. Clin Immunol 2006;120:1–9. [PubMed: 16563867]

Vuckovic S, Gardiner D, Field K, Chapman GV, Khalil D, Gill D, Marlton P, Taylor K, Wright S, Pinzon-Charry A, Pyke CM, Rodwell R, Hockey RL, Gleeson M, Tepes S, True D, Cotterill A, Hart DN. Monitoring dendritic cells in clinical practice using a new whole blood single-platform TruCOUNT assay. Development of a clinical assay to evaluate toll-like receptor function. J Immunol Methods 2004;284:73–87. [PubMed: 14736418]

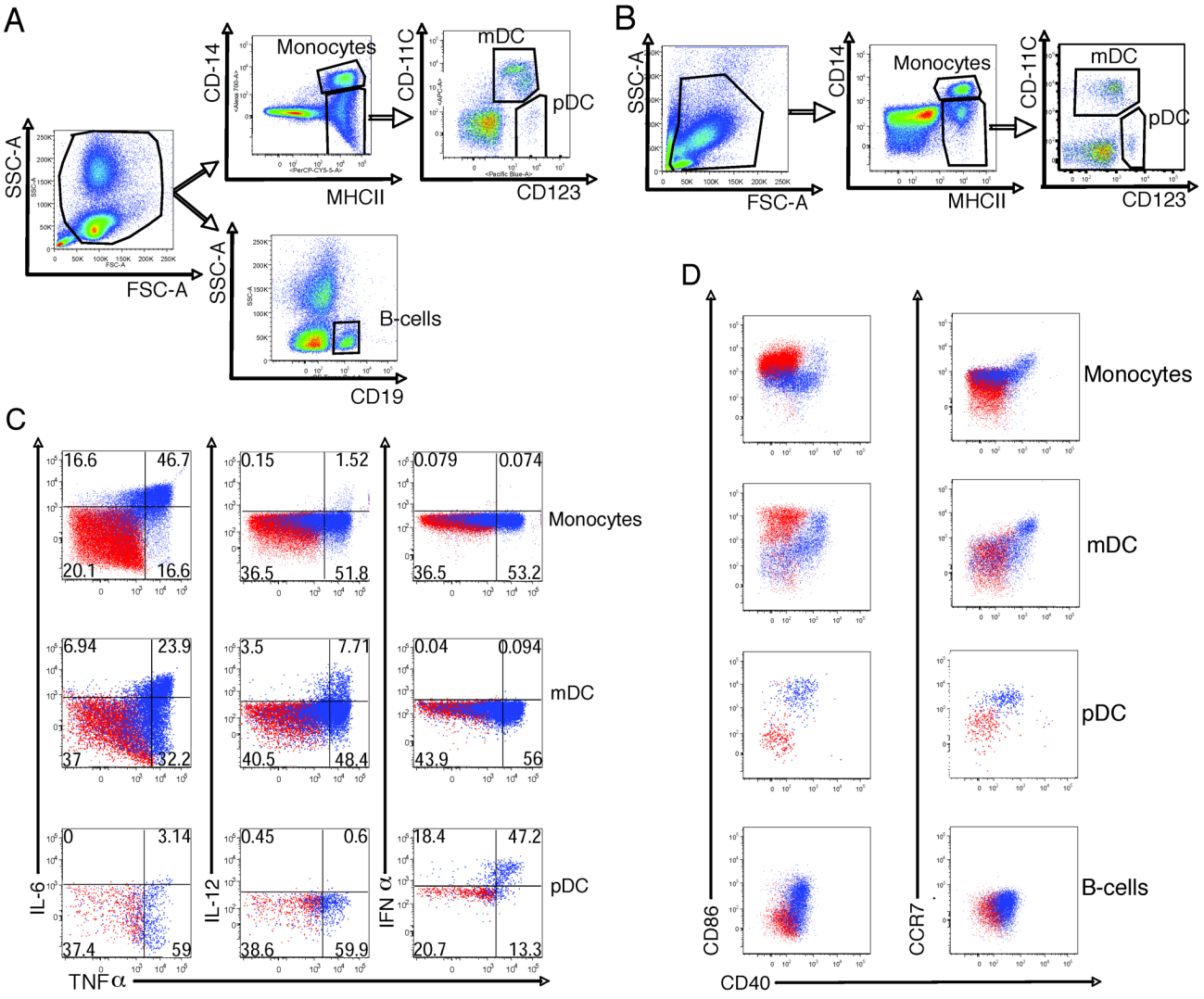

Figure 1. Gating Strategy & Staining Profile for 8-color flow cytometry

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 (MHC-IIpos, CD14pos), B cells (MHC-IIpos, 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 uM of the TLR7/8 ligand 3M-003 (blue) for 6 hours 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. C. Shown is a PBMC sample from the same adult subject, either unstimulated (red) or stimulated with TLR7/8 ligand 3M-003 (blue) for 18 hours without any Golgi-blocker. Innate immune cell subsets were identified as indicated above, then analyzed for expression of CD86, CCR7 and CD40.

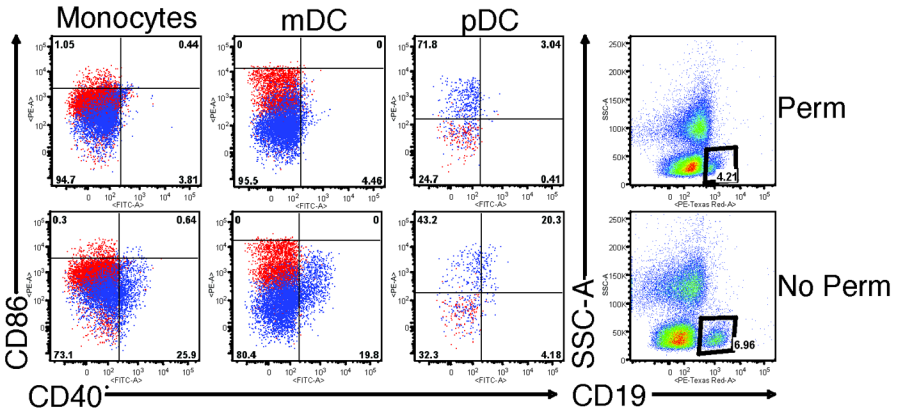

Figure 2. Permeabilization Impacts Surface Marker Detection

Adult PBMC were either left unstimulated (red) or stimulated with 10 μM of the TLR7/8 ligand 3M-003 (blue) for 18 hours 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 Figure 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.

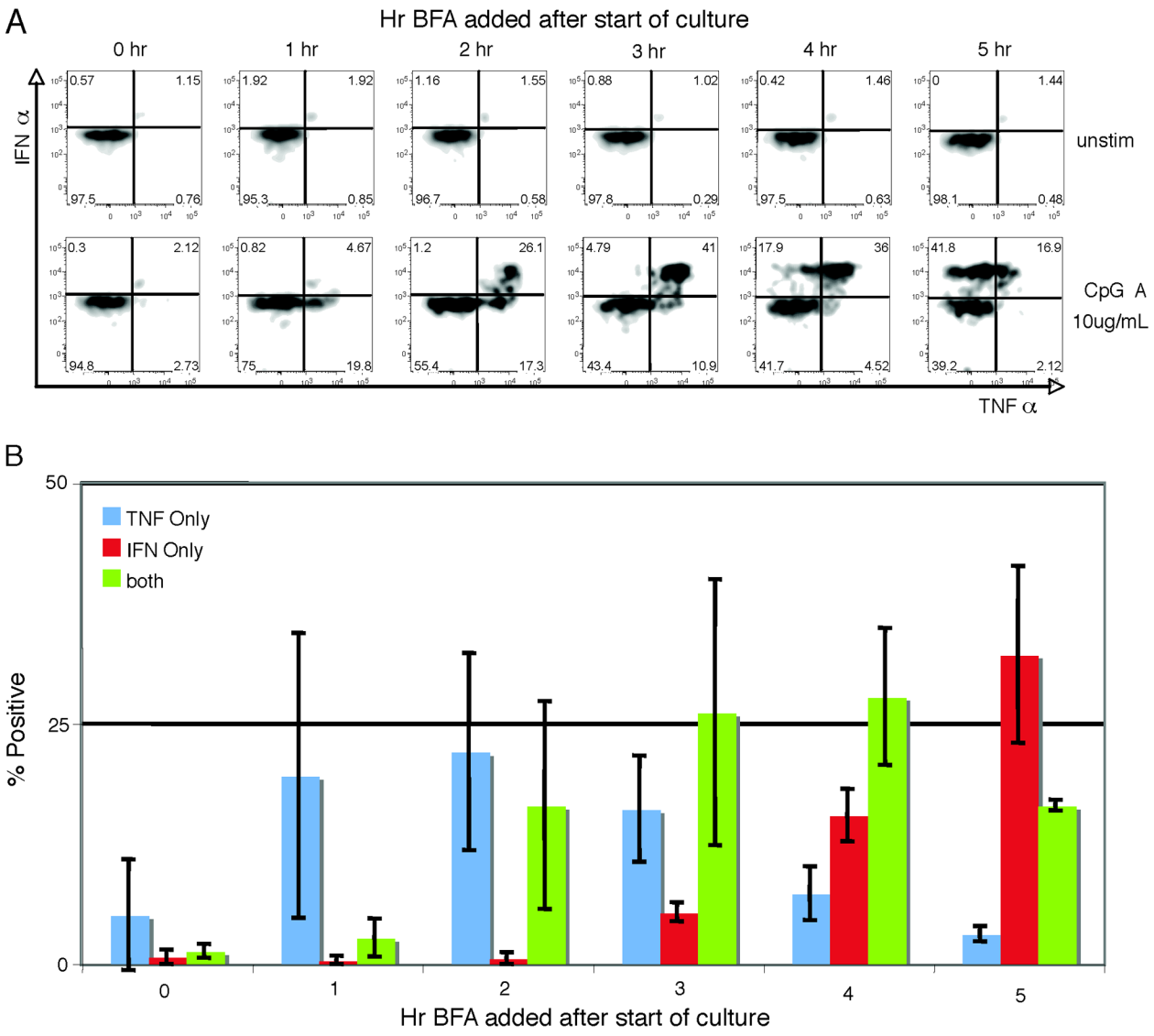

Figure 3. Timing of Brefeldin A Addition has Significant Impact on Responses to CpGs

A. Adult PBMC were either left unstimulated (top row), or stimulated with 10 ug/ml of the TLR9 ligand CpGA (lower row) for 6 hours. The Golgi-blocker Brefeldin A (BFA) was added hourly right from the start (0 hours) or up to 5 hours later. All cells were incubated for the same total time of 6 hours, 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.

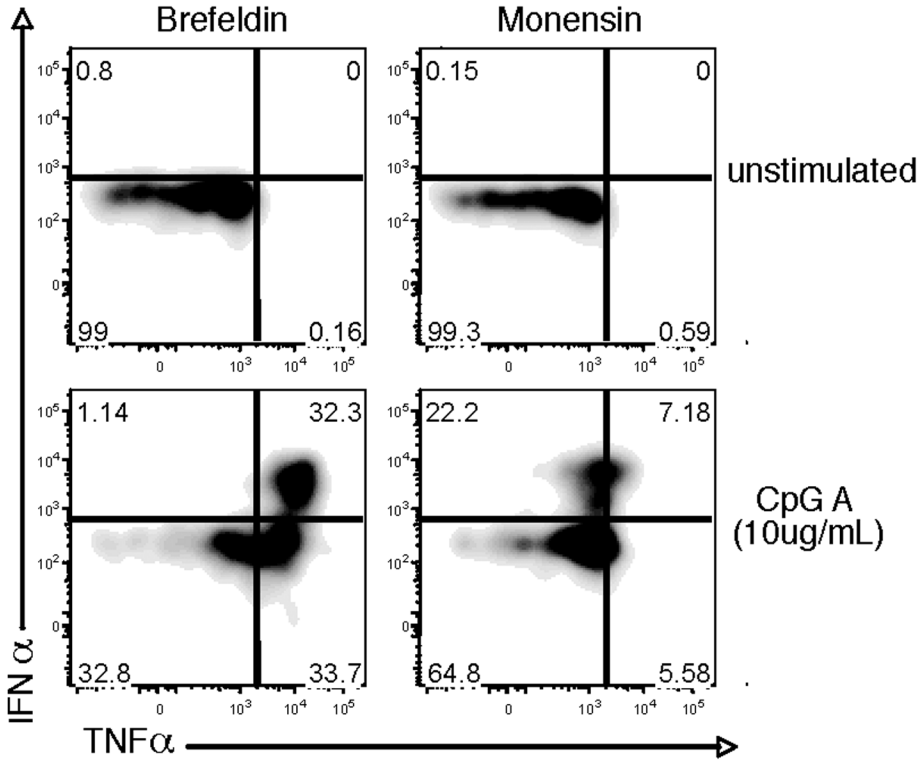

Figure 4. Brefeldin A is the better Golgi Blocking Reagent for ntracellular Cytokine Analysis in Innate Immune Cells

Adult PBMC were either left unstimulated (top row) or were stimulated with 10 μg/ml of the TLR9 ligand CpGA (lower row) for 6 hours. The Golgi-blockers Brefeldin A (BFA) or Monensin were added 3 hours into the incubation period. Cells were incubated for another 3 hours, then processed further for intracellular expression of cytokines. Shown is the co-expression of TNF-α and IFN-α in plasmacytoid DCs.

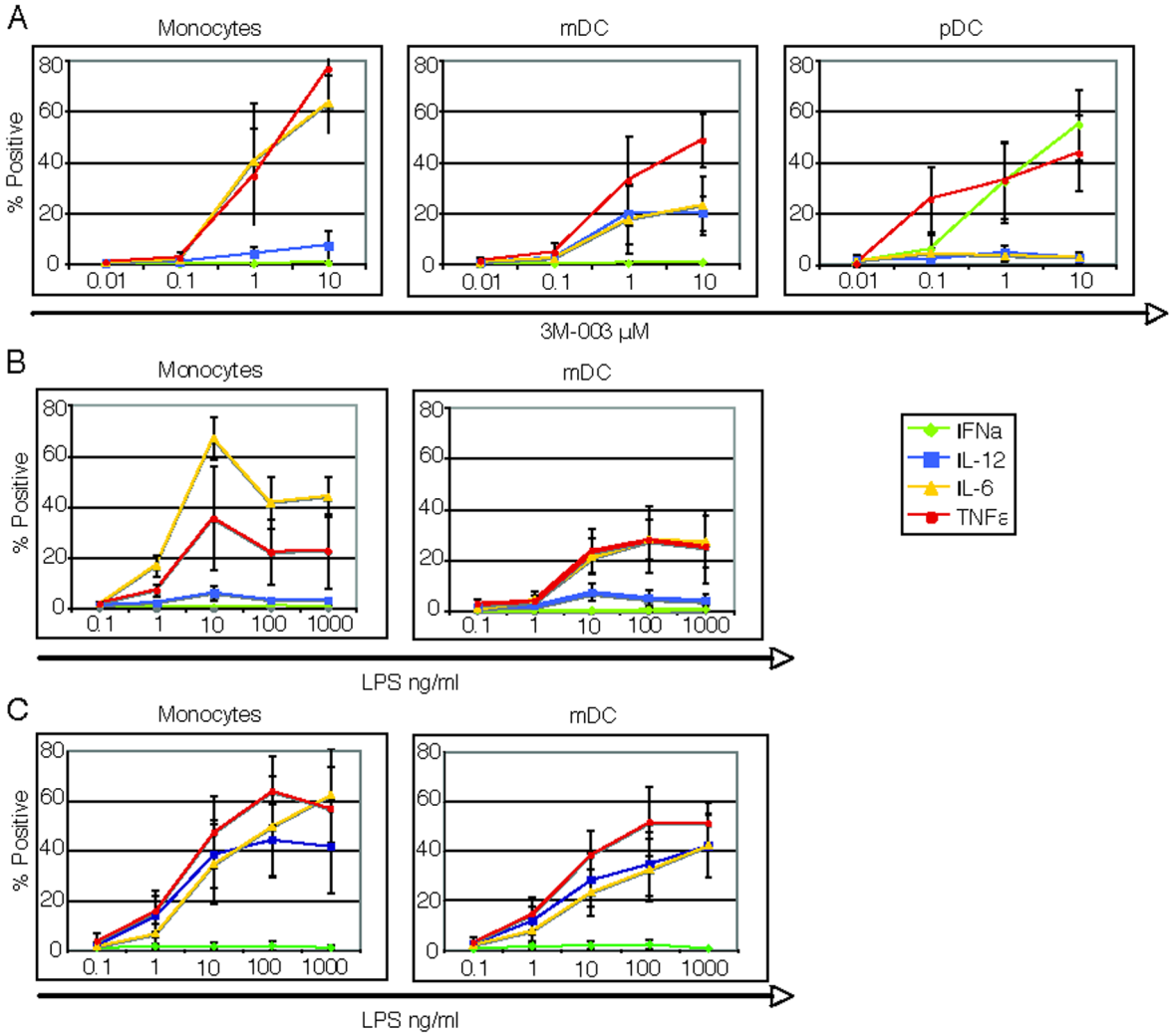

Figure 5. Dose Response Profiles for 3M-003 and LPS

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. Shown in all graphs is the result of intracellular cytokine detection in samples stimulated for 6 hours 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).

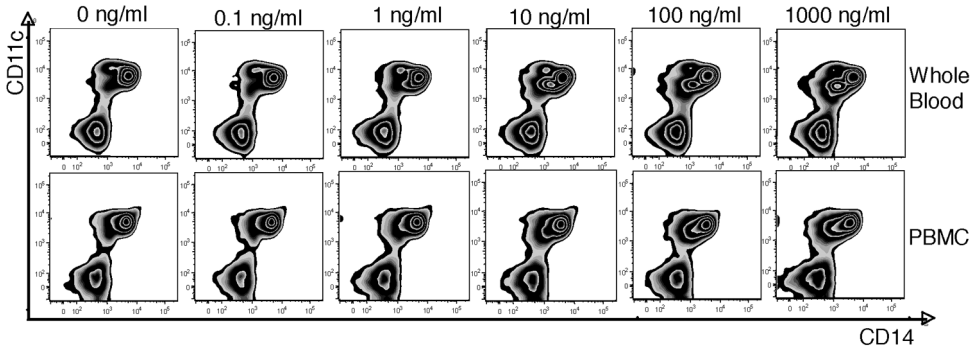

Figure 6. Lineage Marker Expression is Altered with TLR stimulation

Adult whole blood (top row) or PBMC (bottom row) were either left unstimulated or stimulated for 6 hours 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.

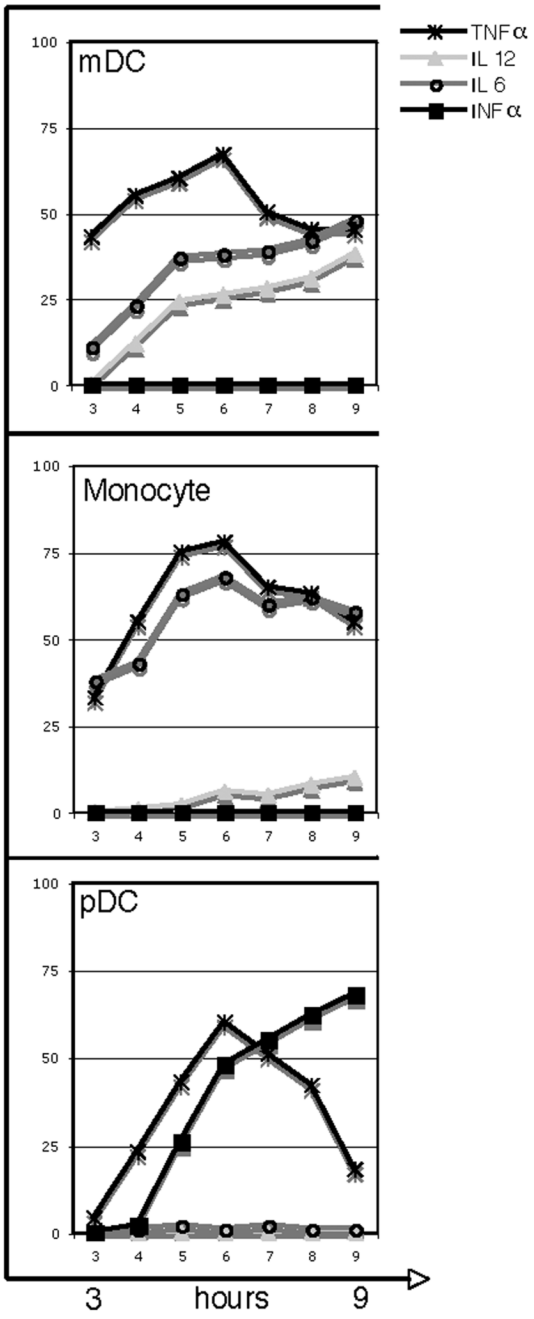

Figure 7. Optimal Time Point of Total Incubation Length

Adult PBMC were either left unstimulated or stimulated with 10μM 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 hours 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.

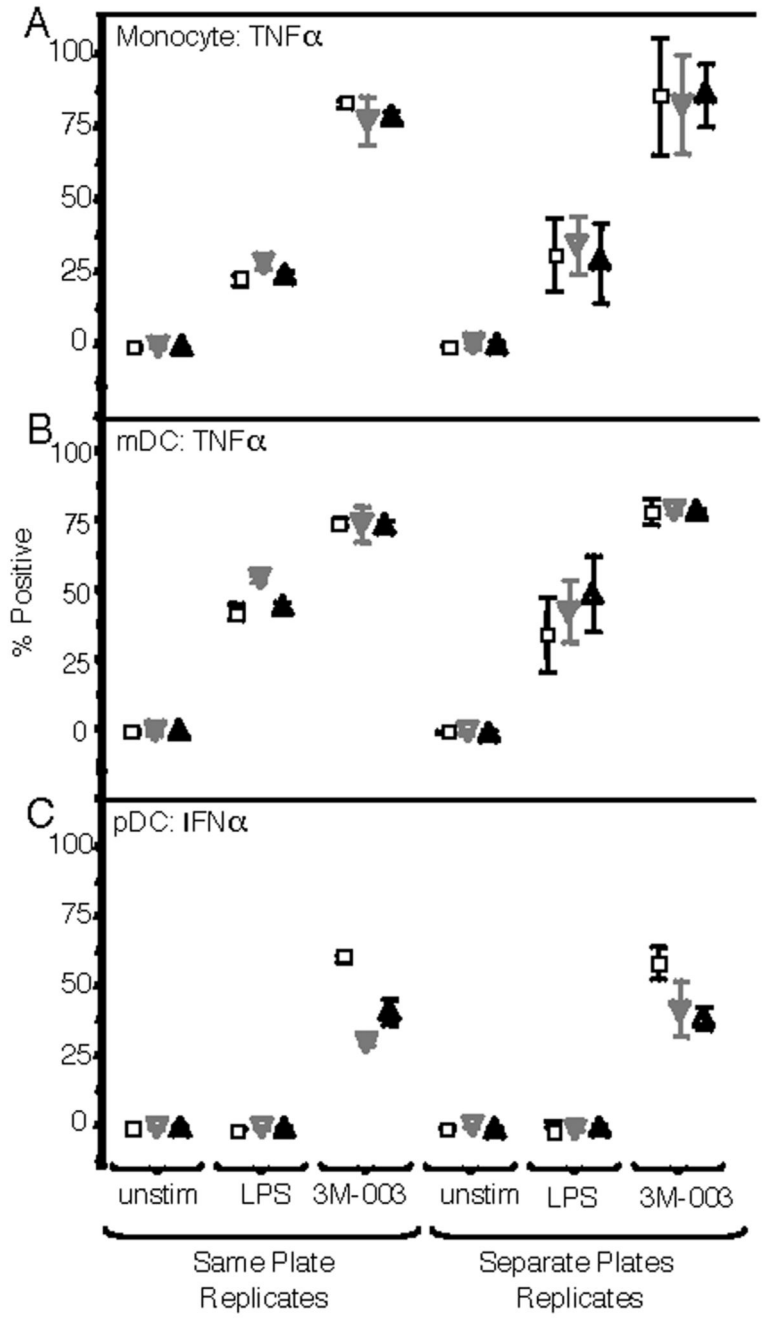

Figure 8. Precision of the Assay

PBMC from 3 different adults were either left unstimulated or stimulated with 10 μM of the TLR7/8 ligand 3M-003 or 10ng/ml of the TLR4 ligand LPS for 6 hours 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 = standard deviation.

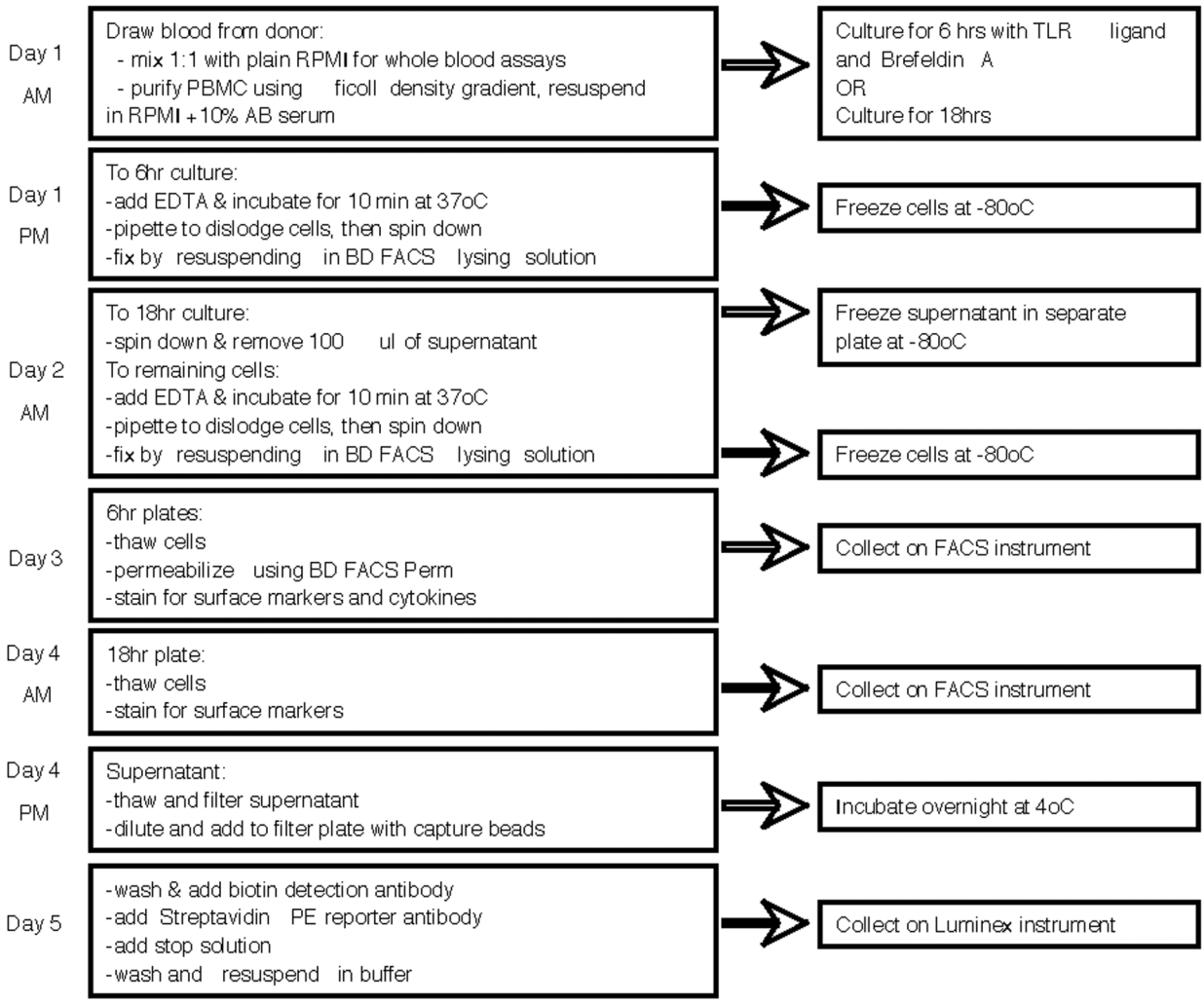

Figure 9. Summary of the protocol

Table 1

TLR ligand panel used to stimulate innate immune cells

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

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

Table 2

Staining panel used for polychromatic flow cytometry

A) Panel used for intracellular cytokine staining

B) Panel used for cell surface staining

A

	Characteristic Being Measured	Antibody Name Clone Name	Vendor cat# dilution used
VIOLET			
AmCyan	Cell Surface Protein	CD123 AmCyan (9F5)	BD#custom 1:50
Pacific Blue	Intracellular Protein	IL12p40/70 (eBio: C8.6)	eBio#577129 1:100
RED			
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	TNFa (Mab11)	BD#557996 1:100
BLUE			
FITC/OG	Intracellular Protein	IFNa (A11)	Antigenix#MC100133 1:100
PerCPCy5.5	Cell Surface Protein	MHCII TU36	BD#custom 1:100
PE-Cy7	Cell Surface Protein	CD14 (M5E2)	eBio #25-0149 1:50

B

	Characteristic Being Measured	Antibody Name Clone Name	Vendor cat# dilution used
VIOLET			
Pacific Blue	Cell Surface Protein	CD 123 (9F5)	BD#custom 1:100
RED			
APC	Cell Surface Protein	CD11c (5HCL3)	BD#340714 1:50
Alexa 700	Cell Surface Protein	CD14 (M5E2)	BD#557923 1:50
BLUE			
FITC/OG	Cell Surface Protein	CD40 (5C3)	eBio #11-0409 1:100
PE	Cell Surface Protein	CD86 (IT2.2)	eBio #12-0869 1:100
Per CPCy5.5	Cell Surface Protein	MHCII TU36*	BD#custom 1:100
PE-TexRed	Cell Surface Protein	CD19-biotin (HIB19) streptavidin PE-TR	BD#555411 BD#551487 1:50
PE-Cy7	Cell Surface Protein	CCR7 (3D12)	BD#557648 1:100