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Toll-Like Receptor 2^{bright} Cells Identify Circulating Monocytes in Human and Non-Human Primates

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Abstract

Polychromatic flow cytometry is a useful tool for monitoring circulating whole blood monocytes, although gating strategies often vary depending on the study. Increased analyses of the myeloid system have revealed monocytes to be more plastic than previously understood and uncovered changes among surface markers previously considered to be stable. The myeloid system has also been found to have disparate surface markers between mouse, human, and non-human primate studies, which further complicates examination between species. This study has found bright Toll-like receptor 2 (TLR2) expression to be a consistent surface marker of circulating whole blood monocytes in humans and two species of macaques. Furthermore, within our pigtailed macaque model of HIV-associated CNS disease, where monocyte surface markers have previously been shown to reorganize during acute infection, TLR2 remains stably expressed on the surface of classical, intermediate, and non-classical monocytes. Our findings demonstrate that TLR2 is a useful surface marker for including all monocytes during other phenotypic changes that may alter the expression of common surface receptors. These results provide a practical tool for studying all types of monocytes during inflammation and infection within humans and macaques.

Key terms

monocytes; Toll-like receptor 2; macaca; flow cytometry

Monocytes maintain a variety of functions, including defense against pathogens, tissue repair, and macrophage homeostasis. Increased capabilities of flow cytometry have contributed to the classification of monocytes and their role within the myeloid system, which continues to be expounded in nomenclature and in function (1). Continued investigation of myeloid cells has improved their characterization beyond the basic definitions of the previously established mononuclear phagocyte system (MPS) and new approaches suggest a classification system focused initially on ontogeny, then function, location, and phenotype (2). In blood, specifically, myeloid committed precursors originate from hematopoietic stem cells and are further matured and diversified into specialized groups of cells, including dendritic cells, monocytes, and myeloid-derived suppressor cells (1). Phenotypic markers frequently used to distinguish these cells within the MPS can overlap or change with inflammation and immune responses. In addition, monocytes themselves can present phenotypes that change in number and activation state during a range of infections and inflammatory conditions (3).

During whole blood analysis, monocytes are frequently separated from lymphocytes and granulocytes based on morphometric and cytometric characteristics (4). Monocytes in human whole blood are often subdivided into three phenotypically and functionally diverse groups based on surface expression of CD14 and CD16,

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with classical monocytes as CD14+ CD16−, intermediate monocytes as CD14+ CD16+, and non-classical/patrolling monocytes as CD14low/− CD16+ (4). However, this strategy, combined with FSC and SSC, does not precisely separate all cell populations. For example, NK cells and non-classical monocytes both express similar levels of surface CD16, and overlap morphometrically and cytometrically (4). In addition, non-classical monocytes are low or negative for CD14 expression, limiting the utility of CD14 alone to identify monocytes (5). The MHC class 2 molecule HLA-DR, in combination with a cocktail of other lineage markers is also used to distinguish human monocytes, although it has been shown to be downregulated in a variety of immune responses, including systemic inflammation (6–9). There is also evidence that expression of common monocyte markers varies within primate species. A recent article discussing the diversity of bronchoalveolar lavage cells found HLA-DR expression on phagocytic mononuclear cells among healthy controls to be lower on macaques than on humans (10).

During a brief investigation of human monocyte phenotypes in a cohort of coronary artery disease (CAD) probands and their relatives, we included Toll-like receptor 2 (TLR2) as a potential marker for cell activation. TLR2 is generally thought to be expressed on the cell surface and is involved in the recognition of several microbial membranes, including those from bacteria, fungi, parasites, and viruses (11). TLR2 induces mainly inflammatory cytokines via the sorting adaptor TIRAP and the MyD88 pathway (11). Instead of changes in cell surface expression, we identified bright, surface TLR2 as a unique identifier of blood monocytes. We continued to examine TLR2 in our non-human primate model of simian immunodeficiency virus (SIV) (12) with similar results. Here, we demonstrate that using TLR2 in flow cytometry panels improves the gating strategy for the analysis of monocytes in circulating whole blood from human and non-human primates. We also demonstrate that surface HLA-DR does not consistently stain monocyte subsets in pigtailed macaques and therefore should be used with caution for the characterization of myeloid cells in blood of this species. This finding further establishes the benefits of alternate gating strategies.

Materials and Methods

Human Samples

Human peripheral whole blood samples were collected from eight participants of the GeneSTAR (Genetic Study of Atherosclerosis Risk), a 30-year cohort study of initially healthy first-degree relatives of probands with early-onset CAD. All volunteers were healthy and consented to blood donation according to the IRB protocol approved by the Johns Hopkins Office of Human Subject Research. Whole blood was collected in a vacutainer tube with acid-citrate-dextrose (ACD) solution (BD, Franklin Lakes, NJ) at room temperature and antibody stained for flow cytometry within 2 h.

Macaque Samples

Peripheral whole blood was drawn from four healthy juvenile male pigtailed macaques (*Macaca nemestrina*) and four rhesus macaques (*Macaca mulatta*) (data not shown) following sedation with 10 mg/kg ketamine. Whole blood was collected i.v. in ACD tubes at room temperature and antibody stained for flow cytometry within 2 h. Whole blood from four pigtailed macaques dual inoculated with SIV/17E-Fr and SIV/DeltaB670 was also assessed (13). Analyses were performed in samples collected before inoculation and at 7 and 14 days post-inoculation (dpi).

Antibody Staining

Both human and macaque whole blood was stained with pre-titrated amounts of monoclonal antibodies for 100 μL of whole blood at room temperature for 20 min. All antibodies and panels used are included in MIFlowCyt Supporting Information. Whole blood samples were then lysed and fixed in 2 mL of FACS Lysing Solution (BD Biosciences, San Jose, CA) for 10 min at room temperature. Samples were collected in a centrifuge at 400*g* for 5 min, washed in 2 mL 1X PBS, and then resuspended in 0.5 mL of PBS for analysis.

Flow Cytometry

Flow cytometry was performed on a BD LSRFortessa (BD Biosciences, San Jose, CA). The instrument was calibrated daily using Cytometer Setup and Tracking (CS&T) Beads (BD Biosciences, San Jose, CA). Application settings were designed and used in conjunction with CS&T beads to maintain consistent fluorescent parameters across longitudinal studies. BD CompBead Anti-mouse Ig, κ/Negative Control Compensation Particles set or BD CompBead Plus Anti-mouse Ig, κ/Negative Control Compensation Particles set (for monocyte/macrophage markers) (BD Biosciences, San Jose, CA) were antibody stained with matched fluorochromes for compensation. For tandem and Brilliant Violet dyes, CompBeads were specifically stained with matched antibody lot and tube. Acquisition event count was set for 10,000 monocytes, as estimated on FSC and SSC parameters. Further details are provided in MIFlowCyt Supporting Information.

Data Analysis

Data were compensated in BD FACSDiva 6.2 software (BD Biosciences, San Jose, CA) using the Compensation feature. Data were analyzed using FlowJo 10.0.8 software (FlowJo, LLC, Ashland, OR). Doublets were first excluded using forward scatter height (FSC-H) and forward scatter area

(FSC-A) parameters. All other gating strategies used are depicted within each figure. Percentages of parent populations were included as shown. Statistical analyses were performed using Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). A Kruskal–Wallis analysis of variance was used to determine significant changes in the percentage of TLR2^{bright} cells expressing HLA-DR during acute SIV infection and compared with pre-infection, followed by Dunn's multiple comparisons test ($P \leq 0.05$).

Results

TLR2^{bright} Staining Distinguishes Monocytes from Other Blood Leukocytes

During a project in which we evaluated activation surface markers in blood leukocytes from eight GeneSTAR participants, we observed that TLR2^{bright} antibody staining identified cells that were predominantly located in the monocyte region based on FSC and SSC parameters. To determine whether TLR2^{bright} only identified circulating monocytes, whole blood samples collected from healthy donors were co-stained with common lineage marker antibodies for other leukocytes and evaluated on a BD LSRFortessa (BD Biosciences, San Jose, CA). In all experiments, singlets were first selected by gating on FSC-H and FSC-A, and TLR2 staining specificity was confirmed by using a matched isotype IgG control (Fig. 1 A). Gating strategy for TLR2^{bright} cells is shown in Figure 1B.

Co-staining with lymphocytic lineage markers confirmed that TLR2^{bright} cells did not encompass T or B lymphocytes (CD3 and CD20) or NK cells (CD159a/NKG2a). Neutrophils, defined by CD66abce+ cells, were TLR2^{dim} and easily separated from those cells expressing high surface levels of TLR2. CD117 expression, which denotes immature and hematopoietic progenitor cells, was also not observed in TLR2^{bright} cells (Fig. 1 C).

Monocytes and dendritic cells can overlap in both surface marker expression and by location in SSC vs. FSC plots. TLR2^{bright} cells were distinct from plasmacytoid dendritic cells (pDCs) based on the expression of CD123. To confirm that TLR2^{bright} cells were not classical (myeloid) dendritic cells (cDCs), we assessed the expression of CD11c, a commonly used cDC marker, and CD16. cDCs are defined as lacking CD16 expression (14). We found that TLR2^{bright} CD11c+ cells also highly expressed CD16, while CD11c+ CD16− cells were TLR2− (Fig. 1 D). Therefore, we conclude that cells identified by TLR2^{bright} staining in whole blood are likely not cDCs.

Phenotypic Markers on the TLR2^{bright} Population Confirm a Myeloid Population

TLR2^{bright} cells were analyzed for the expression of CD14 and CD16 to identify monocyte subsets: CD14+ CD16− as classical, CD14+ CD16+ as intermediate, and CD14low/− CD16+ as non-classical monocytes (Fig. 2A,B). No CD14+ cells were omitted by using TLR2^{bright} as a monocyte prerequisite (Fig. 2C). This gating scheme was used both for healthy pigtailed macaque and healthy human whole blood, as shown in Figure 3. We then compared other commonly used myeloid markers, including CD11b, CD163, CX3CR1, and HLA-DR, in combination with TLR2, CD14, and CD16 across pigtailed macaque and human samples (Figs. 2D and 3D). CD163 showed comparable staining patterns between human and pigtailed macaque, with highest surface levels on classical monocytes and decreasing expression from intermediate into non-classical monocytes. CX3CR1 showed an expected pattern as well, staining intermediate and non-classical monocytes in greater intensity than classical monocytes.

However, CD11b and HLA-DR showed differing patterns of expression between pigtailed macaque and human. For humans, all monocytes appeared positive for CD11b, with classical monocytes having the greatest expression. In pigtailed macaques, the trend is similar, although not all non-classical monocytes appear to express CD11b. HLA-DR staining is the most disparate between the two species. In humans, HLA-DR is expressed on all monocyte subtypes, averaging the highest level of expression on non-classical monocytes, followed by intermediate monocytes, and lastly by classical monocytes. In rhesus macaques, monocytes showed a pattern similar to that of humans (data not shown). In contrast, pigtailed macaques showed highest expression of HLA-DR on intermediate monocytes, followed by classical monocytes, with low to negative staining on non-classical monocytes.

TLR2 Expression in Macaque Monocytes Remains Unchanged During Acute SIV Infection

Some monocyte markers, including HLA-DR, can be downregulated during infections and inflammatory conditions (8,9,15). Specifically, we have previously examined the loss of CCR2, a cell surface marker for classical monocytes, during infection in our accelerated SIV model for HIV-associated neurocognitive disorders (16). Using samples from this SIV model, we further investigated whether TLR2^{bright} was reliable to differentiate pigtailed macaque monocytes during acute SIV infection. Monocyte TLR2 staining in pigtailed macaque whole blood was collected pre-infection and compared with whole blood collected at 7 and 14 dpi. No loss of TLR2 expression on monocytes was observed during SIV infection at either time point (Fig. 4). TLR2^{bright} cells were subdivided in classical, intermediate, and non-classical monocytes as described and HLA-DR expression was examined for each of the subsets. During SIV infection, TLR2^{bright} CD14+ classical monocytes show a significant ($P = 0.0071$) loss in the HLA-DR+ population, corroborating findings from other studies (15). Intermediate monocytes were subject to a more subtle change in HLA-DR expression, while at 7 dpi, non-classical monocytes actually gained HLA-DR expression ($P = 0.0244$), returning to pre-infection levels by the end of acute infection (14 dpi).

Discussion

Here, we demonstrate that surface TLR2 appears ubiquitous in the human monocyte population. In addition, the marker was evaluated in whole blood from pigtailed and rhesus macaques with similar results. We find separating monocytes with TLR2^{bright} to be more robust than FSC and SSC alone, as simply relying on morphometric characteristics may lead to including unwanted cell types or excluding cells of

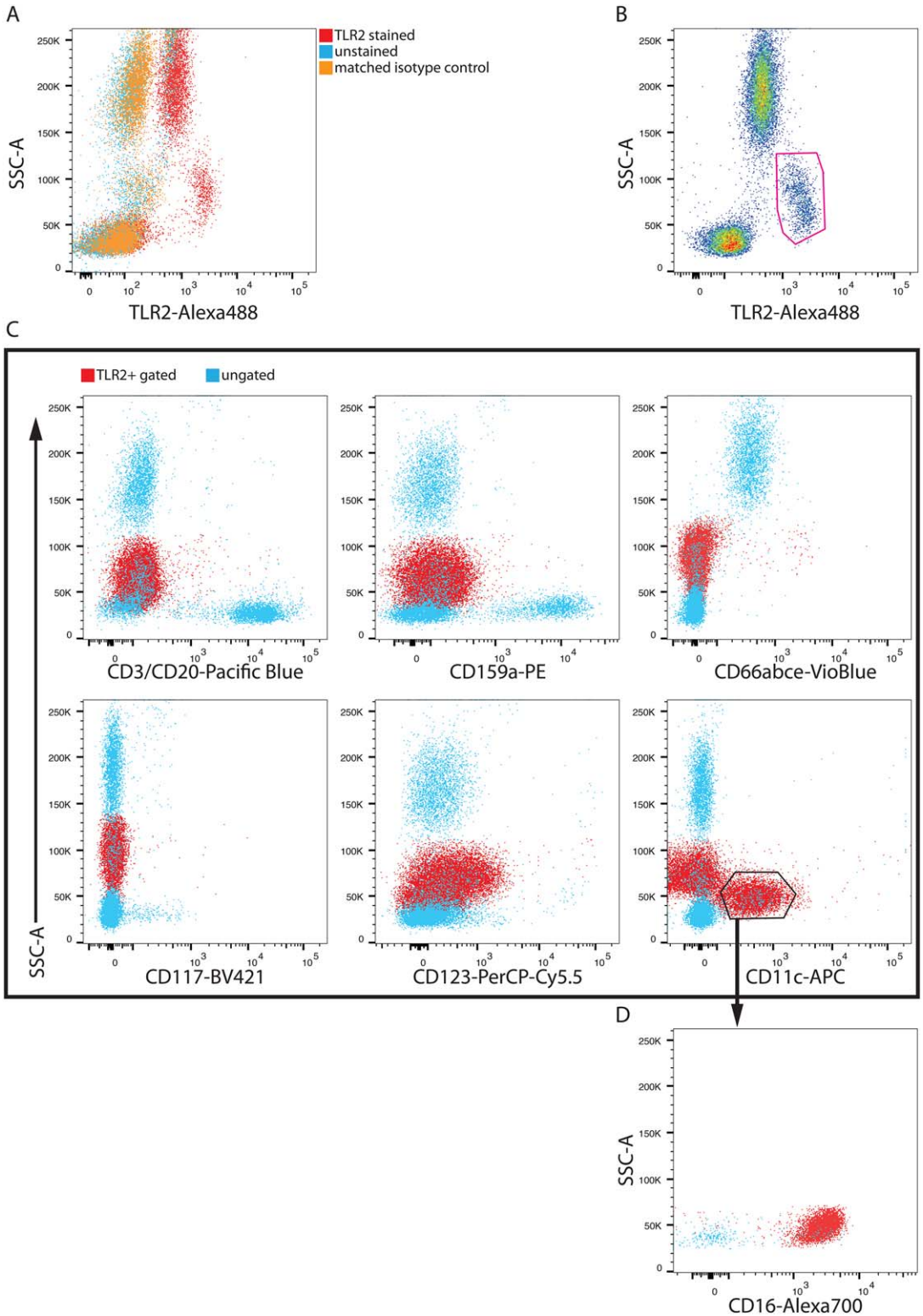

Figure 1. TLR2^{bright} cells do not express lineage markers for non-monocytes. (A) TLR2 stained whole blood is shown in red, unstained in blue, and staining with a matched isotype control in orange. (B) Gating analysis used for TLR2^{bright} cells is shown in whole blood as separated from other leukocyte populations. (C) TLR2^{bright} cells (red) are negative for lymphocyte, NK cell, hematopoietic progenitor, and pDC markers (CD3, CD20, CD159, CD117, and CD123, respectively) and low for neutrophils (CD66abce). (D) CD11c+ TLR2^{bright} cells are also positive for CD16. CD11c+ TLR2− cells are CD16−, and possibly cDCs. Plots are representative of pigtailed macaque whole blood. Analogous results were observed in humans.

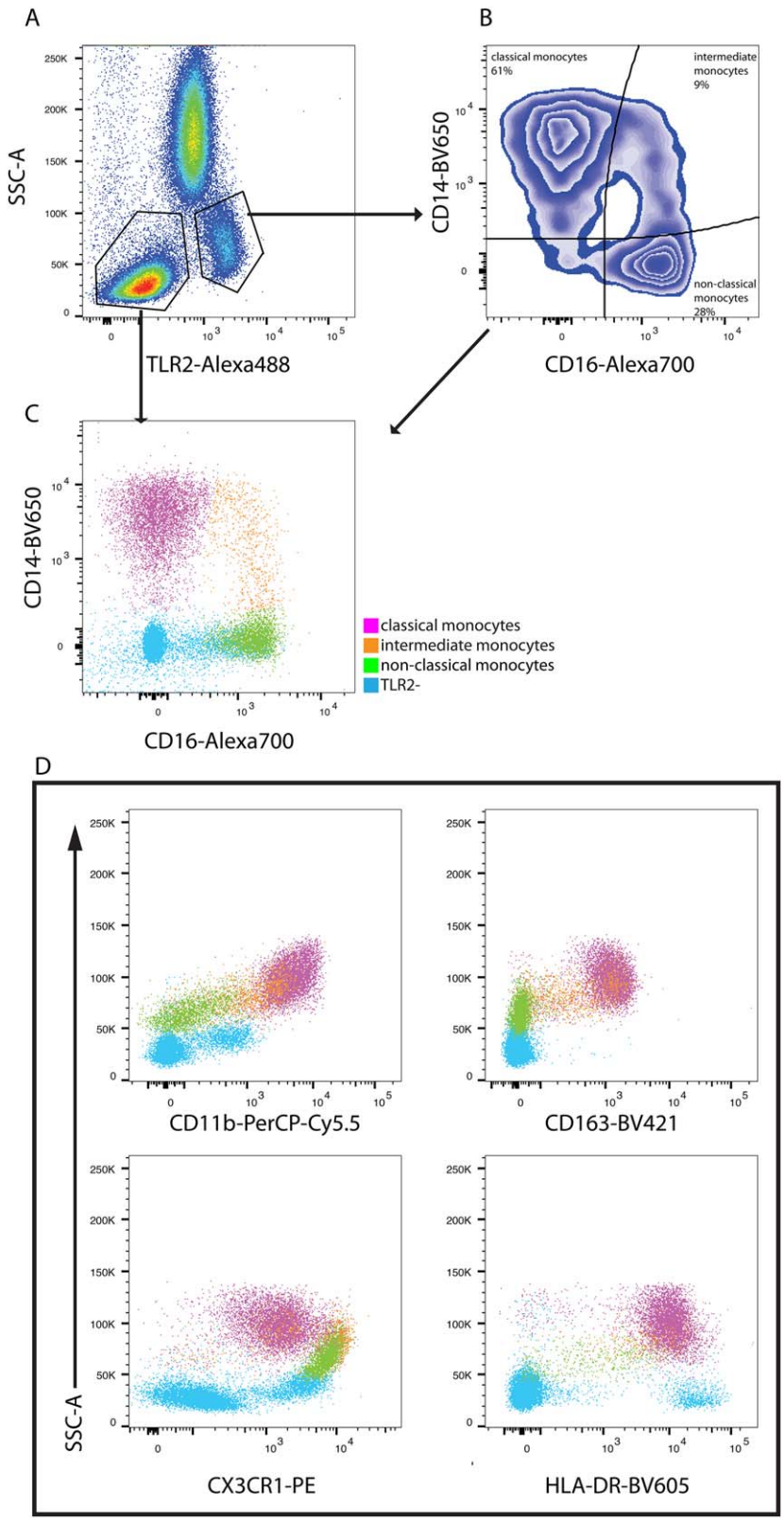

Figure 2. TLR2^{bright} cells express canonical monocyte markers in macaque blood. Pigtailed macaque whole blood analyzed by SSC vs.TLR2 (A) is evaluated for expression of CD14 and CD16 (B) to classify monocyte subsets as classical, intermediate, and non-classical. (C) Monocyte subsets (B) are overlaid with TLR2− mononuclear cells in blue (A). (D) The same gating scheme is used to further analyze common monocyte markers on subsets of TLR2^{bright} pigtailed macaque mononuclear cells.

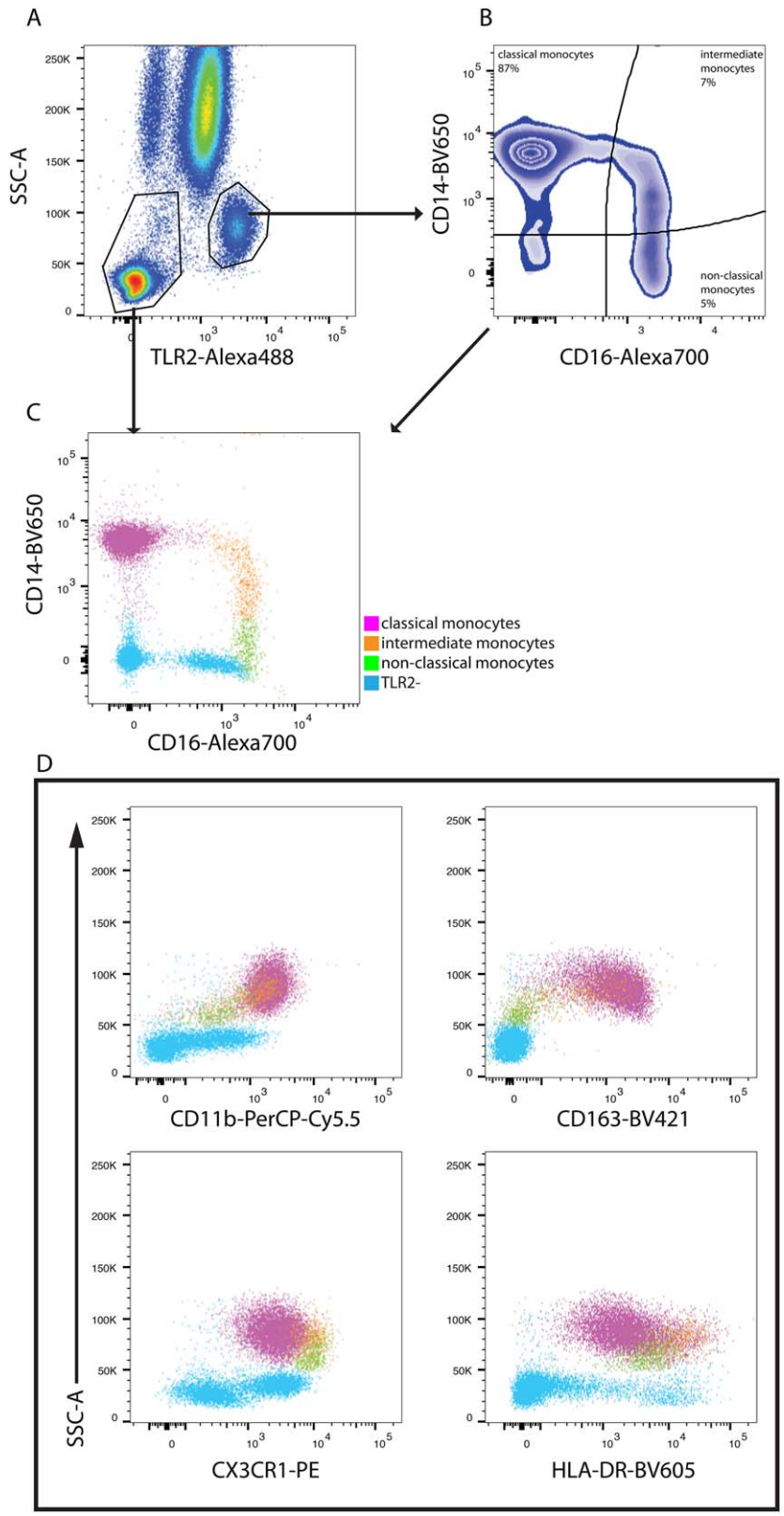

Figure 3. TLR2^{bright} cells express canonical monocyte markers in human blood. Whole blood analyzed by SSC vs.TLR2 (A) is evaluated for expression of CD14 and CD16 (B) to classify monocyte subsets as classical, intermediate, and non-classical. (C) Monocyte subsets (B) are overlaid with TLR2− mononuclear cells in blue (A). (D) The same gating scheme is used to further analyze common monocyte markers on subsets of TLR2^{bright} human mononuclear cells.

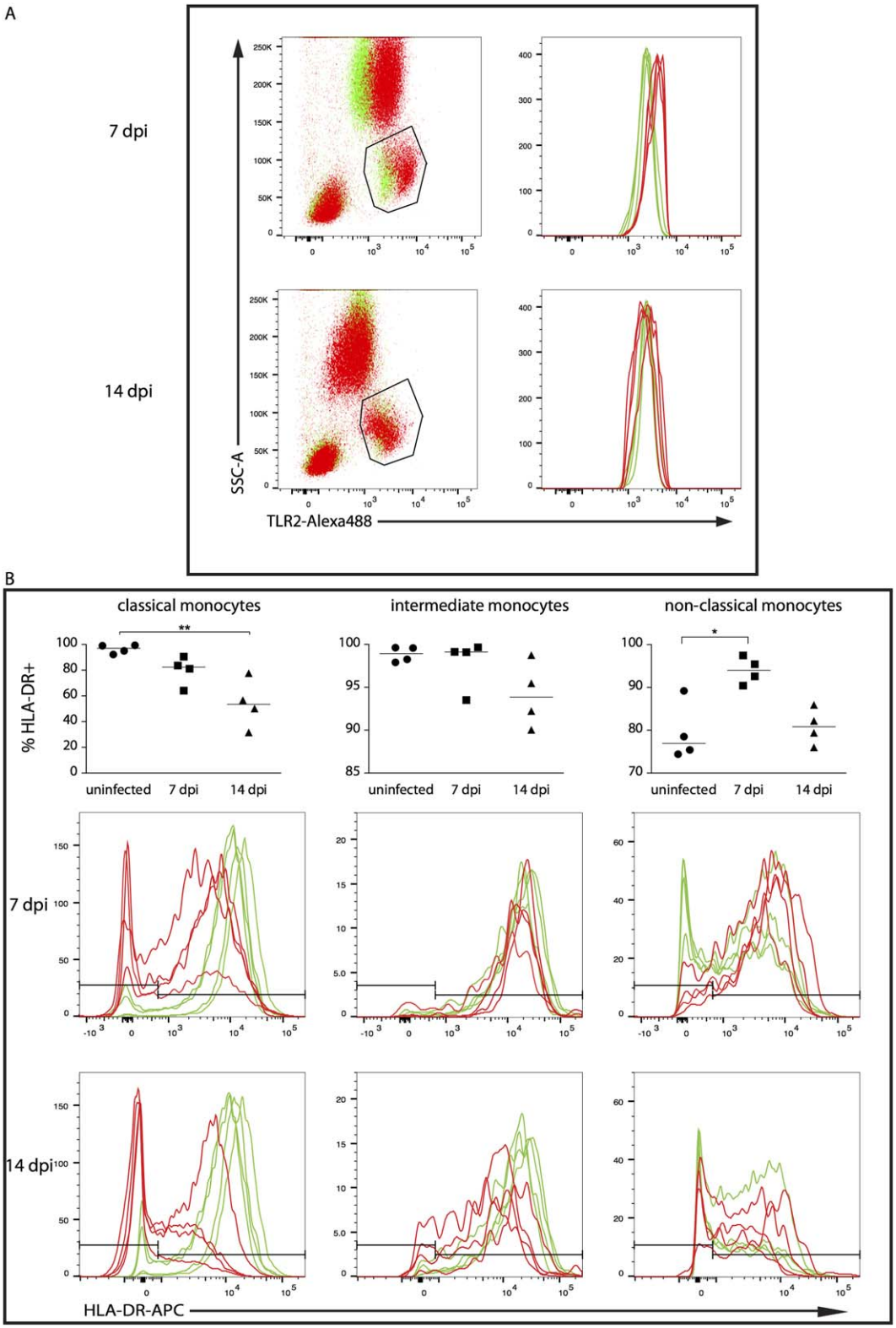

Figure 4. TLR2^{bright} monocytes decrease HLA-DR surface expression during acute SIV infection. Pigtailed macaque whole blood was stained for TLR2 and HLA-DR before SIV infection and 7 and 14 dpi. (A) Whole blood from pre-inoculation (green) is overlaid with results following SIV inoculation (red) at 7 (top row) and at 14 (bottom row) dpi for TLR2 analysis. (B) Surface expression of HLA-DR in gated TLR2^{bright} cells from pre (green) and post-inoculation (red) macaques are separated by CD14 and CD16 as classical, intermediate, or non-classical monocytes at 7 (top row) and 14 (second row) dpi. The final row shows the percentage of HLA-DR negative cells in each subset at each timepoint. $N = 4$.

interest that fall along the edges of estimated gates. Including a variety of lineage markers to exclude unwanted cell types from a broad gate is more costly than positively selecting a population based on TLR2 alone. The variations observed between human monocytes and pigtailed macaque monocytes in HLA-DR and CD11b, two common monocyte markers for separation, also stress the need for thorough review when using animal models. More importantly, we believe, is the potential use of TLR2 as a stably expressed marker of monocytes during immune activation that can be utilized in both human and macaque models of disease.

Studying the myeloid system across various species continues to prove to be a challenge. We have previously observed in our animal model that HLA-DR expression differed between humans and pigtailed macaques. While markers defining classical, intermediate, and non-classical monocytes appear consistent across both species, HLA-DR expression is generally greater on non-classical than on classical monocytes in human whole blood. The opposite is observed in the pigtailed macaque. In mice, which have a comparably well-defined myeloid system, MHC class II expression differs greatly when juxtaposed with human myeloid cells (3). It is possible that various non-human primate models need to be more closely examined before comparisons are made based solely on previously reported human research.

One traditionally used marker of myeloid cells is the MHC class II cell surface receptor HLA-DR. However several studies during disease states, often during severe inflammation and aggressive disease, report the loss of HLA-DR on monocytes (7,8,15,17). In humans, it is fairly well noted that M2-type macrophages do not consistently express HLA-DR (18). Immune responses to bacteria and certain viruses, including HIV and SIV, induce the anti-inflammatory cytokine IL-10, which is a proposed driver of surface HLA-DR loss (9,19,20). As our understanding of the complexity of the myeloid system grows, having more surface markers in our toolbox could be an important component in studying small but potentially critical populations. While we have only been able to follow the marker during one type of inflammatory event, our results suggest that TLR2^{bright} as a marker of monocytes in whole blood can improve the accuracy and quality of myeloid cell analysis in both human and macaque species.

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