

Identification of B Cells Through Negative Gating—An Example of the MIFlowCyt Standard Applied

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• Abstract

Polychromatic flow cytometric analysis takes advantage of the increasing number of available fluorophores to positively identify and simultaneously assess multiple parameters in the same cell (1). Additional parameters may be analyzed through negative identification (i.e., through exclusion of particular stains or antibodies employed). In this report, we tested whether such negative-gating strategy would identify human B lymphocytes in innate immune phenotyping studies. To this end, B cells were identified as the negatively-stained subpopulation from the CD123 vs. CD11c plot of the CD14^{neg-low}, MHC II^{high} human peripheral blood mononuclear cells. To test the specificity of this negative gating approach, we confirmed that negatively gated B cells indeed expressed CD19, the bona fide marker for human B cells. However, a small number of unidentified cells were contained in the negatively-gated B cells. Furthermore, a small percentage cells expressing markers used to identify monocytes and myeloid dendritic cells (mDC) coexpressed CD19. This identifies such negative B-cell gating approach as potentially problematic. When applied to the analysis of Toll-like receptors (TLR) stimulation experiments, we were however able to interpret the results, as B-cells respond to TLR stimulation in a qualitative different pattern as compared to monocytes and DC. This report is presented in a manner that is fully compliant with the Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) standard, which was recently adopted by the International Society for Advancement of Cytometry (ISAC) (2) and incorporated in the publishing policies of Cytometry and other journals. We demonstrate how a MIFlowCyt-compliant report can be prepared with minimal effort, and yet provide the reader with a much clearer picture of the portrayed FCM experiment and data. © 2010 International Society for Advancement of Cytometry

• Key terms

innate immune response; toll-like receptors; activation markers; B cells; MIFlowCyt

HUMAN B cells are known to express pattern recognition receptors, e.g., Toll-like receptors (TLR), and are amenable to stimulation with pathogen-associated molecular patterns such as TLR ligands (3–5). Akin to innate immune cells such as monocytes and dendritic cells, B cells are believed to play a role in the initial response to infection or vaccination and take part in the complex interactions directing the ensuing adaptive immune response. Polychromatic flow cytometry (FCM) allows assessment of such complex immune interactions. We previously described a high-throughput polychromatic FCM-based platform that enables the rapid interrogation and large-scale screening of human innate cell response to TLR ligands and other immune modulators (6). The power of polychromatic FCM increases exponentially with the number of parameters that can be analyzed simultaneously on the single-cell level. This is most readily accomplished through positive identification (i.e., dyes or fluorochrome coupled antibodies identify their target as present). In addition, targets can potentially be identified through negative identification (i.e., the absence of

signals). For such a negative identification approach to be useful, the collection of absent markers has to be sufficient to unequivocally identify the negative target. For example, most viability dyes identify live cells through the absence (exclusion) of their signal. Here we tested the hypothesis that B cells could be identified through a negative-gating strategy as cells that are MHC II⁺, CD14^{neg-low}, CD11c^{neg}, and CD123^{neg}. If successful, such negative-gating strategy would leave one fluorescence channel free for an additional functional read-out.

This report also provides an example of the ease and value of applying the Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) standard, which was recently adopted by the International Society for Advancement of Cytometry (ISAC) (2) and incorporated in the publishing policies of *Cytometry A* and the Nature Publishing Group journals. MIFlowCyt states the minimum information required to report FCM experiments in the published literature. In Supporting Information we provide a structured representation of the information provided in a MIFlowCyt compliant report. And in Supporting Information we demonstrate where in the free-flowing text of our manuscript specific parts of the MIFlowCyt standard are incorporated (this is indicated by providing the MIFlowCyt section numbering in brackets { }); this numbering would not be necessary in actual submissions of manuscripts containing FCM data but is shown in the Supporting Information of the manuscript to aid authors in their preparation of FCM data for publication).

MATERIALS AND METHODS

Subjects and Blood Samples

All studies were approved by the Clinical Research Ethics Board of the University of British Columbia, and the Institutional Review Board of the University of Washington Medical Center. We obtained blood from five healthy human adults (22–50 years old) for both the intracellular cytokine (ICC) expression and the costimulatory cell-surface marker experiments. Blood was drawn in May and June 2007 via sterile venipuncture into vacutainers containing 143 USP units of sodium-heparin [Becton Dickinson (BD) catalog no. 8019839]. Blood and all reagents for peripheral blood mononuclear cells (PBMC) isolation were kept at room temperature throughout the purification. PBMC were isolated by density gradient centrifugation as previously described (6). PBMC were passed through a 70 μ m filter resuspended in cRPMI (RPMI 1640 (Invitrogen catalog no. 72400-047) containing 10% human AB serum (Gemini Bio-Products catalog no. 100-512) and 1% Penicillin-Streptomycin (Invitrogen catalog no. 15140-122)) at a density of 2.5×10^6 PBMC/ml.

TLR Stimulation of PBMC

Two hundred microliters of PBMC were added to wells of 96-well plates containing 10 μ l of RPMI alone (Unstim) or 10 μ l of RPMI containing the following TLR ligands: Pam3CSK4 (TLR2/1 agonist; EMC microcollections catalog no. L200), ultrapure *E. coli* 0111:B4 LPS (TLR4 agonist; InvivoGen catalog no. tlr1-pelps), and CpG-A ODN 2336 (TLR9

agonist; Coley Pharmaceutical). The final concentrations of the ligands were 1 μ g/ml, 100 ng/ml, and 25 μ g/ml for Pam3CSK4, LPS, and CpG-A, respectively. The TLR stimulations were for 6 hours for the ICC assays, and 18 hours for the costimulatory cell-surface marker assays. For the former, Brefeldin A (Sigma catalog no. B-6542) was present at the final concentration of 10 μ g/ml from the beginning of the stimulation except for the CpG-A wells to which it was added 3 hours later. At the end of the stimulations, adherent cells were detached by adding EDTA to each well at a final concentration of 2 mM for 10 minutes at 37°C. The plates were spun and the supernatants removed. The PBMC pellets were resuspended in 100 μ l of 1 \times FACSlyse solution (BD catalog no. 349202). The plates were then covered with aluminum plate sealers and stored at -80°C until staining.

Flow Cytometry

The frozen plates containing cells in FACSlyse solution were thawed and permeabilized as previously described (6). Cells were stained in a final volume of 100 μ l PBSAN for 30–60 minutes at room temperature. We used the following antibodies for our ICC staining: anti-CD123-AmCyan (clone 9F5; BD custom-made; used at 1:50 dilution), anti-CD11c-APC (clone S-HCL3; BD catalog no. 340544; used at 1:50 dilution), anti-MHC II-PerCP-Cy5.5 (clone TU36; BD custom-made; used at 1:100 dilution), anti-CD14-PE-Cy7 (clone M5E2; eBioscience catalog no. 25-0149; used at 1:50 dilution), anti-IL6-APC-Cy7 (clone AS12; BD custom-made; used at 1:100 dilution), anti-IL12p40/70-Pacific Blue (clone C8.6; eBioscience catalog no. 577129; used at 1:100 dilution), anti-IFN- α -FITC (clone A11; Antigenix catalog no. MC100133; used at 1:100 dilution), and anti-TNF- α -Alexa700 (clone Mab11; BD catalog no. 557996; used at 1:100 dilution). After two washes with PBSAN, the cells were resuspended in PBS containing 1% paraformaldehyde and immediately analyzed using an unmodified BD FACS ARIA flow cytometer that was set up according to published guidelines (7). Costimulatory cell-surface markers were identified by using their corresponding antibody-fluorochrome conjugates as follows: anti-CD11c-APC, anti-MHC II-PerCP-Cy5.5, anti-CD123-Pacific Blue (clone 9F5; BD custom-made; used at 1:100 dilution), anti-CD14-Alexa700 (clone M5E2; BD catalog no. 557923; used at 1:50 dilution), anti-CD19-biotin (clone HIB19; BD catalog no. 555411; used at 1:50 dilution), anti-CD86-PE (clone B7-2; eBioscience catalog no. 11-0577; used at 1:100 dilution), and anti-CD40-FITC (clone 5C3; eBioscience catalog no. 11-0409; used at 1:100 dilution). After two washes in PBSAN, cells were incubated with 100 μ l PBSAN containing streptavidin-PE-TexasRed (BD catalog no. 551587; used at 1:400 dilution) for 30 minutes at room temperature. After two further washes with PBSAN, the cells were resuspended in PBS containing 1% paraformaldehyde and immediately analyzed using the BD FACS ARIA flow cytometer described above. Unstained cells, single fluorochrome stained cells, and cells stained as fluorescence-minus-one (FMO) controls were used to set-up the machine. Compensation beads (CompBeads; BD catalog no. 552843) were used as quality control across experi-

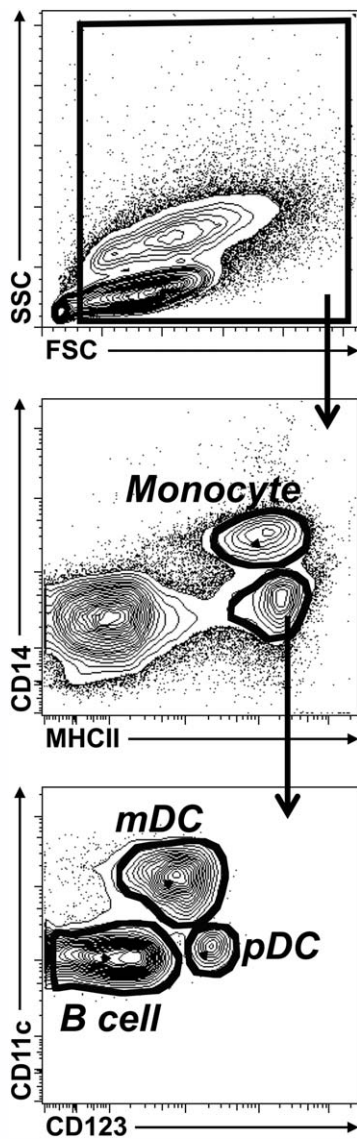


Figure 1. Gating strategy to identify innate cells in PBMC. After gating on target cells based on FSC vs. SSC plot, monocytes were identified as MHC II⁺, CD14^{high} cells. The MHC II⁺, CD14^{neg-low} cells were further classified into mDC, pDC and B cells by plotting them on CD123 vs. CD11c plot. The mDC and pDC are the CD11c^{high} and the CD123⁺ subpopulations, respectively. The distinct subpopulation that expresses neither CD123 nor CD11c is composed mostly of B cells.

ments as described (8,9). For each single-stain control, 3 μ l of anti-mouse Ig CompBeads and 3 μ l of anti-FBS negative control CompBeads were added to 94 μ l of PBSAN in the same plate as the cells, and stained with 3 μ l of antibody. For the rest of the protocol, CompBeads were treated in the same way as the cells. Two hundred thousand cells per well and 5,000 CompBeads per well were acquired uncompensated. Compensation was set in FlowJo (Tree Star) and samples were analyzed compensated. Cytometer optimization and calibration were performed according to published guidelines (7).

RESULTS

Figure 1 shows our negative-gating strategy to identify B cells. After gating on target cell populations in the FSC vs. SSC plot, innate cells including monocytes, dendritic cells, and B cells were identified by gating on MHC II-expressing cells, a marker expressed on all antigen-presenting cells. Amongst these cells, monocytes were identified as CD14^{high} cells. We then mapped the CD14^{neg-low} and MHC II⁺ cells in a CD123 vs. CD11c plot. This allowed us to positively identify the myeloid dendritic cells (mDC) and the plasmacytoid dendritic cells (pDC) as the CD11c^{high} and the CD123⁺ subpopulations, respectively. Additionally, a distinct cell population that expressed neither CD123 nor CD11c can be seen in the lower

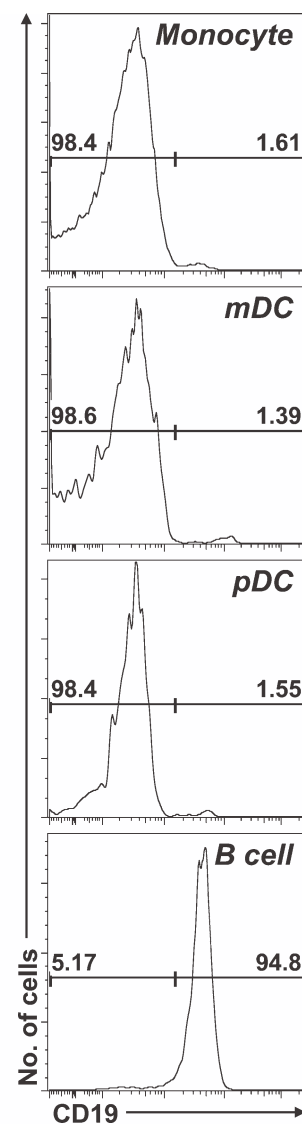
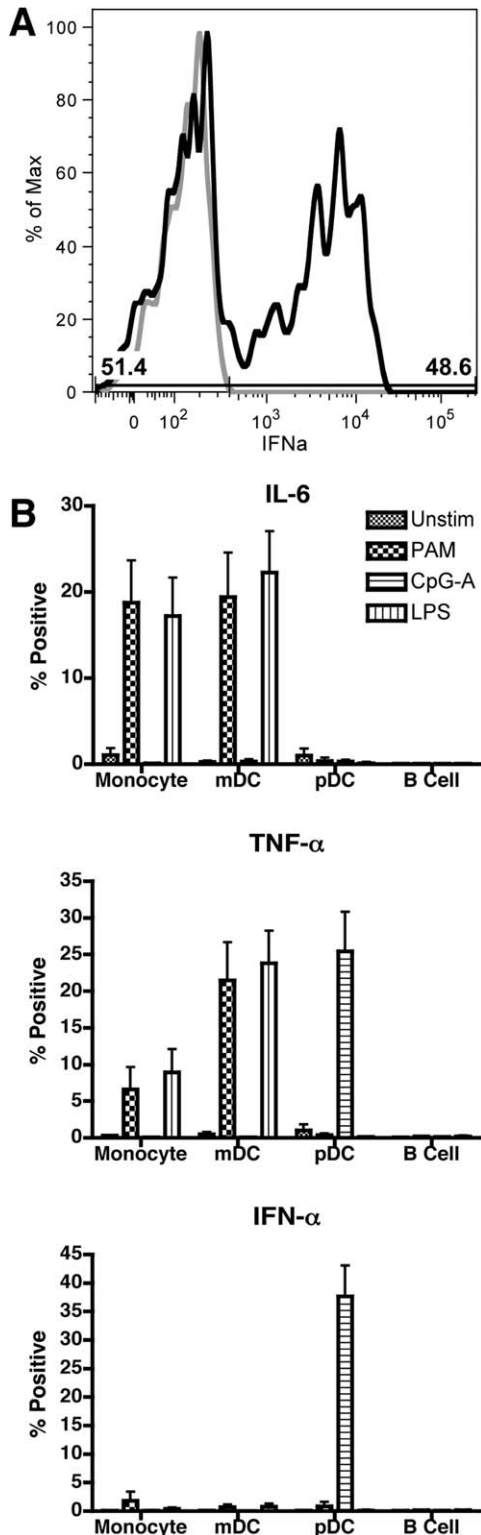


Figure 2. CD19 expression of the negatively-gated B cell population confirms their identity. The four distinct innate cell populations in Figure 1 were checked for CD19 expression. Greater than 95% of the negatively-gated B cells are positive for CD19 expression; a small percentage of monocytes, mDC, and pDC also stained positive for CD19.

left quadrant of this graph. These CD123^{neg}, CD11c^{neg} cells were the negatively gated B lymphocytes (i.e., CD14^{neg-low}, MHC II⁺, CD123^{neg}, CD11c^{neg}). We confirmed this in separate staining experiments through positive staining and gating,



where we included CD19, the canonical B cell surface marker in the stain (Fig. 2): About 95% (range: 94 to 98%) of the CD123^{neg}, CD11c^{neg} cells were B cells based on their expression of CD19 (i.e. CD14^{neg-low}, MHC II⁺, CD123^{neg}, CD11c^{neg}, CD19⁺). A small fraction of the positively identified monocytes (average of 1.4%; ranges from 0.8 to 1.6%), mDC (2.6%; 1.4 to 3.7%), and pDCs (3.5%; 1.6 to 5.3%) also stained positive for CD19. Specific gate boundaries are available upon request for each of the analyzed subjects.

We next assessed the responses of these human B cells to TLR stimulation. PBMC from five individuals were left unstimulated or stimulated for 6 hours with the following TLR ligands: Pam3CSK4 (recognized by the heterodimer of TLR2 and TLR1), LPS (TLR4), and the CpG-A ODN 2336 (TLR9). The Golgi transport blocker Brefeldin A was added in the culture media either at the start of stimulation or at the 3-hr mark (for the CpG-A-treated wells). Cells left unstimulated did not express any of these cytokines (Fig. 3). This allowed us to count in each of the four populations, as gated in Figure 1, the number of cells that express the cytokines IFN- α , IL-6, TNF- α (Fig. 3), and IL12p40/70 (data not shown). None of the negatively-gated B cells expressed these cytokines in response to TLR stimulation. Both monocytes and the mDC responded robustly to Pam3CSK4 and LPS by upregulating IL-6 and TNF- α expression, but did not respond to the CpG-A. In contrast, pDC did not respond to either Pam3CSK4 or LPS, but responded to the CpG-A by expressing TNF- α and its signature cytokine, IFN- α .

To determine if TLR stimulation affected the expression of costimulatory cell-surface markers, we assessed the response of cells after 18 hours of stimulation in the absence of Brefeldin A. The B cells responded most robustly to the TLR9 ligand (Fig. 4). About 40% and 25% of CpG-A-treated B cells became positive for CD40 and CD86, respectively, compared to none or only a very low percentage in the unstimulated controls. Interestingly, the two ligands that led to the highest percentage of cytokine-positive monocytes and mDCs—Pam3CSK4 and LPS—decreased the number of CD86⁺ cells from about 75% of unstimulated cells to about 50% of the TLR-stimulated cells. CpG-A, just like in the 6-hr ICC, had no effect on either monocytes or mDCs with respect to CD86⁺ expression. On

Figure 3. Expression of cytokines by innate cells after 6-hour stimulation with TLR ligands. Two hundred microliters of PBMC (2.5×10^6 PBMC/ml) were plated per well on 96-well plates containing 10 μ l of RPMI alone (Unstim) or 10 μ l of RPMI containing the following TLR ligands: Pam3CSK4, LPS (0111:B4), and CpG-A (ODN 2336). The final concentrations of the ligands are 1 μ g/ml, 100 ng/ml, and 25 μ g/ml for Pam3CSK4, LPS, and CpG-A, respectively. Brefeldin A was present at the start of the stimulation except for the CpG-A wells which received it after 3 hours. (A) shows an overlaid histogram of pDC expressing IFN- α in resting (gray line) vs. CpG-A stimulated (black line) PBMC with the bisector line indicating the histogram regions chosen as IFN- α negative vs. positive cells, while (B) shows the % of Monocytes, mDC, pDC, and B cells expressing a given cytokine (IL-6 top, TNF- α -middle, IFN- α bottom) above the respective unstimulated control sample. Error bars indicate SEM ($n = 5$).

the other hand, pDC appeared more like B cells in that they responded most strongly to TLR9 engagement by upregulating CD86. With CpG-A treatment, the number of CD40⁺ pDC increased from almost nil to about 40%; pDC also showed the

highest number of CCR7⁺ cells (>60%) among all four cell-types.

DISCUSSION

Polychromatic FCM has tremendously accelerated our progress in understanding the multifaceted activities of immune cells. The power of polychromatic FCM lies in the number of parameters that can be analyzed simultaneously on a single-cell level. Here, we tested the hypothesis that the number of functions that can be read out in an innate immune FCM-based platform can be further increased by identifying B cells through a negative-gating approach. The strategy appeared to work in that negatively-gated B cells (i.e., CD14^{neg-low}, MHC II⁺, CD123^{neg}, CD11c^{neg}) did not appreciably contaminate the minor cell populations of monocytes, mDC or pDC. However, when this was directly tested through positive identification via CD19 staining, a small fraction of CD19⁺ cells were present in the monocyte, mDC, and pDC populations. It is possible that some non-B cells might express CD19 and/or some B cells might express CD11c, CD14 and/or CD123, which might explain the presence of CD19⁺ cells in the monocyte, mDC and pDC populations. We are currently in the process of investigating this. Furthermore, a small proportion of cells that are not B cells (i.e., not CD14^{neg-low}, MHC II⁺, CD123^{neg}, CD11c^{neg}, CD19⁺), nor monocytes (i.e., CD14^{high}, MHC II⁺, CD19^{neg}), mDC (i.e. CD14^{neg-low}, MHC II⁺, CD123^{neg}, CD11c⁺, CD19^{neg}), or pDC (i.e., CD14^{neg-low}, MHC II⁺, CD123⁺, CD11c^{neg}, CD19^{neg}) based on the panel of lineage markers employed, were found to contaminate the negatively-gated B cells. The identity of this small population is currently not clear, but may adversely affect strategies that aim to identify e.g. dendritic cells based on the absence of lineage markers such as CD19 (12).

The low-level contamination of negatively-gated B cells at first glance reduces the applicability of this negative gating strategy, and would disprove our hypothesis. Only at the qualitatively extreme ends of a response spectrum (i.e., either none of the B cells respond to a given innate stimulation or all/most B cells respond but not monocytes, mDC or pDC) would this B cell negative-gating strategy supply acceptable data. This extreme response difference was indeed the case, when measuring cytokine production upon TLR activation, as B cells did not express any of the cytokines tested under the conditions we employed. Following the same reasoning, this negative-gating strategy also appeared acceptable in our experiments to measure changes in the expression of costimulatory cell-surface molecules where B cells did respond

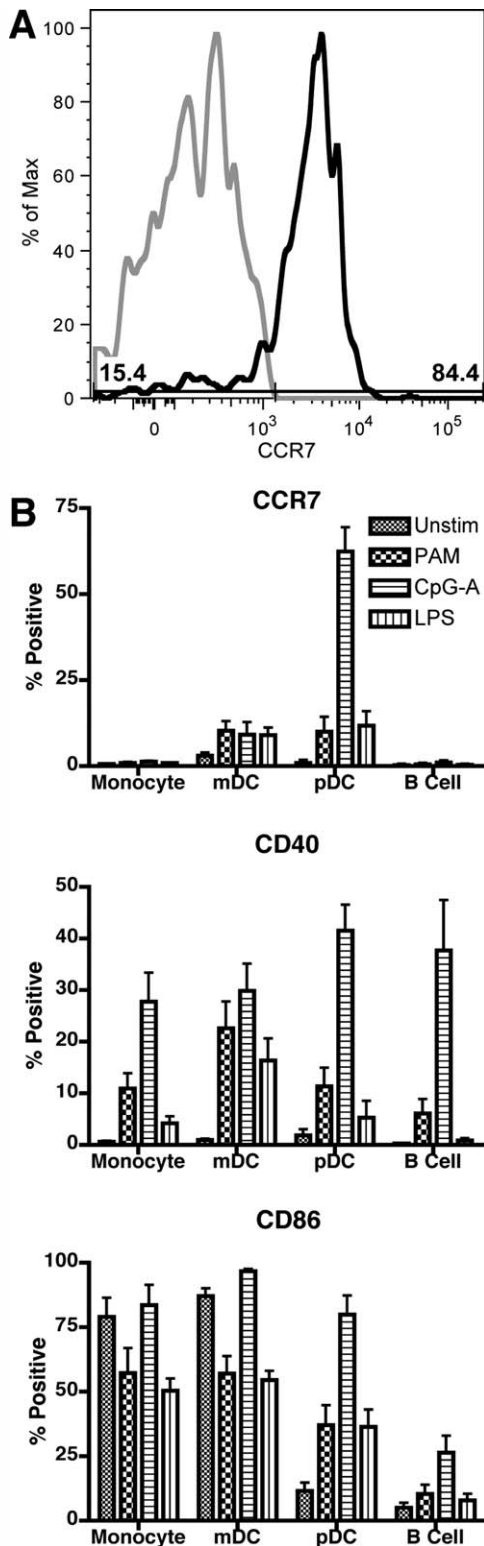


Figure 4. Expression of costimulatory cell-surface markers by innate cells after 18-hour stimulation with TLR ligands. Plates were set up as in Figure 3 except Brefeldin A was not used. (A) shows an overlaid histogram of pDC expressing CCR7 in resting (gray line) vs. CpG-A stimulated (black line) PBMC with the bisector line indicating the histogram regions chosen as CCR7 negative vs. positive cells, while (B) shows the % of Monocytes, mDC, pDC, and B cells expressing a given surface marker (CCR7 top, CD40 middle, CD86 bottom) above the respective unstimulated control sample. Error bars indicate SEM (n = 5).

robustly. However, if both B cell and non-B cell populations would respond in low frequency, a negative-gating strategy as outlined in this manuscript would potentially be difficult to interpret. Overall we conclude that a negative B-cell gating strategy as outlined here can only be applied if pilot experiments using positive identification of B cells (e.g., using CD19) indicate a qualitatively different response patterns between B-cells and the other target cell populations.

The secondary aim of this article was to serve as an example for the application of the ISAC-adopted MIFlowCyt standard for FCM experiments, one of an increasing number of community-developed publication checklists (10). Community opinion, journals and funding agencies increasingly favor that such regularized set of the available metadata ('data about the data') pertaining to an experiment be associated with the results, enhancing communication by making explicit both the biological and methodological contexts (11,13,14). Following the MIFlowCyt standard required minimal ~5% of the total effort extra work to the description of the research, yet helped ensure appropriate information was included to interpret the experimental design, methodology, results and conclusions.

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