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CD163 positive subsets of blood dendritic cells: The scavenging macrophage receptors CD163 and CD91 are coexpressed on human dendritic cells and monocytes

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Abstract

CD163 and CD91 are scavenging receptors with highly increased expression during the differentiation of monocytes into the anti-inflammatory macrophage phenotype. In addition, CD91 is expressed in monocyte-derived dendritic cells (MoDCs), where the receptor is suggested to be important for internalization of CD91-targeted antigens to be presented on the dendritic cell surface for T-cell stimulation. Despite their overlap in functionality, the expression of CD91 and CD163 has never been compared and the expression of CD163 in the monocyte-dendritic cell lineage is not yet characterized. CD163 expression in dendritic cells (DCs) was investigated using multicolor flow cytometry in peripheral blood from 31 healthy donors and 15 HIV-1 patients in addition to umbilical cord blood from 5 newborn infants. Total RNA was isolated from MACS purified DCs and CD163 mRNA was determined with real-time reverse transcriptase polymerase chain reaction. The effect of glucocorticoid and phorbol ester stimulation on monocyte and dendritic cell CD163 and CD91 expression was investigated in cell culture of mononuclear cells using multicolor flow cytometry. We identified two $CD163^+$ subsets in human blood with dendritic cell characteristics, $CD163^{lo}$ and $CD163^{hi}$, together constituting a substantial fraction of DCs. Both subsets were characterized as $[lin]^-CD4^+ILT3^+HLA\text{-}DR^+CD11c^+$ by flow cytometry, and CD163 mRNA was readily detectable in MACS purified human DCs. CD163 on DCs was upregulated by glucocorticoid, and treatment by phorbol ester significantly decreased surface expression. Overall, the expression of CD163 on DCs was significantly increased in HIV-1 patients (19.3% [95% CI: 14.7–26.3%]) compared to healthy patients (10.5% [95% CI: 8.0–12.5]) $p<0.001$. The $CD163^{lo}$ subset was $CD16^+$, whereas the $CD163^{hi}$ subset was $CD16^-$. Both subsets were $CD91^+$, thereby constituting a subfraction of the recently described $CD91^+CD11c^+$ dendritic cell subset. Coexpression of CD163 and CD91 was also demonstrated on human monocytes, which upon glucocorticoid treatment exhibited an increase in both CD163 and CD91 expression. We have now shown that CD163 and CD91 are coexpressed and coregulated on human monocytes. In addition, two subsets of $CD163^+$ DCs constituting a fraction of the recently described $CD91^+CD11c^+$ dendritic cell subset have been

Abbreviations: α_2M, $\alpha_2$macroglobulin; APC, antigen-presenting cells; DCs, dendritic cells; DEX, dexamethasone; FACS, fluorescence-activated cell sorting; Hp–Hb, haptoglobin–hemoglobin; Hx-heme, hemopexin–heme; LRP, low density lipoprotein-related protein; mAbs, monoclonal antibodies; MNC, mononuclear cells; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate

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identified. The CD163 expression pattern suggests that if antigens are targeted to CD163 they may induce an immunostimulatory response like that of CD91-targeted antigens.

Keywords: $\alpha_2$macroglobulin; CD163; CD91; Dendritic cells; Monocytes

Introduction

Dendritic cells (DCs) are bone marrow-derived antigen-presenting cells (APC) that are characterized by their unique capacity to induce and regulate primary immune responses. These rare cells have a remarkable ability to stimulate naïve T lymphocytes and generate memory T lymphocytes (MacDonald et al., 2002; Banchereau and Steinman, 1998; Banchereau et al., 2000; Hart, 1997).

Phenotypically, DCs are classified by a high expression of MHC class II molecules and the absence of lineage (Lin)-specific markers (MacDonald et al., 2002; Hart, 1997). Alternatively, DCs can be defined by expression of CD85k/ILT3, high expression of MHC class II and the absence of CD14 (Hart, 1997; MacDonald et al., 2002). At least two distinct human DC subsets, myeloid DCs (DC1) and plasmacytoid DCs (DC2), have been characterized (Wu et al., 2001). DC1 and DC2 are distinct based on their phenotypes $CD11c^+/CD123^{lo}$ and $CD11c^-/CD123^{hi}$, respectively (Robinson et al., 1999).

DC mediated antigen uptake is controlled by a number of dedicated surface molecules, such as Toll-like receptors, scavenger receptors, and mannose receptors that are each responsible for the binding of antigens sharing a common molecular motif through pattern recognition (Banchereau and Steinman, 1998; Hart, 1997; Kaisho and Akira, 2003; Banchereau et al., 2000).

CD91 (LRP, $\alpha_2$macroglobulin (α_2M) receptor) present on a subset of $CD11c^+$ DCs provides a back-up system for internalization of foreign antigens lacking a molecular motif targeted by pattern recognition receptors. CD91 binds activated $\alpha_2$macroglobulin in a complex that includes the foreign activating antigen. Endocytosis of the complex by DCs leads to antigen presentation and activation of T-cells (Hart et al., 2004).

CD91 is also expressed in macrophages and recently, important functions of the two endocytic macrophage receptors CD163 and CD91 have been identified. CD91 is important for clearance of a broad spectrum of ligands including hemopexin–heme (Hx-heme) complexes resulting in cellular heme uptake and lysosomal hemopexin degradation (Hvidberg et al., 2005). In this way, it backs up the yet only known function of CD163, namely to scavenge haptoglobin–hemoglobin (Hp–Hb) (Kristiansen et al., 2001).

CD163 has been considered to be expressed exclusively on the surface of monocytes and tissue macrophages (Pulford et al., 1992; Backe et al., 1991). The similar functionality of CD91 and CD163 led us to hypothesize that CD163 like CD91 was expressed in DCs. In this work, we present evidence for the existence of two $CD163^+$ DC subsets opening the possibility of an immunostimulatory role of CD163 besides its role in hemoglobin scavenging and anti-inflammation.

Materials and methods

Samples

EDTA stabilised peripheral blood samples were obtained by standard venipuncture from 15 HIV-1 patients and 31 healthy donors. Furthermore, 5 cord blood samples were collected using umbilical venipuncture. The samples were collected in Venoject® vacutainers (Terumo Europe NV, Leuven, Belgium). Additionally, leukocyte-rich buffy coats were separated from units of whole blood (approximately 472 ml) donated by healthy volunteers. The blood was anticoagulated with CPD-A (Baxter, Munich, Germany). The buffy coats were immediately processed as described below.

Monoclonal antibodies

The following monoclonal antibodies (mAbs) were used in combinations for multicolor flow cytometric analysis ([1] BD Biosciences, San Diego, CA, USA; [2] Diatec.com A/S, Oslo, Norway; [3] DAKO A/S, Glostrup, Denmark; [4] IOTest®, Beckman and colter, Marseille, France; [5] Trillium Diagnostics, LLC, Scarborough, Maine, USA; [6] Miltenyi Biotec, Bergisch Gladbach, Germany): Fluoroscein isothiocyanate (FITC)-conjugated anti-CD3 (UCHT1; [1]), anti-CD11c (KB90; [3]), anti-CD14 (RMO52; [4]), anti-CD16 (3G8; [1]), anti-CD19 (SJ25C1; [1]), anti-CD20 (CAT 13.6E12; [2]), anti-CD56 (NCAM16.2; [1]), anti-CD91 (A2MR-α2; [1]) and anti-HLA-DR (EDU-1; [2]); R-Phycoerythrin (R-PE)- conjugated anti-CD163 (GHI/61; [1]), anti-CD163 (MAC2-158; [5]) and anti-HLA-DR (EDU-1; [2]); Phycoerythrin-Cy5 (PE-Cy5)-conjugated anti-ILT3/CD85k (ZM3.8; [4]); Peridinin chlorophyll protein (PerCP)-conjugated anti-HLA-DR (L234; [1]); Allophycocyanin (APC)-conjugated anti-CD4 (EDU-2; [2]) and anti-CD14 (18D11; [2]); MicroBeads-conjugated anti-CD14 (TÜK4; [6]), anti-CD19 (LT19; [6]),

anti-BDCA-3 (AD5-14H12; [6]) and anti-BDCA-4 (AD5-17F6; [6]); Biotin-conjugated anti-CD1c (AD5-8E7; [6]). R-PE-conjugated Mouse IgG_1, κ isotype control (MOPC-21; [1]) was used as a negative control for anti-CD163 PE and analysis for nonspecific antibody binding.

Cell preparation, culture and stimulation

Human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were isolated from blood donor buffy coats by gradient separation using Accuspin System Histopaque® – 1077 (Sigma–Aldrich Denmark A/S, Broendby, Denmark) according to the manufacturer's protocol.

Dendritic cells

DCs were purified from PBMC by a 2-step magnetic cell sorting (MACS® technology) using Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. In brief, PBMC were incubated with FcR Blocking Reagent (human IgG) and MicroBeads-conjugated mAb (anti-CD14 and anti-CD19) and Biotin-conjugated anti-CD1c on ice for 15 min, followed by two washes (10 min, 4 °C, 400 g) in a washing buffer containing PBS (phosphate buffered saline) pH 7.2, 0.5% bovine serum albumin (BSA) and 2 mM EDTA. Cells were resuspended in washing buffer and passed over an LD Column placed in a magnet in order to deplete non-DCs. The unlabeled pre-enriched DC fraction was then centrifuged (10 min, 4 °C, 400 g), resuspended in washing buffer and incubated with MicroBeads-conjugated anti-BDCA-3, anti-BDCA-4 and anti-biotin on ice for 15 min, followed by two washes (10 min, 4 °C, 400 g) in washing buffer. Cells were resuspended in washing buffer and passed over an MS Column placed in a magnet. DCs were recovered by flushing cells out with a plunger after removing the column from the magnet.

Cell culture and stimulation

PBMC (approximately 1×10^7 cells) were cultured for 3 days in 5% CO_2 and 37 °C in RPMI 1640 media (RPMI 1640 + 25 mM HEPES + L-glutamine) (Invitrogen Corporation, Carlsbad, CA, USA) with 20% FCS. When indicated, 200 nM dexamethasone (DEX) (Merck KGaA, Darmstadt, Germany) was added during culture to increase CD163 expression. In some cultures, cells were finally incubated for 30 min with 10 nM phorbol 12-myristate 13-acetate (PMA) (Sigma–Aldrich Denmark A/S, Broendby, Denmark), which previously has been shown to induce CD163 shedding. Cells were washed in RPMI 1640 media with 10% FCS, resuspended and stained for CD163 expression as described below.

Fluorescence-activated cell sorting (FACS) analysis of cell surface CD163 expression

Cells (100 µl whole blood or 300 µl PBMC [approximately 3×10^6 cells]) were stained with isotype-matched control antibody or a relevant antibody for 15 min at room temperature (RT) in the dark. Cells were then washed with 1 ml RPMI 1640 media containing 10% FCS, resuspended in 400 µl FACSflow (Becton Dickinson, San Jose, CA, USA) and analyzed with a FACSCalibur™ flow cytometer using CELLQuest software™ (BD Biosciences). At least 100,000 events were acquired to ensure an adequate number of cells for analysis. On a 2-parameter correlated Dot Plot of forward scatter [FSC] versus side scatter [SSC], a gate was set around the mononuclear cells (MNC) clusters. The gated MNC were re-plotted using different 4-color staining protocols and cell definition strategies as indicated in the text. For CD163 density quantitation, flow cytometric estimation of antibodies bound/cell (ABCs) was performed using Quantibrite PE beads (Becton Dickinson, San Jose, CA, USA). The Quantibrite PE beads were run at the same instrument settings as the assay, and the linear regression obtained using the Quantibrite PE beads was used to convert the FL2 linear fluorescence staining of cell population into the number of (CD163) PE molecules bound per cell (ABC). All stainings were controlled using non-specific mAbs, and data was compensated for spectral overlap between fluorochromes using single stained controls and analyzed via FlowJo for Macintosh software Version 6.3 (Tree Star, San Carlos, CA, USA).

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) assay

Total cellular RNA was extracted from whole blood and DCs with QIAamp® RNA Blood Mini (Qiagen, Albertslund, Denmark) according to the manufacturer's protocol, and stored at −80 °C until further use.

Reverse transcription was performed by adding 1 µl of the extracted mRNA to a reaction mixture consisting of 2 µl 10 × PCR buffer II (Applied Biosystems, Naerum, Denmark) supplemented with 6.3 mM $MgCl_2$, 0.3 mM of each of the four deoxyribonucleoside triphosphates (dATP, dTTP, dGTP, dCTP), 2.5 mM 16mer oligo dT nucleotide, 20 U RNase inhibitor, and 50 U MULV reverse transcriptase in a total volume of 20 µl (All reagents from Applied Biosystems, Naerum, Denmark). The cDNA synthesis was carried out in a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Naerum, Denmark) at 42 °C for 30 min followed by

99 °C for 5 min. The resulting cDNA provided template for the real-time qPCR assay. The synthesized cDNA was stored at −20 °C.

Two μl of cDNA were used as template for real-time qPCR in a reaction mixture containing 10 pmol of each primer (CD163 WT; forward primer 5′-ACA TAG ATC ATG CAT CTG TCA TTT G -3′; reverse primer 5′-CAT TCT CCT TGG AAT CTC ACT TCT A-3′; MWG Biotech AG, Edersberg, Germany), 1.0 μl Light-Cycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics, Hvidovre, Denmark), containing FastStart Taq DNA Polymerase, reaction buffer, deoxyribonucleoside triphosphates (dATP, dUTP, dGTP, dCTP), SYBR Green I dye, and 10 mM of $MgCl_2$. The volume was adjusted to 10 μl with nuclease-free H_2O.

The real-time hot-start qPCR was performed in a LightCycler® System (Roche Diagnostics, Hvidovre, Denmark) with an initial denaturation step of 95 °C for 15 min, then 50 cycles with a 95 °C denaturation for 10 s followed by 65 °C annealing for 10 s and 72 °C extension for 5 s. Amplification specificity was checked by melting curve analysis.

Data and statistical analysis

Results are given as mean (95% CI). Differences between values were analysed for statistical significance with Student's *t*-test. For comparisons between smaller groups without Gaussian distribution of values, the non-parametric Wilcoxon signed-ranks test was used. Differences were considered significant at $p<0.05$. Statistical calculations were performed with SPSS® 13 standard version for Windows (SPSS Inc., Chicago, IL) software.

Results

Identification of CD163 positive peripheral blood cells with dendritic cell characteristics

Whole blood was stained with specific mAbs defining DCs as $CD14^-ILT3^+HLA\text{-}DR^+$ and monocytes as $CD14^+ILT3^+HLA\text{-}DR^+$. As shown in Fig. 1C, a substantial fraction of monocytes (88.0% [95% CI: 85.0–91.0%]) stained positive for CD163 (GHI/61 mAb), as expected. The median fluorescence intensity (MFI) was 22.3 (95% CI: 19.2–25.4). Most DCs stained negative for CD163, however, 10.5% (95% CI: 8.0–12.5) stained positive for CD163 (GHI/61 mAb). The $CD163^+$ DCs segregated into two populations; a subset with staining intensity comparable to monocytes ($CD163^{hi}$, MFI = 34.6 [95% CI: 30.5–40.7]) and a weaker staining subset ($CD163^{lo}$, MFI = 4.2 [95% CI: 3.7–5.1]) (Fig. 1C). For comparison, the MFI of an isotype-matched non-specific IgG staining in the same subset was 1.23 [95% CI: 1.14–1.46] (Fig. 1D).

In order to exclude unspecific cross-reactivity, the experiments were repeated using an alternative CD163 mAb (MAC2-158). Both the MFI (107.5 [95%CI: 74.7–140.3] versus 22.3 [95% CI: 19.2–25.4]) and fraction (32.3 [95% CI: 19.6–45.1] versus 10.5 [95% CI: 8.0–12.5]) of positive DCs were significantly higher ($p<0.001$ and 0.01, respectively) using this antibody, however, again two populations of $CD163^+$ DC were identified (Fig. 1E). Also, the MFI and fraction of positive monocytes were significantly higher using MAC2-158.

DC CD163 gene expression was assessed by reverse transcriptase RT-qPCR using MACS purified DCs from peripheral blood. A significant synthesis of CD163 mRNA was detected in DCs (Fig. 1G).

Surface antigen characteristics of CD163 positive dendritic cells

To investigate surface antigen characteristics of the two identified $CD163^+$ DC populations, CD163, HLA-DR, ILT3, CD11c, and CD16 expression were investigated by flow cytometric analysis. The $CD163^{lo}$ subset was $HLA\text{-}DR^{lo}$ and $ILT3^{lo}$, whereas the $CD163^{hi}$ subset was $HLA\text{-}DR^{hi}$ and $ILT3^{hi}$ (Figs. 1E and 2A). Both subsets expressed CD11c, which indicates that they both belong to the myeloid subtype (DC1) of DCs (Fig. 2B). Interestingly, only the $CD163^{lo}$ population stained positive for CD16 (the low-affinity receptor for aggregated IgG, FcγRIII) (Fig. 2C). In order to verify the existence of the two $CD163^+$ DC subsets, an alternative gating procedure was used. In this selection, DCs were defined as lineage[CD3,CD14,CD16,CD19,CD20, CD56]$^-CD4^+HLA\text{-}DR^+$. Fig. 3D shows the presence of only one $CD163^+$ DC population ($CD163^{hi}$). However, when repeating the experiment with the exclusion of the CD16 antibody from the lineage cocktail, both $CD163^+$ populations were identified (Fig. 3E, compare with Fig. 1E). Therefore, the $CD163^{lo}$ subset was characterized as being [CD3,CD14,CD19,CD20,CD56]$^-$ $CD4^+ILT3^{lo}HLA\text{-}DR^{lo}CD11c^+CD16^+$ and the $CD163^{hi}$ subset characterized as [CD3,CD14,CD16,CD19,CD20, CD56]$^-CD4^+ILT3^{hi}HLA\text{-}DR^{hi}CD11c^+$. Backgating analysis showed the $CD163^+$ DCs ($Lin^-CD4^+HLA\text{-}DR^+$) as a distinct subset of cells localized between lymphocytes and monocytes (Fig. 3G).

Regulation of dendritic cell CD163 expression by glucocorticoid and phorbol ester

To assess whether different stimuli could influence CD163 expression on DCs as on monocytes, freshly isolated PBMC were cultured for 3 days with or without DEX (3 days) or PMA (30 min). The cells were then

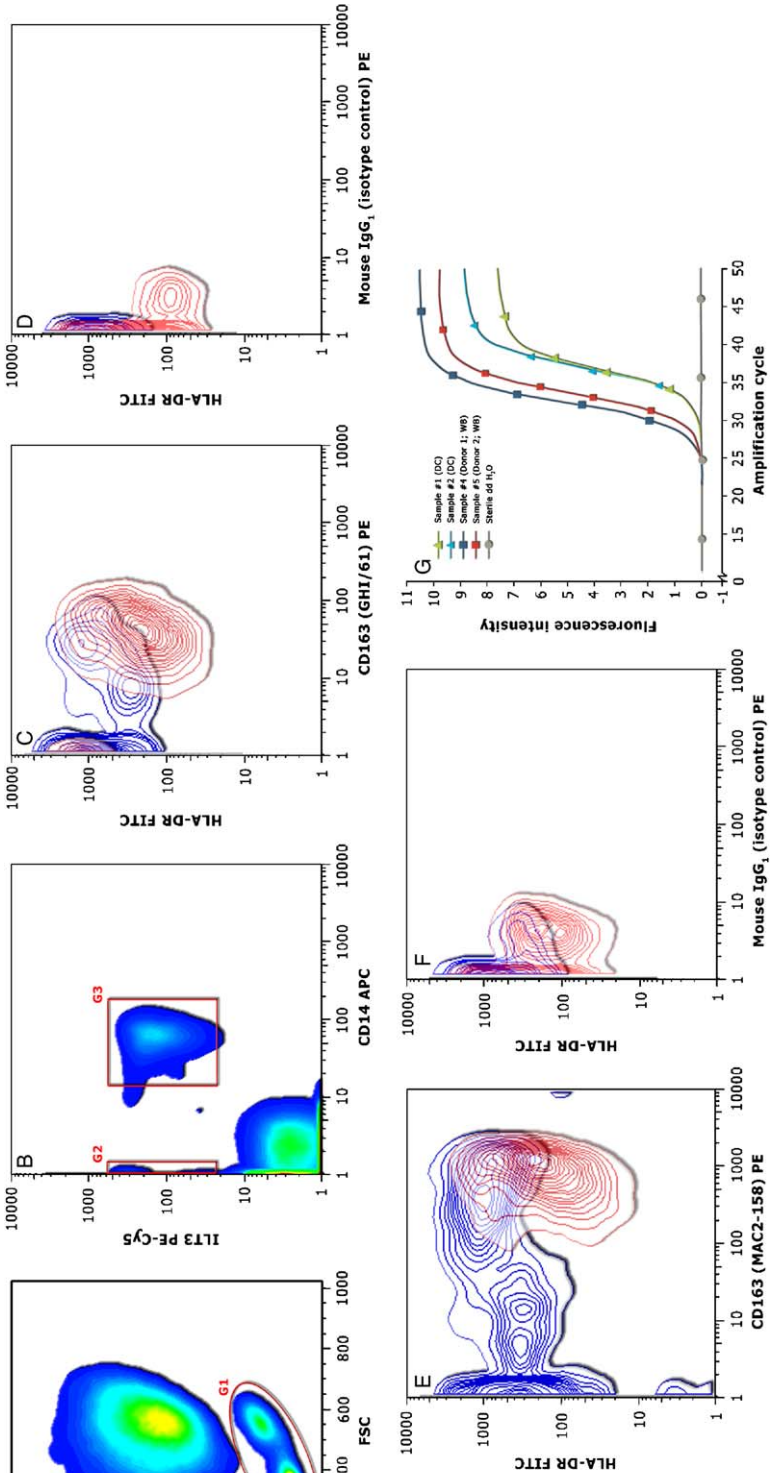

Fig. 1. Two subsets of human dendritic cells express CD163. Flow cytometric analysis of CD163 expression on dendritic cells and monocytes in peripheral blood. In a forward scatter [FSC] versus side scatter [SSC], the mononuclear cell cluster was gated (G1) (A), and the gated cells were re-plotted with CD14 APC (FL4) versus ILT3 PE-Cy5 (FL3) (B). A gate was sat around $CD14^{-}ILT3^{+}$ cells (G2; dendritic cells) and $CD14^{+}ILT3^{+}$ cells (G3; monocytes). The gated cells were re-plotted with CD163 PE (Clone GHI/61) (FL2) versus HLA-DR FITC (FL1) depicting dendritic cells in a blue contour plot with a red overlay contour plot of monocytes (C). The negative control staining of similarly gated cells using isotype matched non-specific PE-conjugated IgG is shown for dendritic cells (blue) and monocytes (red) (D). Panel (E) shows the same experiment using an alternative CD163 antibody (Clone MAC2-158). The negative control staining of similarly gated cells using isotype matched non-specific PE-conjugated IgG is shown for dendritic cells (blue) and monocytes (red) (F). CD163 mRNA expression in MACS purified dendritic cells and whole blood was detected by real-time quantitative reverse transcription polymerase chain reaction (G). A color version of this figure is available at: http://dx.doi.org/10.1016/j.imbio.2006.05.019.

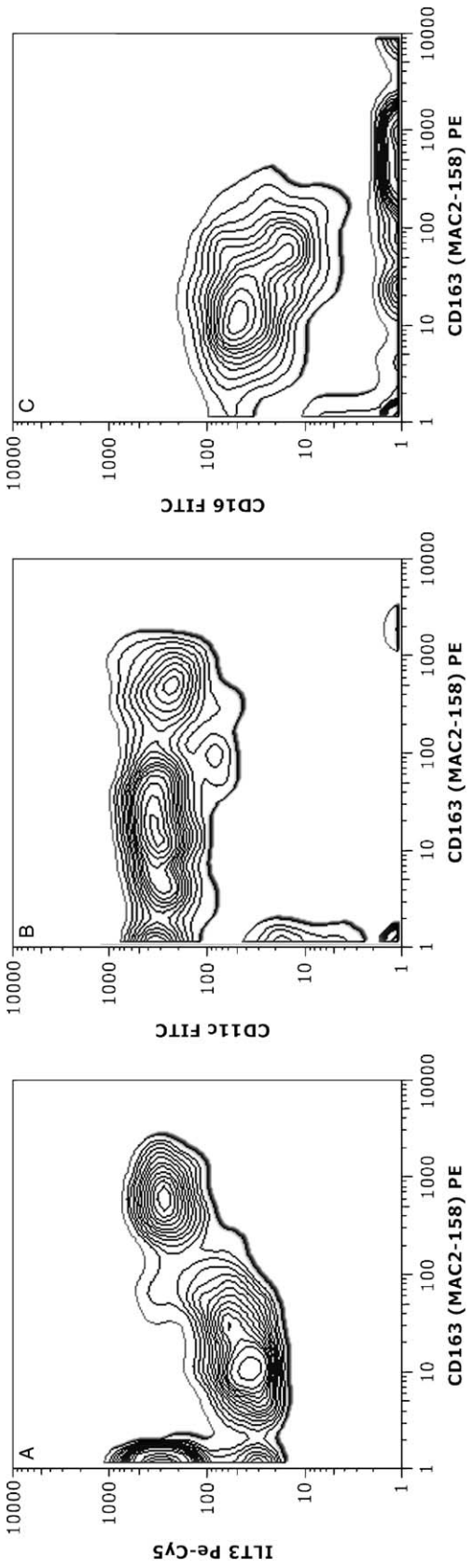

Fig. 2. Surface antigen characteristics of CD163 positive dendritic cell subsets. Flow cytometric analysis of CD163, ILT3, CD11c, and CD16 expression on dendritic cells in peripheral blood (using CD163 MAC2-158 antibody). After gating of mononuclear cells and dendritic cells ($CD14^{-}ILT3^{+}$), gated cells were re-plotted with CD163 PE (FL2) versus ILT3 PE-Cy5 (FL3) (A), CD163 PE (FL2) versus CD11c FITC (FL1) (B), and CD163 PE (FL2) versus CD16 FITC (FL1) (C). No significant staining was observed using isotype matched non-specific PE-conjugated IgG (not shown).

stained and subjected to flow cytometry. Analysis of DCs defined as $CD14^{-}ILT3^{+}HLA\text{-}DR^{+}$ showed that CD163 expression increased significantly when stimulated with DEX (Fig. 4). Phorbol ester is known to induce shedding of the extracellular part of CD163 in monocytes, and a significant decrease in CD163 expression was also observed in DCs when treated with PMA (Fig. 4).

Dendritic cell CD163 expression in healthy controls and in HIV-1 infection

Overall, the fraction of $CD163^{+}$ DCs in HIV-1 infected patients as assessed by the GHI/61 clone (19.3% [95% CI: 14.7–26.3%]) was significantly higher than in healthy controls (10.5% [95% CI: 8.0–12.5]) ($p<0.001$) (Fig. 5A). Fifteen of 15 HIV-1 infected patients expressed both populations of $CD163^{+}$ DCs ($CD163^{lo}$ and $CD163^{hi}$), whereas 16 of 31 healthy controls only expressed one of the populations (Fig. 5B). Furthermore, in controls that expressed the $CD163^{hi}$ subset (75%), the mean expression was significantly lower than in the HIV-1 patients. ($p<0.001$) (Fig. 5C). For comparison, the CD163 expression on monocytes was also determined and showed a significantly higher CD163 expression in HIV-1 infected patients as compared to controls ($p<0.01$) (not shown).

Additionally, the existence of $CD163^{+}$ DCs was demonstrated in umbilical cord blood from 5 individuals. In one individual, both $CD163^{lo}$ and $CD163^{hi}$ subsets of $CD163^{+}$ DCs were present, whereas only $CD163^{hi}$ subset was present in 4 samples (not shown).

Coexpression of CD163 and CD91 on dendritic cells and monocytes

In the macrophage iron metabolism, key roles for both CD163 and CD91 have been described. As CD91 recently was described on a subset of DCs performing important roles in the adaptive immune responses, we wanted to investigate if the two molecules were coexpressed on monocytes and DCs.

As illustrated in Fig. 6A, flow cytometric analysis showed that DCs ($CD14^{-}ILT3^{+}HLA\text{-}DR^{+}$) are segregated into at least 4 subsets when whole blood was stained for both CD163 and CD91 ($CD91^{-}CD163^{-}$, $CD91^{+}CD163^{-}$, $CD91^{+}CD163^{lo}$, $CD91^{+}CD163^{hi}$). This finding indicates that the two $CD163^{+}$ subsets constitute a subfraction of $CD91^{+}$ DCs. On the other hand, analyzes of monocytes ($CD14^{+}ILT3^{+}HLA\text{-}DR^{+}$), showed one uniform population of $CD91^{+}$ $CD163^{+}$ cells (Fig. 6B).

To investigate whether CD163 and CD91 are coregulated on human monocytes, freshly isolated PBMC were cultured with or without DEX or PMA.

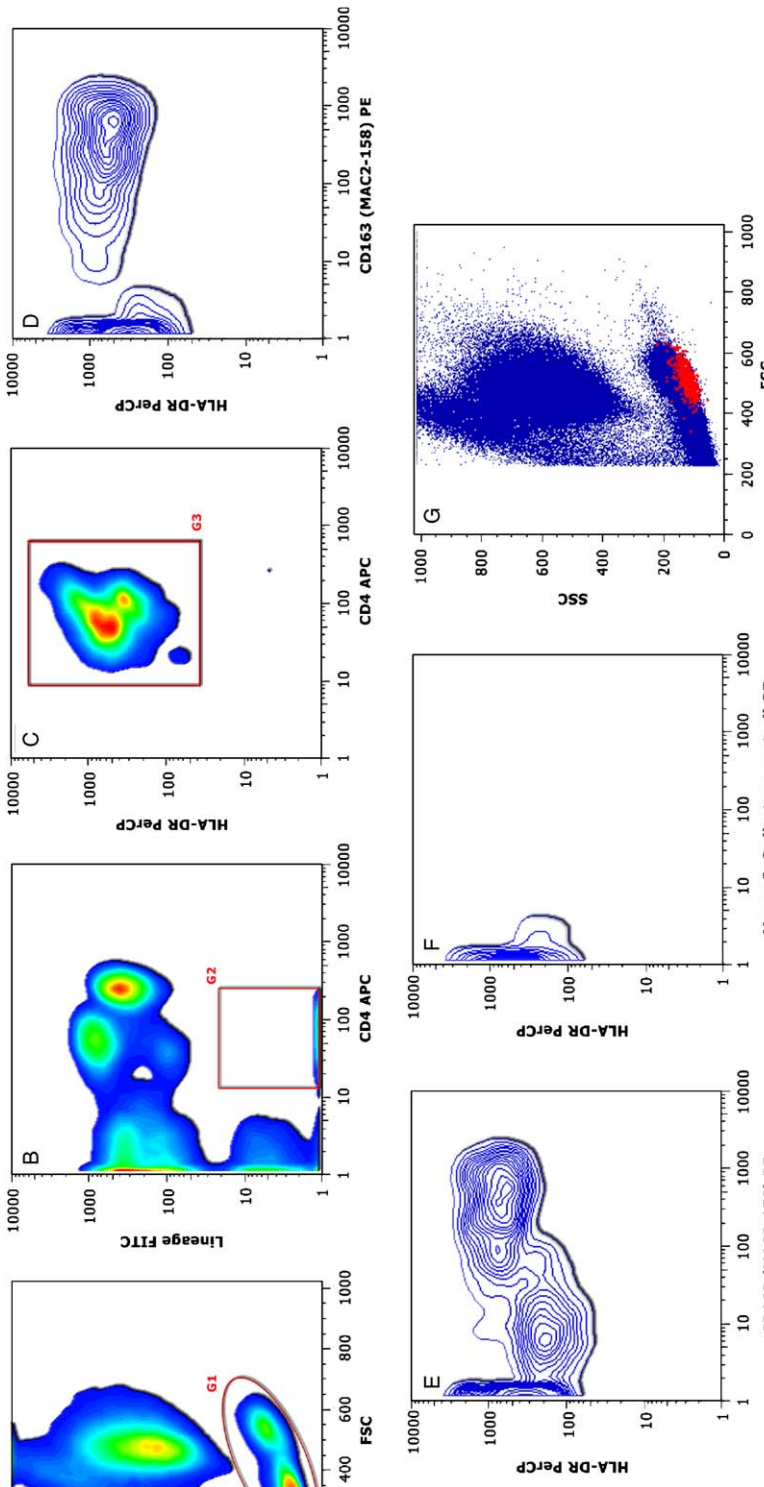

Fig. 3. CD163 expression on dendritic cells defined as lineage negative cells. In order to verify the existence of $CD163^+$ dendritic cells, an alternative staining and gating procedure was used. Gated mononuclear cells (G1) (A) were re-plotted with CD4 APC (FL4) versus Lin(CD3, CD14, CD16, CD19, CD20, CD56) FITC (FL1), and a gate (G2) was sat around Lin^-CD4^+ cells (B). The gated cells were re-plotted with CD4 APC (FL4) versus HLA-DR PerCP (FL3), and a gate (G3) was sat around $CD4^+HLA\text{-}DR^+$ cells (dendritic cells) (C). The gated dendritic cells ($Lin^-CD4^+HLA\text{-}DR^+$ cells) were re-plotted with CD163 PE (FL2) versus HLA-DR PerCP (FL3) (D). The experiments were repeated in the absence of CD16 antibody in the lineage cocktail and using the same gatings (E), revealing the presence of the $CD163^{lo}$ subset when allowing the $CD16^+$ cells in the dendritic cell gate. The negative control staining of similarly gated cells was performed using isotype matched non-specific PE-conjugated IgG (F). Backgating of $Lin^-CD4^+HLA\text{-}DR^+$ cells (red dot plot overlay) in an FSC/SSC plot (G) revealed the expected localization of dendritic cells between lymphocytes and monocytes. A color version of this figure is available at: http://dx.doi.org/10.1016/j.imbio.2006.05.019.

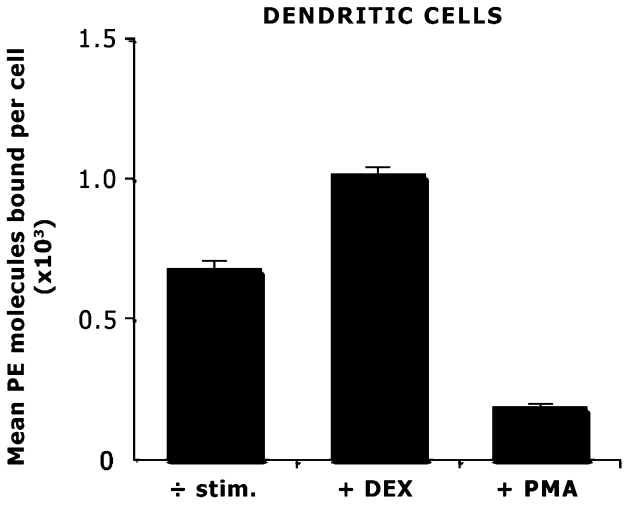

Fig. 4. Effect of glucocorticoid and phorbol ester on dendritic cell CD163 expression. Mononuclear cells were isolated from human whole peripheral blood and cultured for 3 days with or without dexamethasone (DEX) (3 days), or phorbol 12-myristate 13-acetate (PMA) (30 min). Washed cells were stained and analysed by flow cytometry. Gated dendritic cells ($CD14^{-}ILT3^{+}$) were re-plotted with CD163 PE (FL2) versus HLA-DR FITC (FL1), and Quantibrite PE beads were used to convert the FL2 linear fluorescence staining of cell population into the number of CD163 PE molecules (GHI/61) bound per cell. Results (representative of three independent experiments) are expressed as mean ± standard error (SE).

As expected, CD163 expression increased significantly on monocytes ($CD14^{+}ILT3^{+}HLA\text{-}DR^{+}$) upon treatment with DEX ($p<0.01$) and decreased significantly when stimulated with PMA ($p<0.001$) (Table 1). Interestingly, CD91 expression also increased significantly on monocytes ($CD14^{+}ILT3^{+}HLA\text{-}DR^{+}$) when incubated with DEX ($p<0.01$) and decreased significantly when stimulated with PMA ($p<0.001$) (Table 1).

Discussion

We have recently identified CD163 and CD91 as essential players in iron-metabolism by scavenging hemoglobin and heme from plasma (Hvidberg et al., 2005; Kristiansen et al., 2001), functions that are linked to secondary anti-inflammatory functions (Moestrup and Moller, 2004). In the present work, we show that the two molecules are coexpressed and coregulated on human monocytes and describe two subsets of $CD163^{+}$ DCs constituting a fraction of the recently described $CD91^{+}CD11c^{+}$ DC subset. Monocytes from peripheral blood ($CD14^{+}HLA\text{-}DR^{+}$) stained positive for both CD163 and CD91 in flow cytometric analysis, and both were significantly upregulated by glucocorticoid. This coexpression and coregulation seems appropriate in situations of intravascular hemolysis, where the CD163 scavenging system becomes saturated, and free heme in the circulation can be removed by CD91 (Rother et al., 2005).

Besides its role in scavenging heme, CD91 binds and mediates uptake of a multiple other ligands including α_2M-proteinase complexes. α_2M becomes activated by foreign proteases which upon cleavage in α_2M become encapsulated, and endocytosed together with α_2M upon binding to CD91 (Moestrup, 1994). CD91 has recently been described on a subset of myeloid-derived DCs, which is of particular interest because endocytosis by the α_2M-CD91 pathway in DCs can lead to antigen presentation and T-cell activation (Hart et al., 2004).

CD163 belongs to the class B SRCR receptors that, among others, include CD5, CD6, gp-340, M160, and CD163 (Graversen et al., 2002; Gronlund et al., 2000). Whereas most members are expressed in various cell types and exhibiting a broad range of structurally different ligands, CD163 is expressed selectively in monocytes and tissue macrophages (Pulford et al., 1992; Backe et al., 1991). Currently, there is strong evidence, however, that CD163 also is expressed on leukemic blast cells and monocyte-derived dendritic cells (MoDC) (Walter et al., 2003; Chamorro et al., 2004), and a small subset of $CD14^{-}CD163^{+}$ cells has been identified in peripheral blood that has been interpreted as $CD14^{-}$ monocytes (Zarev and Davis, 2004). Although Hp–Hb is the only known ligand for CD163 (Kristiansen et al., 2001), the many scavenger receptor repeats in CD163 may function as targets for other ligands. Therefore, it is tempting to hypothesize that CD163 through pattern recognition is involved in processing of foreign antigens.

To further approach this question, we investigated whether CD163, as CD91, is present on subsets of DCs. We identified two subsets of CD163 positive DCs, $CD163^{lo}$ and $CD163^{hi}$. The two subsets constituted a substantial fraction of peripheral blood DCs in healthy persons. About 10% of DCs stained positive with the monoclonal anti-CD163 GHI/61, whereas as much as 32% stained positive with the clone MAC2-158. This difference is probably partly due to sterical hindrance for binding of the GHI/61 antibody, since it binds to domain 7 of CD163, located in proximity to the cell membrane, whereas MAC2-158 binds to domain 1 at the exposed end of CD163 (Madsen et al., 2004). Both subsets were present in approximately 50% of healthy and in all tested HIV-1 infected patients. Both $CD163^{+}$ subsets were $CD11c^{+}$, which refer them to the myeloid lineage (DC1). Furthermore, we demonstrated that the two $CD163^{+}$ subsets constitute a fraction of the recently described $CD91^{+}CD11c^{+}$ DC subset. This emphasizes the relation between CD163 and CD91, since they are coexpressed not only on monocytes but also on DCs.

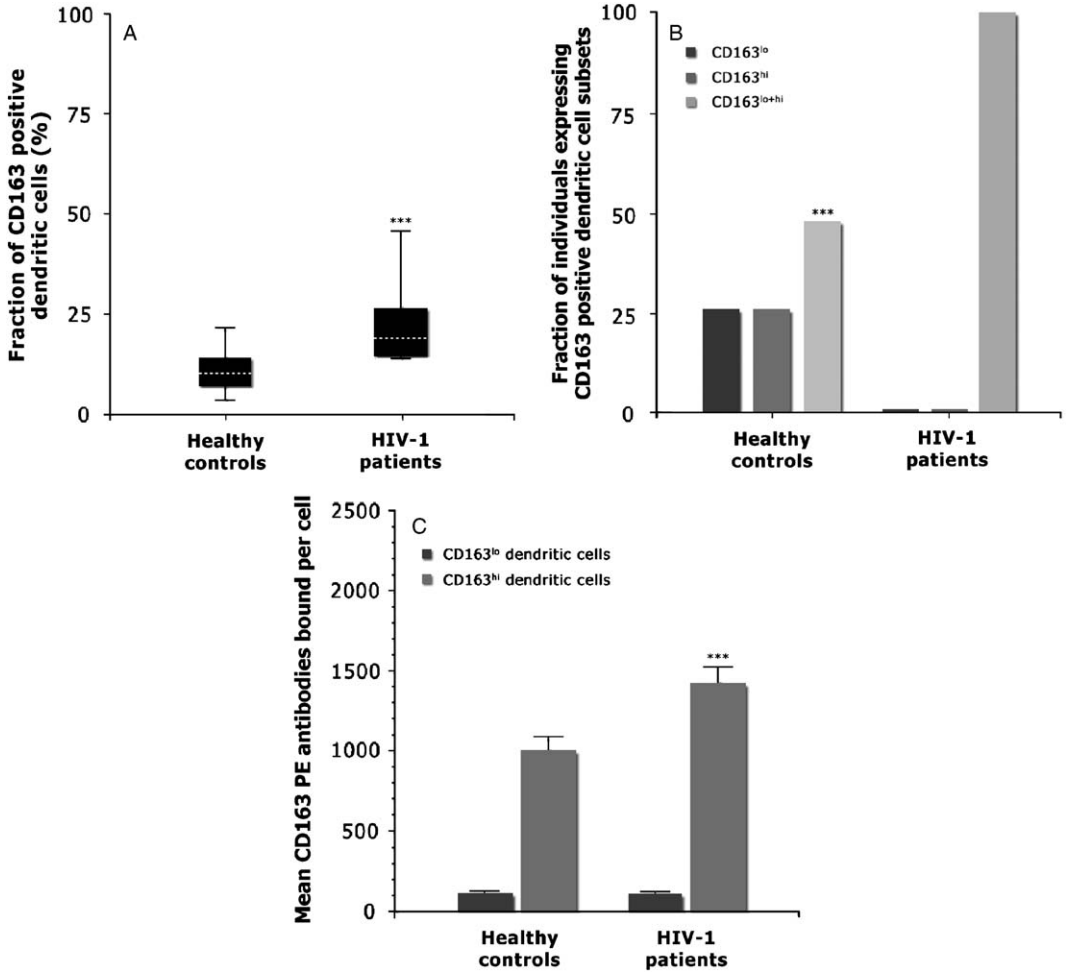

Fig. 5. Dendritic cell CD163 expression in HIV-1 infection. Flow cytometric analysis of CD163 expression on dendritic cells in peripheral blood from HIV-1 patients compared to healthy adults. Gated dendritic cells ($CD14^{-}ILT3^{+}$) were re-plotted with CD163 PE (FL2) versus HLA-DR FITC (FL1): (A) Fraction of $CD163^{+}$ dendritic cells in normal controls ($n = 31$) and HIV-1 infected patients ($n = 15$). Box plots indicate median, 25–75 percentiles, and range. (B) Fraction of control- and patient-samples expressing the $CD163^{lo}$, $CD163^{hi}$, or both subsets. (C) Level of expression of CD163 in $CD163^{lo}$ and $CD163^{hi}$ subsets in controls and HIV-1 infected patients. Quantibrite PE beads were used to convert the FL2 linear fluorescence staining of cell population into the number of CD163 PE molecules (GHI/61) bound per cell. Results are expressed as mean ± standard error (SE). $^{***}p < 0.001$.

The DC expression of CD163 was upregulated by glucocorticoid as has previously been shown for CD163 expression in monocytes. Moreover, the shedding of CD163 from monocytes by phorbol ester and other inflammatory mediators (Droste et al., 1999) seems to be mirrored in DCs that showed a significant decrease in surface-expression of CD163 after treating with phorbol ester. The identity of the cells as being DCs was confirmed in two different staining protocols; one based on the phenotype $CD14^{-}ILT3^{+}HLA\text{-}DR^{+}$, the other based on $Lin^{-}HLA\text{-}DR^{+}$.

Heterogeneity of DC phenotypes is well-known, and functional differences between phenotypically distinct subsets are anticipated or in some instances documented. The concept of constitutively inflammatory versus tolerogenic DC subsets has been forwarded, whereas plasticity of the cellular response during induction or resolution of inflammatory processes as recently demonstrated in macrophages is perhaps more likely (Porcheray et al., 2005).

Nevertheless, the correlation of CD163 intensity to ILT3 and HLA-DR surface membrane expression is noteworthy, since ILT3 density in DCs has been shown in vitro to mirror a tolerogenic capacity (Manavalan et al., 2003). If an extrapolation of immunophenotypes obtained in vitro is accepted, weakly CD163 stained DCs in this context might represent an anti-inflammatory subset. The $CD16^{+}$ $CD163^{lo}$ subset is $ILT3^{lo}$, and $CD16^{+}$ DCs have been reported to constitute a significant proportion of myeloid DCs (MacDonald et al., 2002), and micro array analysis has proposed that toll-like receptor 8 (TLR8) is predominant in these cells (Lindstedt et al., 2005), suggesting a primary role in ssRNA responses.

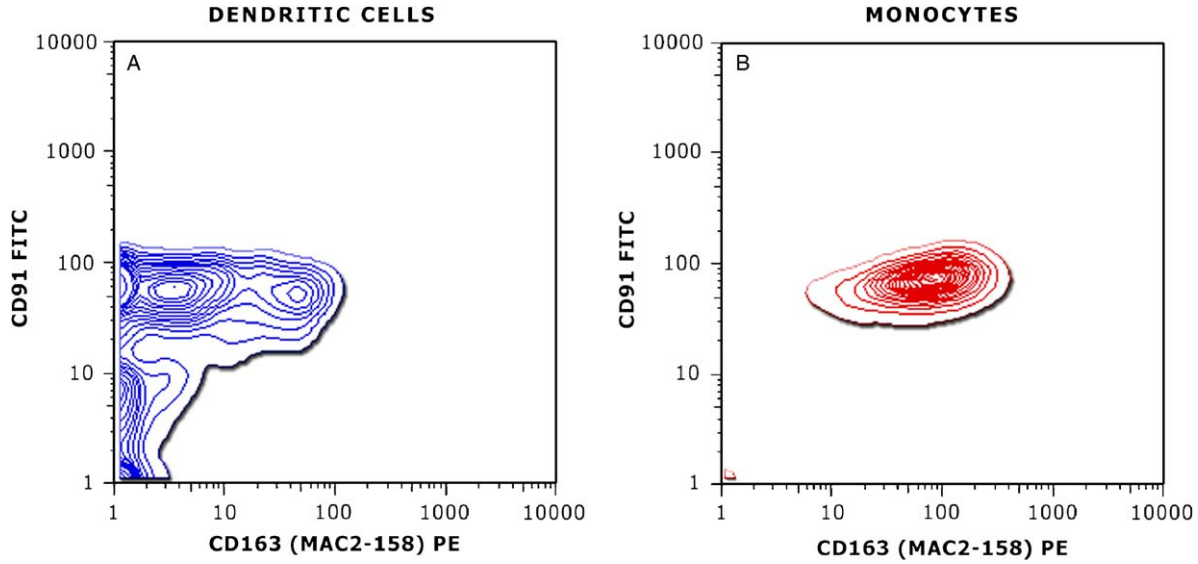

Fig. 6. Coexpression of CD163 and CD91 on monocytes and dendritic cells. Flow cytometric analysis of CD163 and CD91 expression on monocytes and dendritic cells in peripheral blood. Gated dendritic cells ($CD14^{-}ILT3^{+}$) (A) and monocytes ($CD14^{+}ILT3^{+}$) (B) were re-plotted with CD163 (MAC2-158) PE (FL2) versus CD91 FITC (FL1). No significant staining was observed using isotype-matched non-specific FITC- and PE-conjugated IgG (not shown).

Table 1. Coregulation of CD163 and CD91 on monocytes

	Median fluorescence intensity							
	Unstimulated	95% CI	+PMA	95% CI	*p*	+DEX	95% CI	*p*
CD163 PE	472.67	228.46–716.88	8.80	6.53–11.06	<0.01	1211.01	1058.21–1363.79	<0.001
CD91 FITC	40.07	38.49–41.64	27.43	23.33–31.53	<0.001	60.33	53.57–67.10	<0.001

Mononuclear cells were isolated from human peripheral blood and cultured for 3 days with or without dexamethasone (DEX) (3 days), or phorbol 12-myristate 13-acetate (PMA) (30 min). Monocytes were stained and analyzed by flow cytometry as described in Fig. 6B. Results are representative of three independent experiments.

Direct targeting of antigens to DCs in vivo via specific surface receptors is a promising method to enhance immunogenicity of vaccines (Gamvrellis et al., 2004). This approach often employs the use of humanized antibodies coupled to the selected antigen (Tacken et al., 2005). The restricted expression of CD163 on DCs and other antigen presenting cells would make CD163 a putative candidate for such strategies. Moreover, CD163 may be targeted not only by antibodies (Van den Heuvel et al., 1999), but also by antigen coupled to Hp–Hb complexes or parts thereof capable of binding to the CD163 receptor.

In conclusion, we have shown that CD163 and CD91 are coexpressed and coregulated on human monocytes, and we have identified two subsets of $CD163^{+}$ DC that constitute a fraction of the recently described $CD91^{+}CD11c^{+}$ DC subset. However, it remains to be shown whether CD163 has a direct immunostimulatory role by binding of foreign antigen as has been demonstrated for the α_2M-CD91 pathway.

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