Supplementary Figures

Kollmann *et al*., Neonatal innate TLR-mediated response are distinct from those of adults.

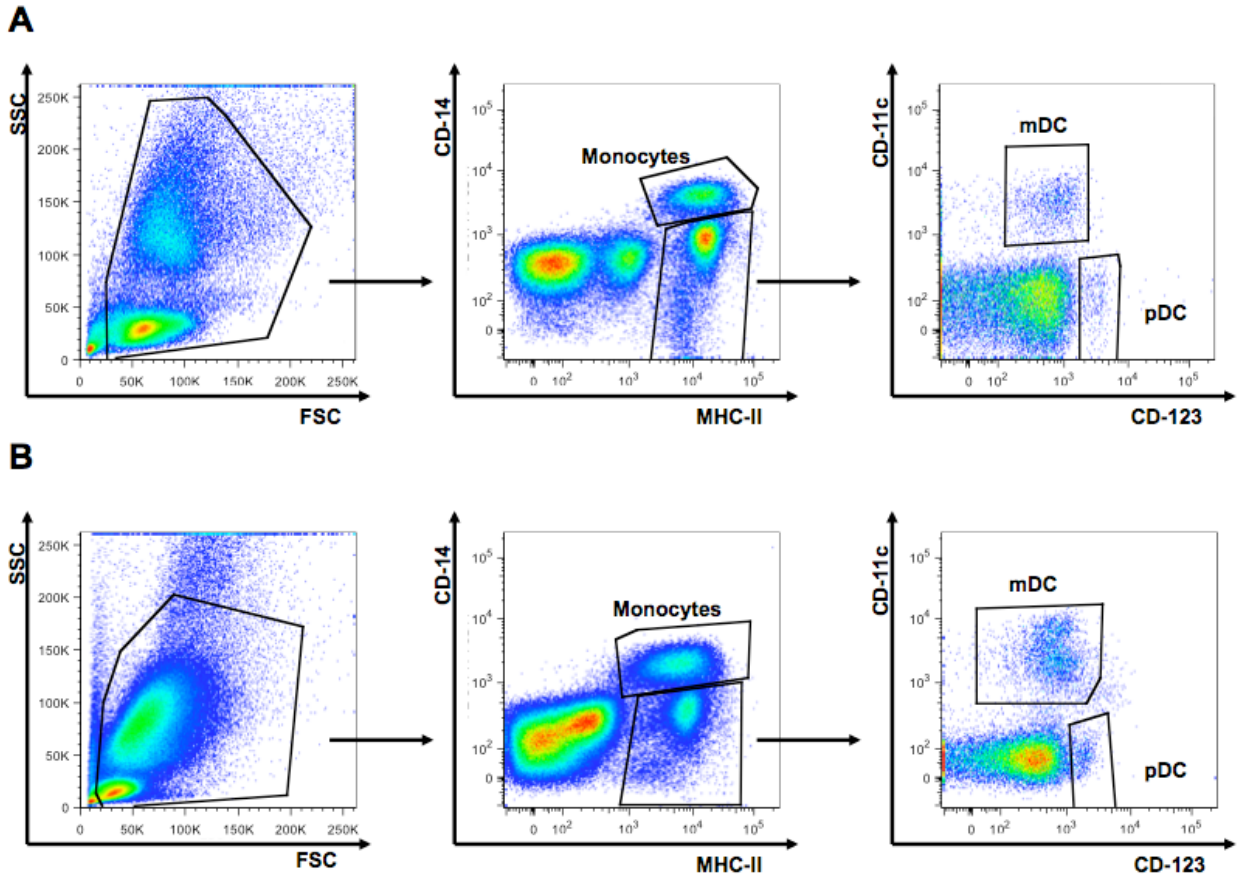

Figure S1. Gating strategy for antigen-presenting cell subsets in MC and WB. Shown is a neonatal MC sample in A, and a neonatal WB sample in B. The gating strategy to identify various innate immune cell subsets was as follows: monocytes (MHCIIpos, CD14pos/high), conventional DCs (MHCIIpos, CD14neg/low, CD123neg, CD11cpos) and plasmacytoid DCs (MHCIIpos, CD14neg/low, CD11cneg, CD123pos).

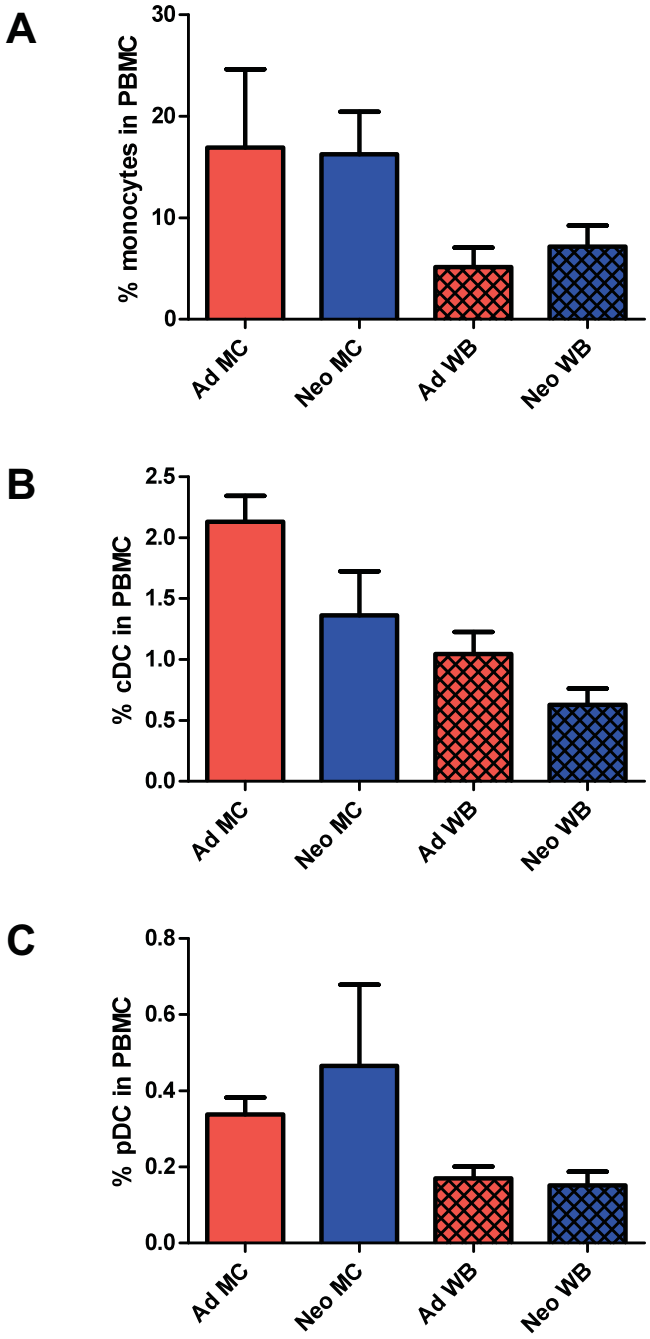

Figure S2. Antigen-presenting cell composition is similar but not identical between neonatal and adult MC and WB samples. Antigen presenting cell percentages for 25 adult and 25 neonatal samples, determined using the gating strategy shown in Supplementary Figure 1. Results shown are the mean ± SD.

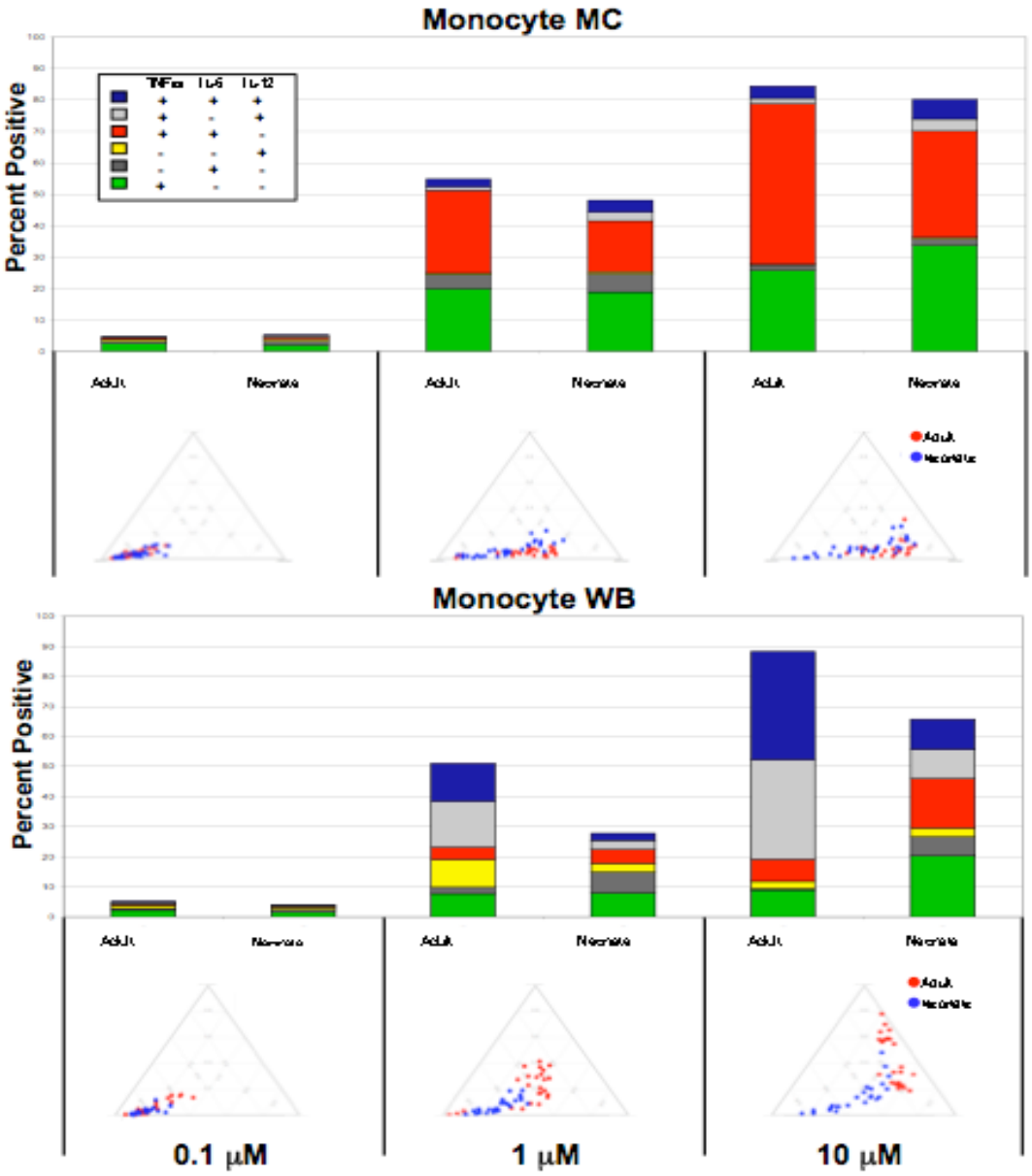

Figure S3. Monocytes in neonatal WB but not MC samples are less responsive and less polyfunctional than adult monocytes to the TLR7/8 ligand 3M-003 at all concentrations. The difference was most pronounced at the highest concentration.
MC or WB samples from adults (A) and neonates (N) (n=25 each) were stimulated with increasing concentrations (0.1, 1 and 10 µM) of the TLR7/8 ligand 3M-003 for 6 hours in the presence of Brefeldin A. Intracellular TNF-a, IL-6, and IL-12/23p40 in monocytes of these samples were determined by multiparameter flow cytometry. Percentage of monocytes expressing one or a combination of these cytokines are shown in the stacked bar graphs. Degree of polyfunctionality depicted in ternary plots. Each circle represents monocytes from one individual (blue = neonate; red = adult). Circles closer to the left lower corner of the triangles are monocytes that expressed any one of these cytokine, circles closer to the right lower are monocytes that expressed any 2, and those closer to the top corner expressed all 3.

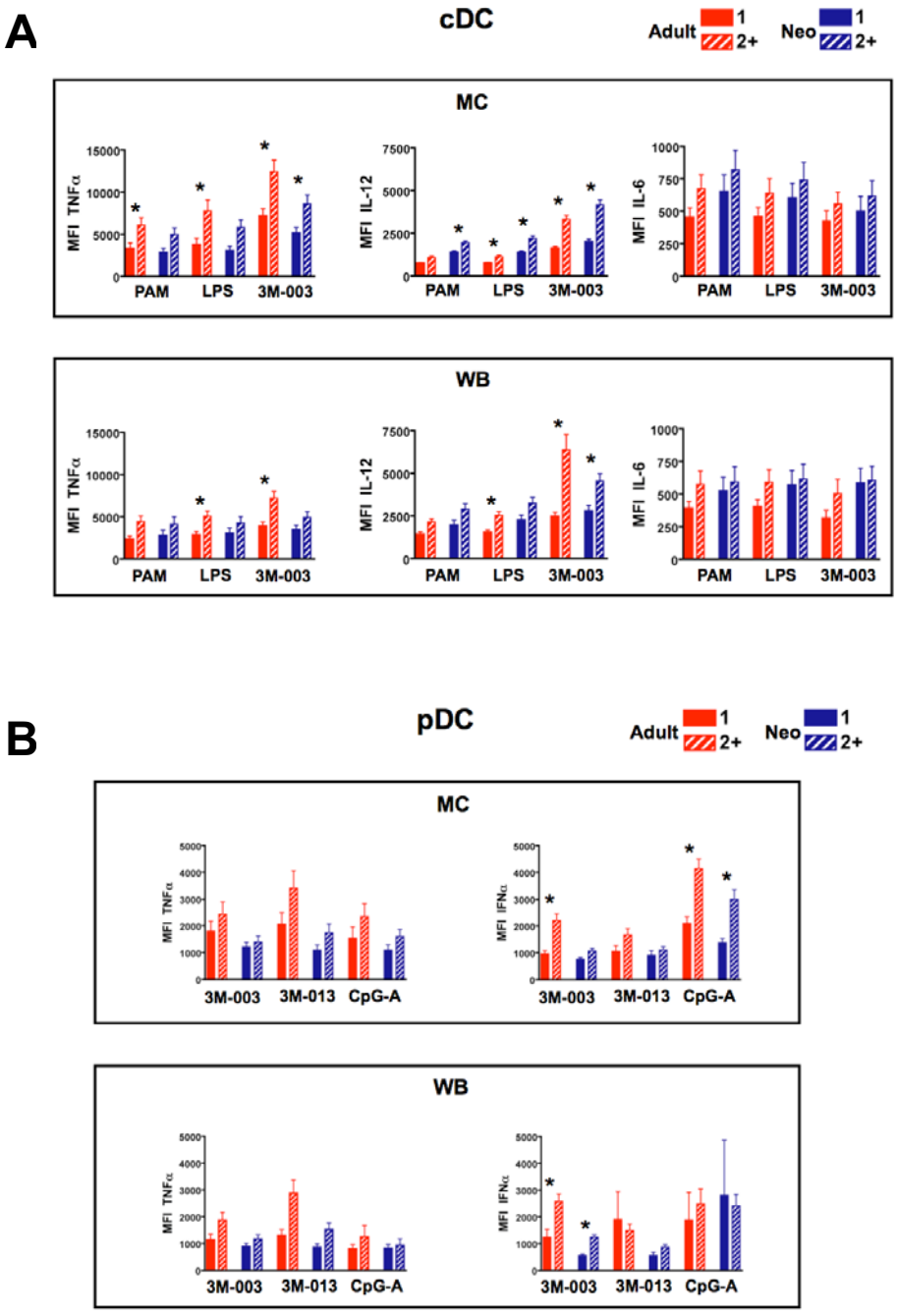

Figure S4. The degree of polyfunctionality may be a specifically regulated function for some but not all cytokines. Blood samples (n = 25 each) from adult or neonate were stimulated with indicated TLR ligands (only top concentration for each ligand is shown) for 6 hours. cDC (**A**) and pDC (**B**) were identified, and expression of intracellular TNFa, IL-6, IL-12/23p40, and IFNa assessed as indicated above. The y-axis depicts the MFI (mean fluorescent intensity) for the indicated cytokine expressed in cells only expressing this one cytokine (solid bar) or in cells expressing this one cytokine plus any of the others (hatched bar), comparing adult (red) to neonate (blue).

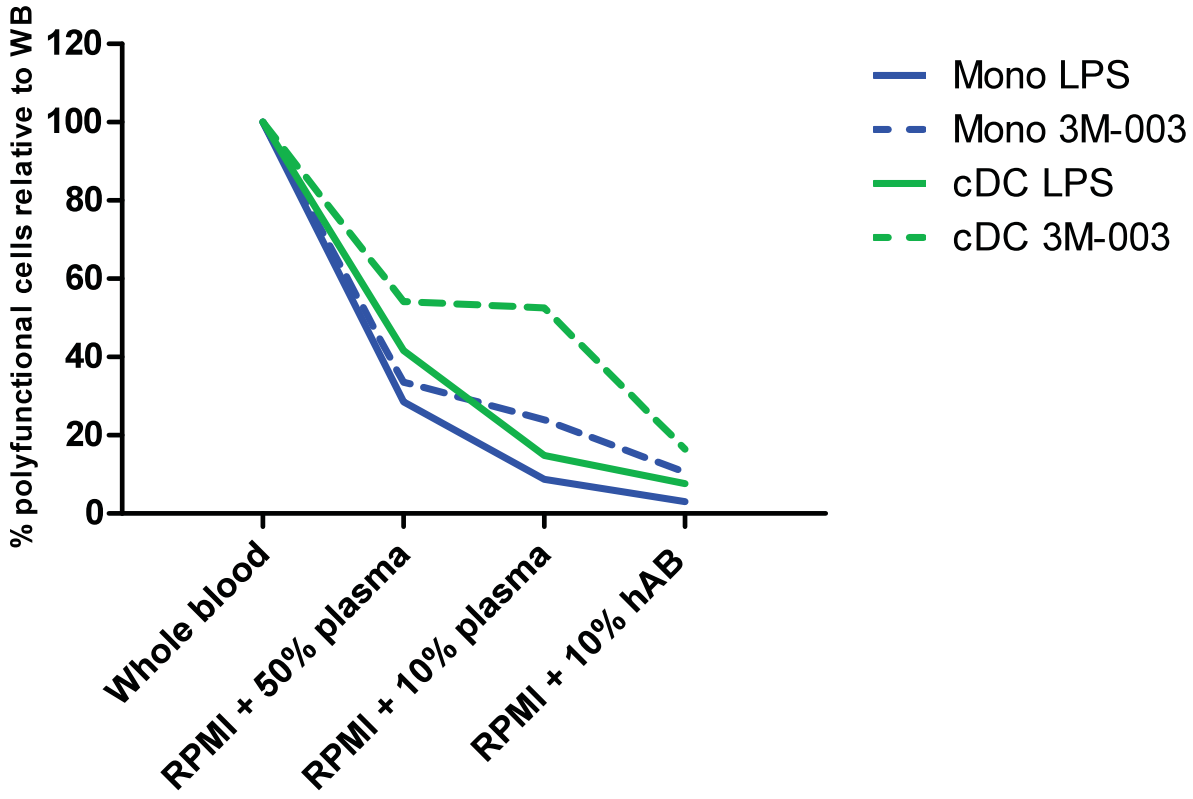

Figure S5. Polyfunctionality is influenced by both, soluble factors in plasma as well as cell-cell interaction in whole blood. The fraction of cells expressing all 3 cytokines (TNF, IL-6, IL-12p40), i.e. cells that are Pfx3**,** was always the highest in WB. We thus set the % of Pfx3 cells in WB to 100%. The impact of other culture conditions on Pfx3 was compared. Cells were gated on monocytes (Mono) or cDC, and analyzed by intracellular flow cytometry, after 6 hr stimulation with either TLR4 ligand LPS (100 ng/ml) or TLR7/8 ligand, 3M003 (10 μM). WB = whole blood, mixed 1:1 with plain RPMI (our WB set-up); hAB = PBMC in RPMI with 10% pooled human AB serum (our PBMC set-up); 10% = PBMC in RPMI with 10% autologous plasma; 50% = PBMC in RPMI with 50% autologous plasma. Shown are the results for one representative adult blood sample out of 3 subjects analyzed.

Supplementary Data
MIFlowCyt standard compliant information for submitted flow cytometric data.

1. **Experiment overview.**
   - **1.1. Purpose**:
     The purpose of the experiment was to compare the response of monocytes, plasmacytoid dendritic cells, and conventional dendritic from neonatal cord blood to adult peripheral blood to TLR stimulation. We also compared purified peripheral white blood mononuclear cells (PBMC) to whole blood (WB) to assess the contribution of factors contained in the plasma or non-PBMC cellular fraction. We hypothesized that cord blood responses to TLR would differ from adult blood responses and that PBMC would differ from WB.
   - **1.2. Keywords:**
   - **1.3. Organization:**
     - **1.3.1.** Wilson Lab, Department of Immunology
       University of Washington
     - **1.3.2.** H459 Health Sciences Building
       1959 NE Pacific St
       Seattle, WA 98195
   - **1.4. Primary Contact:**
     - **1.4.1.** P.I. Dr. Christopher B. Wilson cbwilson@u.washington.edu
     - **1.4.2.** P.I. Dr. Tobias Kollmann tkollmann@cw.bc.ca
     - **1.4.3.** Technicians Juliet Crabtee: jnc3@u.washington.edu; and Annie Rein-Weston: arein@u.washington.edu.
   - **1.5. Date:**
     Experiments were **set up from 5/24/07 to 6/5/07 and stained from 6/6/07; to 6/22/07.**
   - **1.6. Conclusions:**
     Cord blood responses to TLR stimulation differ significantly from adult blood responses. The difference between cord and adult blood is more pronounced in WB as compared to PBMC.
   - **1.7. Quality Control Measures:**
     Unstimulated controls were set up for each condition tested.
     Single stain controls were set up by staining 3 ul of Anti-Mouse Ig CompBeads (BD #552843) and 3 ul of anti-FBS negative control beads (included with BD #552843) with 3ul of each antibody used.

2. **Flow Sample/Specimen Description**
   - **2.1. Sample/Specimen Material**
     - **2.1.1. Biological Samples:**
       - **2.1.1.1. Biological Sample Name**
         Cord and adult PBMC or WB
       - **2.1.1.2.Biological Sample Source:**
         Healthy human adult male peripheral blood; cord blood obtained and processed within < 4h from a healthy full-term baby born via C-section (no labor).

2.1.1.2.1. Biological Sample Source Organism:

2.1.1.2.1.1. Taxonomy:

Homo sapiens

2.1.1.2.1.2. Age:

birth (for cord blood); 21-45 years old (for adult blood)

2.1.1.2.1.3. Gender:

Male and Female

2.1.1.2.1.4. Phenotype:

healthy (none)

2.1.1.2.1.5. Genotype:

not applicable

2.1.1.2.1.6. Treatment:

PBMCs were isolated from peripheral blood using a Ficoll gradient. After purification, cells were washed twice with DPBS then resuspended at 1 x 10^6 in RPMI supplemented with 10% human AB serum and 1% Pen/Strep. WB was mixed 1:1 with RPMI.

2.1.2. Environmental Samples: not applicable

2.1.3. Control Sample Description:

Single stain controls were set up by staining 3 ul of Anti-Mouse Ig CompBeads (BD #552843) and 3 ul of anti-FBS negative control beads (included with BD #552843) with 3ul of each antibody used.

Sample Treatment Description

Cells were plated in a 96 well plate and cultured for a total of 6 hrs. Cells were stimulated with either nothing, or PAM3CSK4 (TLR2/1, EMC microcollections); poly I:C (TLR3, Amersham); 0111:B4 LPS (TLR4, InVivogen); 3M-003 (TLR/8, 3M); 3M-002 (TLR8, 3M); 3M-013 (TLR7, 3M); CpGA (TLR9, Coley). After culture, cells were treated with a final concentration of 2mM EDTA for 15 min at 37^{o}C, then spun down and resuspended in 100ul of 1x BD FACS Lysing solution (BD 349202) and frozen at -80 ^{o}C.

3. Fluorescence Reagent Description:

	Characteristic Being Measured	Antibody Name *Clone Name*	Vendor cat# *dilution used*
VIOLET			
AmCyan/PO	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 *(AS12)*	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)*	BD #557742 *1:50*

Instrument Details:

3.1. Manufacturer:

BD Biosciences

3.2. Model:

BD LSR II 4 Laser, Blue/Red/Violet/UV cat # 347545

3.3. Instrument Configuration and Settings:

All lasers, filters and mirrors were manufactured by BD Biosciences. All filters and mirrors came with the machine and were installed March 2005.

3.3.1. Light Sources:

The light path, filters and detectors are described below in Table 2. The lasers are listed in the order the cells pass through them. The detectors and filters are listed in the order the light hits them, with the exception of FSC which is measured from light that passes through the cell/bead while all the other 488 detectors detect light that has been scattered 90 ^{o}, in the order listed. For example, for blue laser detector A light passes through or is reflected off of filter 1, 735 LP, then the light passes through filter 2, 780/60 BP, then it hits the PMT detector. Light that is reflected off the long pass goes to detector B and so on. For parameters used in this experiment, it is indicated whether Area (-A), Height (-H) or Width (-W) was used.

Abbreviations:

PMT = photomultiplier tube

PD = photodiode,

BP = band pass filter, first number is center of interval, second number is the width of the interval.

LP = long pass filter, lets light waves through that have a longer wavelength than the number specified. All LP filters are dichroic and reflect at an angle of incidence at 11.25^{o}.

Table 2

Laser	Detector Name (Type)	Filter 1 (1st filter)	Filter 2 (2nd Filter)	Parameter detected	Detector voltage	Amplification Type
Blue Laser (488 nm)	FSC (PD)	488/10 BP	na	FSC-A	390	LINEAR
Solid state Coherent Sapphire blue laser 20 mW	488 A (PMT)	735 LP	780/60 BP	PE-Cy7-A	625	LOG
	488 B (PMT)	685 LP	712/21 BP	PerCP-Cy5.5-A	750	LOG
	488 C (PMT)	655 LP	670/14 BP	PerCP	na	
	488 D (PMT)	595 LP	610/20 BP	PE-TexRed	na	
	488 E (PMT)	550 LP	575/26 BP	PE	na	
	488 F (PMT)	505 LP	530/30 BP	FITC, Ax488-A	450	LOG
	488 G (PD)	blank	488/10 BP	SSC-A	410	LINEAR
	488 H (PMT)	blank	na	blank	na	
Violet Laser (405 nm)	405 A (PMT)	505 LP	585/42 BP	AmCyan-A	650	LOG
Coherent VioFlame PLUS laser 25mw	405 B (PMT)	blank	440/40 BP	Pacific Blue-A	530	LOG
	405 C (PMT)	blank	na	blank	na	
UV Laser (355 nm)	355 A (PMT)	505 LP	530/30 BP	Ca++ Blue	na	
Solid state Coherent Lightwave Xcyte 20mW	355 B (PMT)	blank	440/40 BP	Alexa350	na	
	355 C (PMT)	blank	na	blank	na	
Red Laser (637 nm)	637 A (PMT)	755 LP	780/60 BP	APC-Cy7-A	700	LOG
Solid State Coherent laser 25 mW	637 B (PMT)	685 LP	710/50 BP	Alexa700-A	600	LOG
	637 C (PMT)	660/20 BP	na	APC-A	505	LOG

4. Data Analysis

4.1. FCS Data File:

To request raw data please contact Dr. Christopher Wilson

cbwilson@u.washington.edu

4.1.1. Total Count of Events:

Recorded within individual FCS files, as keyword $TOT, 200,000 events for PBMC, and 1 millione events for WB.

4.2. Compensation Description:

Compensation was done in FlowJo using BDCompBeads as single stain controls. The matrix is below in Table 3:

Compensation Matrix for one of the sample shown in Supplementary Figure1.

	Am Cyan-A	Pacific Blue-A	APC-A	APC-CY7-A	Alexa 700-A	FITC-A	PerCP-CY5-5-A	PE-CY7-A
Am Cyan-A		1.74	0	0	0	1.78	0	0
Pacific Blue-A	103.5		0	0	0	0	0	0
APC-A	0.212	0		12.15	56.26	0	0.439	0
APC-CY7-A	-0.0876	0.135	4.96		4.17	0	0	0.387
Alexa 700-A	0.412	0.0632	0.378	28.37		0	1.9	0.242
FITC-A	102.7	0.233	-0.0555	-0.132	-0.0785		1.96	0.156
PerCP-CY5-5-A	0.221	0.0675	2.58	9.61	40.22	0		9.84
PE-CY-A	0.274	0	0	35.64	0.541	0.246	2.29	

4.3. Gating (Data Filtering) Description:

4.3.1. Gate Summary Information:

4.3.1.1. -4.3.1.3 Gate Descriptions/subpopulations/statistics:

		Gate Statistics (% Parent Gate)	
Gate Description:	**Qualitative Description of the Subpopulation**	**Unstim (red)**	**R848 stim (blue)**
Live Cells	High cell density excluding lower left corner population	79.6	71.9
Monocytes	CD14 high, MCHII high	24.9	13.2
Other MHCII+ cells	MHCII high, CD14 mid to low	15.3	19.2
Myeloid Dendritic Cells (mDCs)	MHCII high, CD11c high, CD123 low	22.9	26.9
Plasmacytoid Dendritic Cells (pDCs)	MHCII high, CD11c low, CD123 high	2.39	2.42
Monocyte TNF+ IL-6 -	"Monocyte" TNFa high, IL-6 low	0.29	16.6
Monocyte TNF+ IL-6+	"Monocyte" TNFa high, IL-6 high	0.15	46.7
Monocyte TNF- IL-6+	"Monocyte" TNFa low, IL-6 high	0.28	16.6
Monocyte TNF- IL-6-	"Monocyte" TNFa low, IL-6 low	99.3	20.1
Monocyte TNF+ IL-12-	"Monocyte" TNFa high, IL-12 low	0.35	61.8
Monocyte TNF+ IL-12+	"Monocyte" TNFa high, IL-12 high	0.093	1.52
Monocyte TNF- IL-12+	"Monocyte" TNFa low, IL-12 high	0.048	0.15
Monocyte TNF- IL-12-	"Monocyte" TNFa low, IL-12 low	99.5	36.5
Monocyte TNF+ IFNa -	"Monocyte" TNFa high, IFNa low	0.43	63.2
Monocyte TNF+ IFNa+	"Monocyte" TNFa high, IFNa high	0.00757	0.074
Monocyte TNF- IFNa+	"Monocyte" TNFa low, IFNa high	0.033	0.079
Monocyte TNF- IFNa-	"Monocyte" TNFa low, IFNa low	99.5	36.6
mDC TNF+ IL-6 -	"mDC" TNFa high, IL-6 low	0.98	32.2
mDC TNF+ IL-6+	"mDC" TNFa high, IL-6 high	0.018	23.9
mDC TNF- IL-6+	"mDC" TNFa low, IL-6 high	1.66	6.94
mDC TNF- IL-6-	"mDC" TNFa low, IL-6 low	97.3	37
mDC TNF+ IL-12-	"mDC" TNFa high, IL-12 low	0.98	48.4
mDC TNF+ IL-12+	"mDC" TNFa high, IL-12 high	0.018	7.71
mDC TNF- IL-12+	"mDC" TNFa low, IL-12 high	0.32	3.5
mDC TNF- IL-12-	"mDC" TNFa low, IL-12 low	98.6	40.5
mDC TNF+ IFNa -	"mDC" TNFa high, IFNa low	1	56
mDC TNF+ IFNa+	"mDC" TNFa high, IFNa high	0	0.094
mDC TNF- IFNa+	"mDC" TNFa low, IFNa high	0.14	0.04
mDC TNF- IFNa-	"mDC" TNFa low, IFNa low	98.8	43.9
pDC TNF+ IL-6 -	"pDC" TNFa high, IL-6 low	0.69	59.2
pDC TNF+ IL-6+	"pDC" TNFa high, IL-6 high	0.17	3.16
pDC TNF- IL-6+	"pDC" TNFa low, IL-6 high	0.69	0
pDC TNF- IL-6-	"pDC" TNFa low, IL-6 low	98.3	37.6
pDC TNF+ IL-12-	"pDC" TNFa high, IL-12 low	0.86	60.2
pDC TNF+ IL-12+	"pDC" TNFa high, IL-12 high	0	0.6
pDC TNF- IL-12+	"pDC" TNFa low, IL-12 high	0.17	0.45
pDC TNF- IL-12-	"pDC" TNFa low, IL-12 low	98.8	38.8
pDC TNF+ IFNa -	"pDC" TNFa high, IFNa low	0.86	13.4
pDC TNF+ IFNa+	"pDC" TNFa high, IFNa high	0	47.4
pDC TNF- IFNa+	"pDC" TNFa low, IFNa high	0.34	18.5
pDC TNF- IFNa-	"pDC" TNFa low, IFNa low	98.6	20.8

Table 4

4.4. Data Transformation Description:

Data was transformed using FlowJo's "Define BiExponential Transformation" function using the above mentioned compensation matrix, with an additional negative display size set at 0.5 and Positive Decades of "log" Display set at 5.