

Letter to the Editor

Human dendritic cell subset 4 (DC4) correlates to a subset of CD14^{dim/-}CD16⁺⁺ monocytes

To the Editor:

Knowledge about the origin, tissue fate, and precise functions of circulating monocytes and dendritic cells (DCs) in human subjects is still incomplete and requires elucidation. Advances made in the last years have established unequivocally that blood monocytes and DCs are phenotypically Lin⁻HLA-DR⁺ because they express HLA-DR molecules but not lymphocyte lineage markers.^{1,2} Moreover, based on their CD14 versus CD16 expression, monocytes have been recognized to cluster into 3 subsets known as classical CD14⁺⁺CD16⁻, intermediate CD14⁺CD16⁺, and nonclassical CD14^{dim/-}CD16⁺⁺ monocytes,¹ with the latter including CD14^{dim/-}CD16⁺⁺slan⁺ monocytes as major components.^{1,2} Lin⁻HLA-DR⁺ DCs, conventionally defined CD14⁻CD16⁻ cells,² are instead distinguished into CD11c⁻CD123⁺CD303⁺ plasmacytoid dendritic cells (pDCs) and myeloid DCs. Myeloid DCs, in turn, include the CD11c⁺CD141⁺CLEC9A⁺ DCs/conventional dendritic cells (cDC1s) and the CD1c⁺CD11c⁺FcεR1⁺ DCs/cDC2s.^{1,2}

In this context, using single-cell RNA sequencing and unbiased genomic classification performed after cell sorting by means of flow cytometry, Villani et al³ have recently identified new populations of Lin⁻HLA-DR⁺ myeloid cells. Precisely, these authors described 2 new monocyte populations named Mono3 and Mono4, in addition to the classical and nonclassical monocytes (named Mono1 and Mono2, respectively), as well as 3 new DC subsets, namely DC3, DC4, and DC5 in addition to DC1, DC2, and DC6 corresponding to cDC1s, cDC2s, and pDCs, respectively.³ These findings, which were obtained by using the most modern and straightforward methodologies, are undoubtedly fundamental and very exciting in the field. By carefully reading the article,³ we noticed that the authors sorted monocytes from the Lin⁻CD14^{+/lo} cells (Fig 3, A, in Villani et al³) according to the conventional CD14 versus CD16 distribution,^{1,2} whereas they isolated DCs by intentionally opting for a strategy based on the lack of CD14 expression only in HLA-DR⁺ cells (see Fig 1, B, in Villani et al³). The latter approach was justified on the assumption that CD14⁻CD16⁺ cells within the human Lin⁻HLA-DR⁺ fraction have been classified as both monocytes and DCs.³ However, we believe that, as a consequence of such a DC gating strategy, the resulting DC pool used for further sorting and single-cell analysis inevitably contained a contaminant fraction of nonclassical CD14^{dim/-}CD16⁺⁺ monocytes, which ultimately ended up as the newly identified DC4 subset.³ Our belief is clearly demonstrated in Fig 1.

Accordingly, human PBMCs were stained with a panel of fluorochrome-conjugated antibodies (listed in the Methods section in this article's Online Repository at www.jacionline.org), including one (M-DC8) for the CD14^{dim/-}CD16⁺⁺slan⁺ monocytes.^{4,5} CD14^{dim/-}CD16⁺⁺slan⁺ monocytes are more widely known as slanDCs,⁴ as we have also called them for a while,⁵ but recent unsupervised hierarchical clustering using microarray analysis has proved unequivocally that they represent a subset of monocytes.⁶ Fig 1 makes it evident that, after exclusion of doublets, enrichment of CD45⁺ cells, exclusion of Lin⁺ cells (red

gate in Fig 1, A-I), and selection of HLA-DR⁺ cells (red gate in Fig 1, A-II), classical CD14⁺⁺CD16⁻ and nonclassical CD14^{dim/-}CD16⁺⁺ monocytes are recognized by their CD14/CD16 profile (gray gates in Fig 1, B), whereas the cDC1s, cDC2s, and pDCs (green gate in Fig 1, C) reside in the compartment of CD14⁻CD16⁻ nonmonocytic cells (dark blue gate in Fig 1, B). Fig 1 confirms that the CD14^{dim/-}CD16⁺⁺slan⁺ monocytes (gated in the violet rectangle of Fig 1, D-I) constitute part of the nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/Mono2s (violet overlay plot in Fig 1, D-II), as expected.⁵ Fig 1 also shows that, if DCs are selected by excluding only CD14⁺ cells from the Lin⁻HLA-DR⁺ cells (orange gate in Fig 1, E), the resulting DC populations include not only the cDC1s, cDC2s, and pDCs (green gate in Fig 1, F, and green overlay plot in Fig 1, G) but also CD1c⁻CD141⁻CD303⁻ cells (light blue gate in Fig 1, F) corresponding to the DC4 subset.³ However, inclusion of CD16 staining in this analysis demonstrates that DC4s are undoubtedly CD16⁺⁺ (Fig 1, F), as also shown previously,⁴ and that they substantially overlap with the nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/Mono2s (by 31% to 41%; overlay plot in Fig 1, G; n = 10).

The notion that CD16⁺⁺ DC4s are not *bona fide* DCs is also evidenced by other findings. First, 73% ± 4% of CD16⁺⁺ DC4s express the slan antigen (Fig 1, H; n = 7), a feature that strongly supports their monocytic nature.^{5,6} Notably, being a carbohydrate modification of P-selectin glycoprotein ligand 1, slan cannot be detected at the gene level.

Second, CD16⁺⁺ DC4s, but not cDC1s, cDC2s, and pDCs, display remarkable levels of surface colony-stimulating factor receptor 1 (CSF-1R)/CD115 similar to those expressed by CD14^{dim/-}CD16⁺⁺slan⁺ monocytes and nonclassical monocytes/Mono2s (Fig 2, A, and see Fig E1 in this article's Online Repository at www.jacionline.org), which is in line with current knowledge establishing that monocytes, but not circulating DCs, express CSF-1R/CD115.⁷ Consistently, both CD16⁺⁺ DC4s and nonclassical monocytes/Mono2s, but not cDC2s and pDCs, were found to express increased CSF-1R/CD115 mRNA levels (see Figs 1, D, and 3, C, in Villani et al³).

Third, CD16⁺⁺ DC4s behave as CD16⁺⁺slan⁺ monocytes and nonclassical monocytes/Mono2s but differ radically from the cDC2s and classical monocytes/Mono2s if one evaluates either their mRNA expression pattern of DC-specific intercellular adhesion molecule 3–grabbing nonintegrin (CD209), CD206, and CD207 under resting conditions (Fig 2, B) or their mRNA and surface antigen levels of CD86 and CD83 after stimulation with LPS for 20 hours (see Fig E2 in this article's Online Repository at www.jacionline.org).

Fourth, the pattern of IL-12p70, IL-10, and TNF-α production by sorted CD16⁺⁺ DC4s incubated for 24 hours with LPS (or R848, data not shown) in the presence or absence of IFN-γ was found to be qualitatively and quantitatively almost identical to that displayed by nonclassical monocytes/Mono2s and CD14^{dim/-}CD16⁺⁺slan⁺ monocytes, but completely different from those by classical monocytes/Mono1s or cDC2s (Fig 2, C). Similarly, CD16⁺⁺ DC4s, but not cDC2s or Mono1s, behaved exactly as Mono2s and CD14^{dim/-}CD16⁺⁺slan⁺ monocytes in terms of cytokine production, even after a 15-hour mediated “priming” by IFN-γ (Fig 2, C). In fact, the latter molecule is

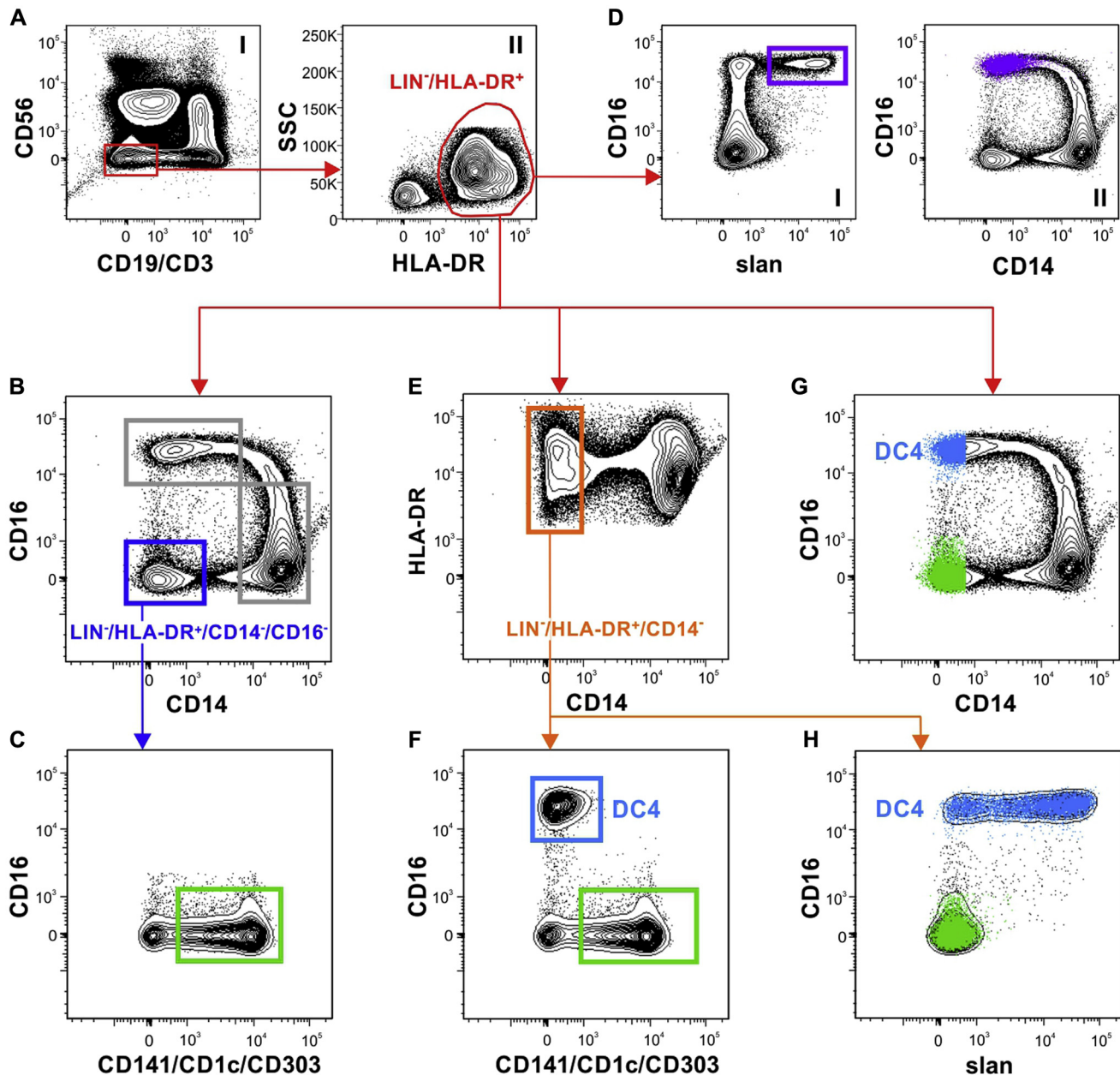


FIG 1. Demonstration that DC4s overlay nonclassical CD14^{dim/-}CD16⁺⁺ monocytes. Flow cytometric plots illustrate (1) cell lineage exclusion (**A-I**) and gating of HLA-DR⁺ cells (**A-II**) from PBMCs; (2) identification of both classical CD14⁺⁺/CD16⁻/nonclassical CD14^{dim/-}CD16⁺⁺ monocytes based on their CD14/CD16 expression (gray rectangles in **B**) and CD14/CD16 double-negative nonmonocytic cells (dark blue box in Fig 1, **B**), containing cDC1s, cDC2s, and pDCs (green rectangle in **C**); (3) identification of CD14^{dim/-}CD16⁺⁺slan⁺ monocytes based on their CD16/slan antigen expression (violet gate in **D-I**) and demonstration that they overlap with nonclassical monocytes (violet overlay plot in **D-II**); (4) gating strategy used by Villani et al³ to identify DC4s, namely the DC pool selection among CD14⁻ cells within the Lin⁻ HLA-DR⁺ cells (orange gate in **E**), consequently including the cDC1s, cDC2s, and pDCs (green gate in **F**) and a unique CD16⁺⁺ cell population (blue rectangle in Fig 1, **F**) corresponding to the DC4s; and (5) demonstration that CD16⁺⁺ DC4s are slan⁺ (**H**) and overlay with nonclassical monocytes (**G**).

well known to dramatically enhance the production of IL-12p70 by Toll-like receptor stimulated CD14^{dim/-}CD16⁺⁺slan⁺ monocytes,⁴ as largely confirmed in Fig 2, **C**.

It should be emphasized that the single-cell RNA sequencing experiments performed in the article by Villani et al³ have uncovered that nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/

Mono2s display a very homogeneous and unique gene expression signature that is fully distinct from that of classical monocytes/Mono1s or DC1s, DC2s, DC3s, DC5s, and DC6s (see Fig 3, **B**, in Villani et al³). Moreover, nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/Mono2s and CD16⁺⁺ DC4s were found to display a similar gene expression signature (see Fig 3, **C**,

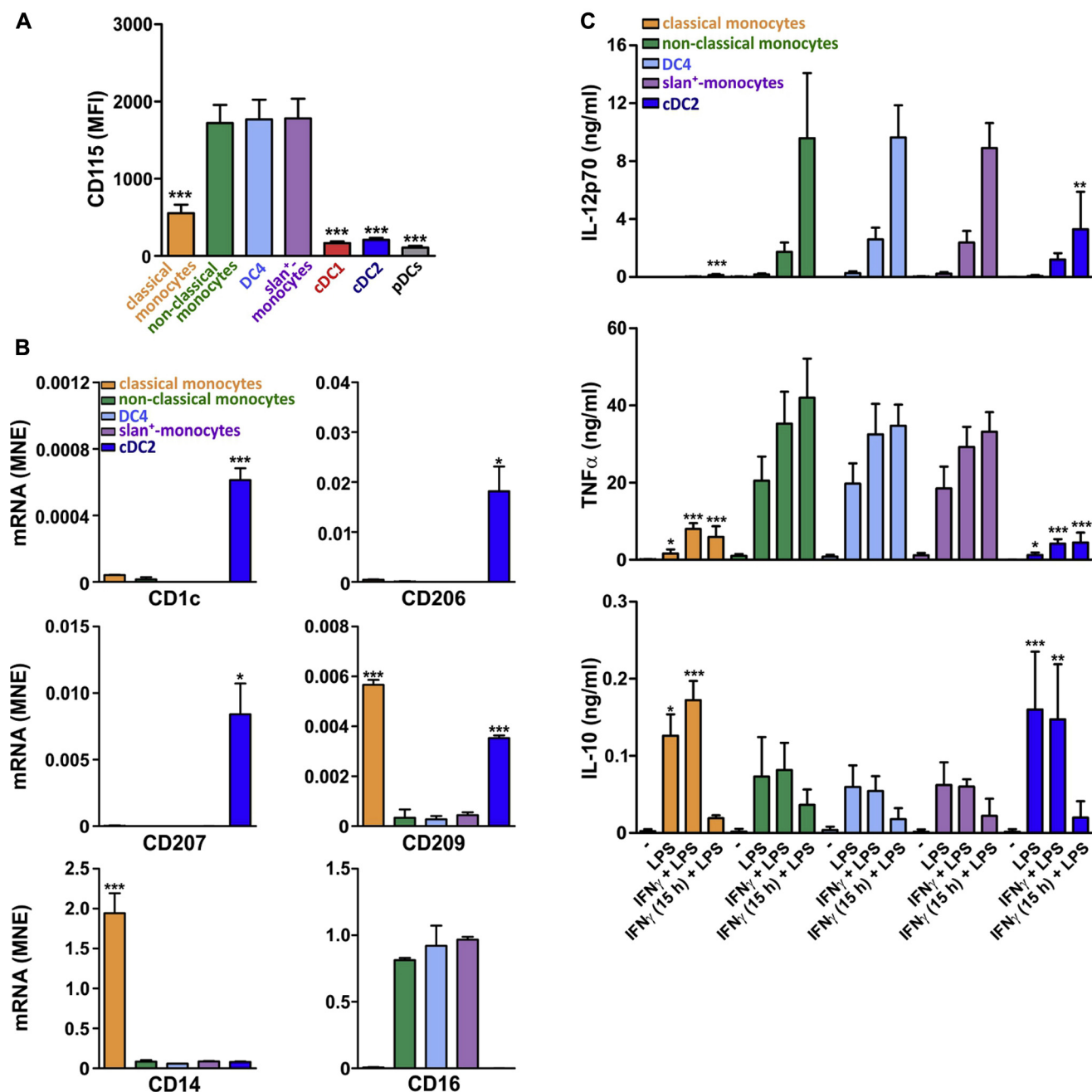


FIG 2. CD16⁺ DC4s behave as nonclassical and slan⁺ monocytes in terms of CSF-1R/CD115 expression and cytokine production. **A**, CSF-1R/CD115 expression in CD16⁺ DC4s, monocytes, and DC populations under investigation. Total PBMCs were stained with a specific panel of mAbs, as described in the [Methods](#) section in this article's Online Repository, for identification of the various monocyte and DC subsets and CSF-1R/CD115 expression levels displayed as mean fluorescence intensity (MFI) $\pm$ SEM (n = 4). **B**, Classical and nonclassical monocytes, slan⁺ monocytes, CD16⁺ DC4s, and cDC2s were sorted from PBMCs and then lysed for investigating CD1c, CD206, CD207, CD209, CD14, and CD16 mRNA expression by using quantitative RT-PCR (mean normalized expression units $\pm$ SEM, n = 3). **C**, IL-12p70, TNF- α , and IL-10 levels in cell-free supernatants (depicted as nanograms per milliliter $\pm$ SEM, n = 5) harvested from 4×10^5 cells/mL classical and nonclassical monocytes, slan⁺ monocytes, CD16⁺ DC4s, and cDC2s sorted from the same donors and incubated for 20 hours with 100 ng/mL LPS, either with or without 200 U/mL IFN- γ or after a 15-hour preincubation with IFN- γ . Asterisks indicate significant differences between CD16⁺ DC4s and other monocyte and DC populations. **P* < .05, ***P* < .01, and ****P* < .001 by means of 1-way ANOVA, followed by the Tukey posttest (Fig 2, A and B) or by means of 2-way ANOVA (Fig 2, C), followed by the Bonferroni posttest.

in Villani et al³), with only minor differences in their transcriptomes (see Table S7 in Villani et al³), which were indicated as sufficient for their discrimination. Remarkably, some of the genes expressed by nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/Mono2s found to selectively discriminate them from CD16⁺⁺ DC4s, namely *CD14*, *S100A8*, *PER1*, *VCAN*, *CD36*, *NCF1*, *LRP1*, *ITGAM*, *TNFAIP3*, *CSF3R*, *NLRP3*, *NAIP*, and *CDA* (see Table S7 in Villani et al³), are also expressed distinctively by classical CD14⁺⁺CD16⁻ monocytes/Mono1s at much higher levels (see Table S5 in Villani et al³). Again, these same genes (along with others) were found to discriminate classical CD14⁺⁺CD16⁻ monocytes/Mono1s from CD16⁺⁺ DC4s (see Table S8 in Villani et al³). These observations suggest that, at least at the gene expression level, CD16⁺⁺ DC4s differ from nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/Mono2s simply because they constitute the most CD14⁻ cells within nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/Mono2s (Fig 1, G). It remains to be explained why, in the single-cell RNA sequencing data set of Villani et al³, CD16⁺⁺ DC4s and Mono2s were not found to be virtually indistinguishable. In such regard, differences might have arisen from batch effects, assuming that DC4s and Mono2s were sorted and sequenced in separate experiments. Alternatively, it has been described⁸ that the normalization method of single-cell sequencing data used by Villani et al³ is not optimal for identification of differentially expressed genes, especially if transcribed at low levels.

The data provided demonstrate that the CD16⁺⁺ DC4s identified by Villani et al³ herein overlap substantially with a fraction of nonclassical CD14^{dim/-}CD16⁺⁺ monocytes/Mono2s and thus cannot be considered a newly discovered DC population. Data argue implicitly for the nonexistence of CD16⁺ DC populations. Supporting the relevance of our findings, even in the field of allergic inflammation, we could draw similar conclusions in reference to the study by Lundberg et al,⁹ who also recognized putative CD16⁺⁺ DCs in allergic subjects. However, CD16⁺⁺ DCs were identified according to a DC gating strategy based on the lack of CD14 expression only, as done by Villani et al.³

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METHODS

Cell purification, sorting, and culture

PBMCs were isolated from buffy coats after Ficoll-Paque density gradient centrifugation and then rinsed twice in PBS to remove platelets. PBMCs were then depleted of T and B lymphocytes by anti-CD3 and anti-CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, with an autoMACS separator (Miltenyi Biotec), and the remaining cells were stained subsequently with the following mAbs: anti-CD14 allophycocyanin (APC; Tuk4; Miltenyi Biotec), anti-CD16 peridinin-chlorophyll-protein complex (PerCP)–Cy5.5 (3G8; BioLegend, San Diego, Calif), anti-CD1c phycoerythrin (PE; AD5-8E7; Miltenyi Biotec), anti-HLA-DR APC-Cy7 (L243; BioLegend), and anti-M-DC8 fluorescein isothiocyanate (FITC; DD1; Miltenyi Biotec) and, as lineage exclusion markers, anti-CD56 Vioblu (AF12-7H3; Miltenyi Biotec), anti-CD3 PE-Vio770 (BW264/56; Miltenyi Biotec), and anti-CD19 PE-Vio770 (LT19; Miltenyi Biotec). Cell sorting was then performed with a Becton Dickinson FACSARIA II, yielding highly pure (>97%) monocyte and DC subsets, as indicated in the text.

Comparison of gating strategies and assessment of cell phenotypes by means of flow cytometry

After isolation, 10^6 human PBMCs were stained with the following mAbs to perform the gating procedures shown in Fig 1: anti-CD45 Brilliant Violet (BioLegend), anti-CD1c, anti-CD141 (Miltenyi Biotec), and anti-CD303 (Miltenyi Biotec) FITC; anti-CD56 PE; anti-CD16 PerCP-Cy5.5; anti-CD3 and anti-CD19 PE-Vio770; anti-CD14 APC; anti-HLA-DR APC-Cy7; and anti-M-DC8 Vioblu. Alternatively, anti-CD14 Vioblu, anti-M-DC8 FITC, anti-CD115 PE, and anti-CD56 APC mAbs were used for the experiments shown in Fig 2, A, and Fig E1. In experiments in which sorted monocyte and DC subsets were incubated in the presence of 100 ng/mL LPS with or without 200 U/mL IFN- γ , 3×10^4 cells were stained with anti-CD83 PE (HB15; Miltenyi Biotec), anti-CD86 PE (FM95; Miltenyi Biotec), or, as an isotype control, mouse IgG₁-PE (IS-21S5; Miltenyi Biotec). In both cases cells were incubated for 15 minutes at room temperature in the presence of 10% complement-inactivated human serum for Fc γ R blocking, and after washing, antigen expression was measured with the MACSQuant Analyzer (Miltenyi Biotec). Data analysis was performed with FlowJo software, (Version 8.8.6; TreeStar, Ashland, Ore).

Cytokine production

After sorting, classical monocytes/Mono1s, nonclassical monocytes/Mono2s, CD14^{dim}/CD16⁺⁺slan⁺ monocytes, CD16⁺⁺ DC4s, and cDC2s were suspended at 5×10^5 /mL in RPMI 1640 medium supplemented with 10% low-endotoxin FBS (<0.5 EU/mL; Sigma, Saint Louis, Mo) and dispensed in 96-well tissue culture plates (Corning, New York, NY) for culture at 37°C in a 5% CO₂ atmosphere. Cells were then either incubated for 20 hours in the presence of 100 ng/mL LPS with or without 200 U/mL IFN- γ or preincubated with IFN- γ for 15 hours and then stimulated with LPS for 20 hours. After culture, cells were collected and spun at 300g for 5 minutes. Cell-free supernatants were immediately frozen in liquid nitrogen and stored at –80°C, whereas the corresponding pellets were either extracted for total RNA or suspended for flow cytometric staining, as described above. Cytokine concentrations in cell-free supernatants were measured by using commercial ELISA kits specific for IL-12p70 (eBioscience, San Diego, Calif), TNF- α (eBioscience), and IL-10 (eBioscience). Detection limits of these ELISAs are 4 pg/mL for IL-10 and 8 pg/mL for both IL-12p70 and TNF- α .

Quantitative RT-PCR

Total RNA was extracted from 4×10^4 cells by using the RNeasy Mini Kit (Qiagen, Venlo, Limburg, The Netherlands), according to the manufacturer's instructions. Purified RNA was then reverse transcribed into cDNA by using Superscript III (Life Technologies, Carlsbad, Calif) and random hexamer primers (Life Technologies). Quantitative PCR was carried out with Fast SYBR GreenMasterMix (Life Technologies) and the following gene-specific primers (purchased from Life Technologies): *MRC1/CD206* forward CTCCTCAAGACAATCCACCAG and reverse GTAATCCACTTTGCTTCCATCC, *CD207* forward TACCTTAAATGCCCAAATCCCA and reverse TATACAGAACTCCTGCTCACTC, *CD209* forward GAGGAGCAGA ACTTCCTACAG and reverse GATTACATTTGTCGTCGTTCCAG, *CD83* forward CCTGGGTCAAGTTATTGGAGG and reverse CATCCTGTCACTCTCAAGATCAC, *CD86* forward TGTCAGTGCTTGCTAACTTCAG and reverse AGAAGATGGTCATATTGCTCGT, *CD1C* forward AGAAGC ACTGTCATATCAGG and reverse TGGGTTCAGTATGGGTCAAGG, *CD14* forward CTAAAGCACTTCCAGAGCCT and reverse CAGCGG AAATCTTCATCGTC, *CD16* forward TGTGTCACTGTCCCAAGTTG and reverse AGTTTCATACTTAACTCAGCG, and *RPL32* forward AGGGTTCGTAGAAGATTCAAGG and reverse GGAAACATTGTGAGCG ATCTC. Data were calculated by using Q-Gene software (<http://www.genequantification.de/download.html>) and expressed as mean normalized expression units after normalization to *RPL32*, a housekeeping control.

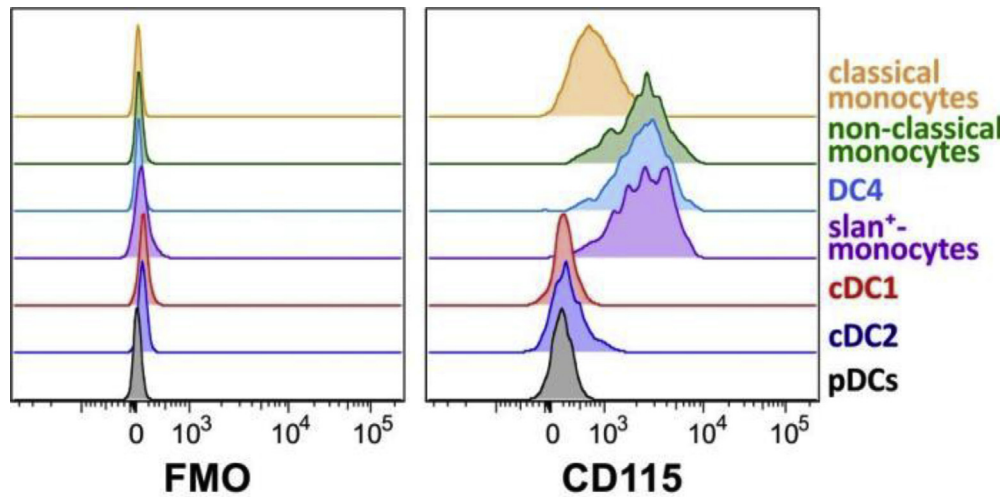


FIG E1. Histograms show representative expression of CSF-1R/CD115, as well as the related fluorescence minus one (*FMO*) control in classical CD14⁺⁺/CD16⁻ and nonclassical CD14^{dim/-}CD16⁺⁺ monocytes, CD14^{dim/-}CD16⁺⁺ slan⁺ monocytes, CD16⁺⁺ DC4s, cDC1s, cDC2s, and pDCs.

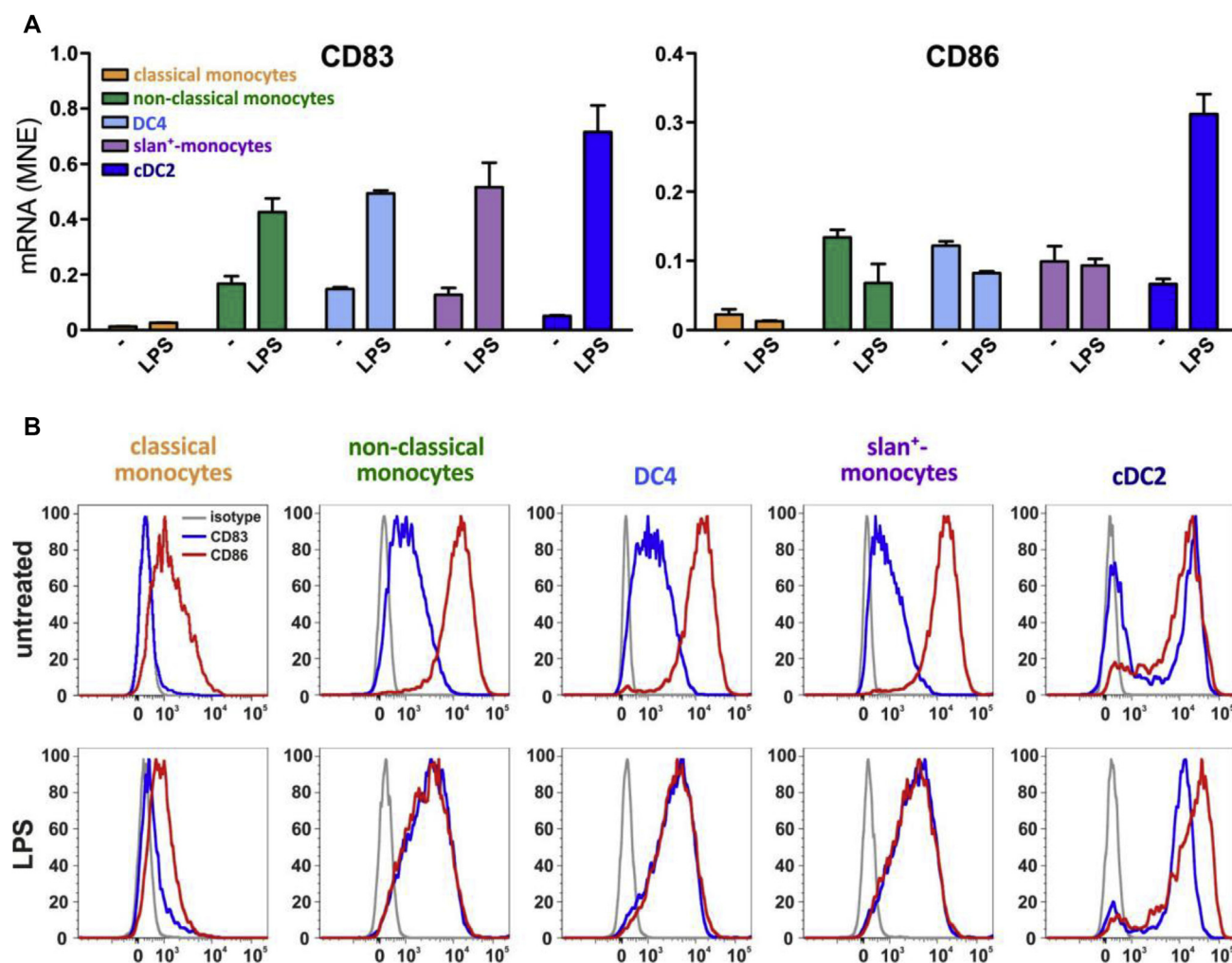


FIG E2. CD83 and CD86 mRNA and surface expression levels in CD16⁺⁺ DC4s and other monocyte and DC populations incubated with LPS. Classical and nonclassical monocytes, CD14^{dim/-} CD16⁺⁺ slan⁺ monocytes, CD16⁺⁺ DC4s, and cDC2s were sorted from PBMCs and then incubated (at 4×10^5 cells/mL) for 20 hours in the presence of 100 ng/mL ultrapure LPS. CD83 and CD86 mRNA (**A**) and surface (**B**) levels were then measured by using quantitative RT-PCR (Fig E2, **A**) and flow cytometry (Fig E2, **B**). Gene expression data are depicted as mean normalized expression units after normalization to RPL32 mRNA. Error bars represent SEs calculated from triplicate quantitative PCR reactions (Fig E2, **A**). One representative experiment of 2 independent experiments with similar results is shown for both Fig E2, **A** and **B**.