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Human CD300 receptors expression, regulation and function in the immune system. Implication in human immunodeficiency virus type 1 infection

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ABBREVIATIONS

ADCC: antibody dependent cellular citotoxicity

AIDS: acquired immunodeficiency syndrome

ALL: acute lymphoblastic leukemia

AML: acute myeloid leukemia

ART: antiretroviral therapy

BAFF: B cell activation factor

BATF: basic leucine transcription factor ATF-like

BCR: B cell receptor

BMMCs: bone marrow-derived mast cells

bnAbs: broadly neutralizing antibodies

CD4+CD45RA-: CD4+RA-

CLP: cecal ligation and puncture

CML: CMRF-35-like molecules

CMV: cytomegalovirus

DAP: DNAX-activating protein

DC: dendritic cells

DENV: dengue virus

DLBCL: diffuse large B cell lymphoma

DSS: dextran sodium sulfate

FcR: Fc repector

GM-CSF: granulocyte-macrophage colony-stimulating factor

Grb2: growth factor receptor-bound protein 2

HIV: human immunodeficiency virus

IFN: interferon

IL: interleukin

ITAMs: immunoreceptor tyrosine-based activating motifs

ITIMs: immunoreceptor tyrosine-based inhibitory motifs

KIRs: killer cell immunoglobulin-like receptors

LMIR: leukocyte mono-Ig-like receptors

LPS: lipopolysaccharide

mAbs: monoclonal antibodies

MAIR: myeloid associated Ig-like receptors

mDC: myeloid dendritic cells

MNV: murine norovirus

MRD: minimal residual disease

NCRs: natural cytotoxicity receptors

NK: natural killer

PC: phosphatidylcholine

pDC: plasmacytoid dendritic cells

PE: phosphatidylethanolamine

PI3K: phosphatidylinositol 3-kinases

PRV: porcine pseudorabies virus

PS: phosphatidylserine

sCD300f: soluble CD300f

SCF: stem cell factor

SHIP: SH2-containing inositol phosphatase

SHP: src homology 2 domain-containin phosphatase

siRNA: small interference RNA

TCR: T cell receptor

TGF: transforming grow factor

TIM: T cell immunoglobulin mucin

TLR: toll-like receptor

TNF: tumor necrosis factor

Treg: regulatory T

WHO: World Health Organization

WT: wild type

1. INTRODUCTION

1.1. CD300 receptor family

The CD300 molecules are receptors mainly expressed on the surface of human and mouse immune cells. In the last years, CD300 receptors have gained importance due to their key role in multiple immune processes, highlighting their potential as targets in order to modulate immune responses. For that reason, the following doctoral thesis has been focused on this family of receptors for a better understanding of how the immune system is regulated in normal conditions and during human immunodeficiency virus (HIV)-1 infection and thus, to contribute to the search of new biomarkers or therapeutic targets.

1.1.1. Structure and mechanisms of signaling

CD300 receptors are type I transmembrane proteins formed by an immunoglobulin (Ig)V-like extracellular domain and a cytoplasmic tail, which could be short or long depending on their signaling capacity. In humans, the CD300 multigene family consists of eight members, which are named alphabetically according to their location on chromosome 17 (Figure 1A). Human CD300 family is divided in two groups: activating receptors (i.e., CD300b, CD300c, CD300d, CD300e and CD300h) and inhibitory receptors (i.e., CD300a and CD300f). Activating receptors have a short cytoplasmic tail with a charged transmembrane residue that facilitates the association with adaptor proteins containing immunoreceptor tyrosine-based activating motifs (ITAMs) such as DNAX-activating protein (DAP)12 and Fc receptor (FcR)γ chain, or phosphatidylinositol 3-kinases (PI3K) binding motifs (YxxM) such as DAP10. By contrast, the inhibitory receptors have a long cytoplasmic tail including immunoreceptor tyrosine-based inhibitory motifs (ITIMs)[1–3]. Tyrosine phosphorylation of the ITIMs is required for the transmission of inhibitory signals. It has been shown that the Src lymphocyte-specific protein tyrosine kinase (lck) is responsible for the phosphorylation of CD300a ITIMs in Jurkat T cells. These phosphorylated ITIMs recruit different phosphatases including src homology 2 domain-containing phosphatase (SHP)-1 and SHP-2 to the phosphorylated intracellular tails of CD300a and CD300f receptors and

SH2-containing inositol phosphatase (SHIP) only to CD300a. Depending on the receptor or cell type, the importance of each phosphatase in the transmission of inhibitory signal is different. For example, in DT40 B cells, SHP-1 deficiency largely abolished CD300a-mediated signal, but not SHP-2 and SHIP, whereas the pharmacological inhibition of both SHP-1 and SHP-2 in the monocytic cell line THP-1 blocked CD300f-mediated signals[4–10]. Although CD300f has been classically considered as an inhibitory receptor, it is also able to transmit activating signals through PI3K-binding motifs and growth factor receptor-bound protein 2 (Grb2)[11,12]. Exceptionally, in addition to its IgV-like domain, CD300g molecule also contains an extracellular mucin-like domain and lacks stimulatory or inhibitory motifs in the intracellular tail[13].

The mouse counterparts of human CD300 receptors are encoded by 9 genes located on chromosome 11, the synthenic region of human chromosome 17 (Figure 1B). The nine members of murine CD300 receptor family are also known as CMRF-35-like molecules (CLM), leukocyte mono-Ig-like receptors (LMIR) and myeloid associated Ig-like receptors (MAIR) or CD300-like (e.g. CD300lf), and with the exception of CD300a and CD300f, they are not perfect functional orthologs of the human receptors[1,2,14].

It has been shown that CD300 molecules have the capacity to associate with other receptors. CD300b is able to regulate lipopolysaccharide (LPS)-induced responses on myeloid cells by forming a complex with toll-like receptor (TLR)4. Moreover, mouse CD300f has the capacity to amplify interleukin (IL)-4-induced responses through its association with IL-4 receptor α (Figure 1C). In addition, CD300 family members can interact with each other to form homo and heterodimers. This interaction depends on their extracellular domains and adds a new layer of complexity to the signaling pathways of CD300 receptors (Figure 1D)[3,15–19].

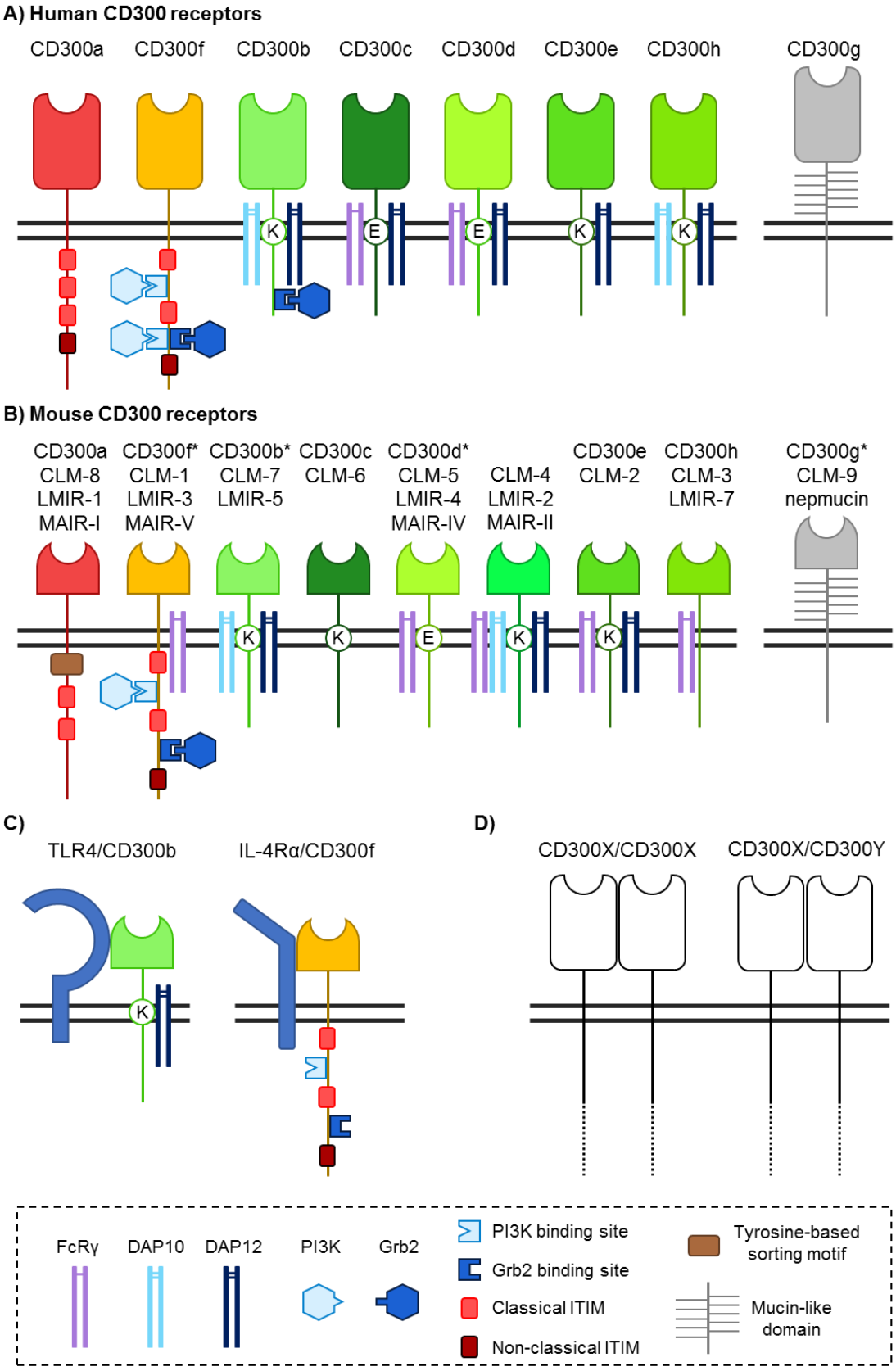


Figure 1. Human and mouse CD300 receptor family members and complexes. The CD300 receptors in humans (A) and mice (B) are differently represented in terms of the shape of the extracellular domain. The receptors with an activating function are shown in green, while the ones with an inhibitory capacity are indicated in red. CD300f, displaying dual function, is

colored in orange and CD300g in gray. The motifs and binding sites from the intracellular tail, and the interaction with adaptor proteins (ITAM-bearing FcRγ and DAP12, DAP10 containing PI3K-binding motif and Grb2) and kinases (PI3K) are also indicated. Mouse CD300 molecules marked with an asterisk (*) are also named as CD300lX (e.g. CD300lf or CD300lb). This figure also shows the capacity of CD300 receptors to form complexes with other molecules, such as TLR4 and IL-4Rα (C), and their ability to interact with each other forming homo- and hetero-dimers (D).Vitallé J *et al., Eur. J. Immunol.* 2019;49:364–374 (Annex 1).

1.1.2. Ligand recognition

It has been described that CD300 molecules are able to bind different ligands (Table 1). Various CD300 receptors are known to bind lipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE)[20–25]. These aminophospholipids are localized in the inner leaflet of the plasma membrane in resting cells, while they are translocated to the outer leaflet when cells undergo apoptosis, are activated, transformed or infected[26–28]. Both CD300a and CD300c receptors recognize PS and PE, albeit with different affinity. Specifically, human CD300a binds PE with higher affinity, while mouse CD300a preferentially binds PS. On the other hand, human CD300c recognize both phospholipids with a similar affinity and its binding to PS is also similar to the one of human CD300a[20–23,29]. Other CD300 molecules known to bind PS include mouse CD300b and CD300f[24,25], although other ligands have been described for these receptors. In fact, other works have demonstrated that human CD300b binds LPS, while mouse CD300b binds LPS, PE, phosphatidylcholine (PC), 3-O-sulfo-β-D-galactosylceramide C24:1 and phytosphingosine[18,30,31]. Regarding CD300f, the human receptor recognizes ceramide and sphingomyelin, while the murine receptor binds PC, ceramide, sphingosylphosphocholine, high-density lipoprotein and low-density lipoprotein[32,33]. Moreover, human and mouse CD300e have also been demonstrated to recognize sphingomyelin[34].

In addition, CD300 molecules are also known to bind non-lipid ligands (Table 1). For example, it has been described that mouse CD300b recognize cholera toxin B subunit, pertussis toxin and T cell immunoglobulin mucin (TIM)-1 and TIM-4 receptors[35–37]. Moreover, pertussis toxin can be also recognized by mouse CD300c[36]. Finally, several publications have demonstrated the ability of CD300 molecules to bind certain viruses through their interaction with lipids or proteins found in those viruses, such as E3/49K protein from adenoviruses[38](see below).

Table 1. Ligands of human and mouse CD300 receptors. Adapted from Vitallé J 2019 (Annex 1).

Specie	**CD300**	**Ligands**
Human	CD300a	PS, PE
	CD300c	PS, PE
	CD300b	LPS
	CD300f	Ceramide, SPH
	CD300e	SPH
Mouse	CD300a	PS, PE
	CD300c	PT
	CD300b	LPS, PS, PE, PC, TIM-1, TIM-4, PT, CT-B, C24:1, phytosphingosine[31]
	CD300f	PS, PC, Ceramide, SPC, HDL, LDL. Norovirus
	CD300d	Norovirus
	CD300e	SPH

PS: phosphatidylserine; **PE**: phosphatidylethanolamine; **PC**: phosphatidylcholine; **LPS**: lipopolysaccharide; **SPH**: sphingomyelin; **SPC**: sphingosylphosphocholine; **HDL**: high-density lipoprotein; **LDL**: low-density lipoprotein; **TIM**: T cell immunoglobulin mucin domain; **PT**: pertussis toxin; **CT-B**: cholera toxin B subunit; **C24:1**: 3-O-sulfo-β-D-galactosylceramide C24:1.

In this introduction, we will focus on four CD300 molecules: two ITIM-bearing receptors, which have been classified as inhibitory receptors (CD300a and CD300f) and two non ITIM-bearing receptors, which display an activating function (CD300c and CD300e). These receptors are the ones that have been studied during this doctoral thesis, with a special emphasis in the CD300a inhibitory molecule.

1.1.3. Expression and function in immune system.

1.1.3.1. ITIM-bearing receptors

Human **CD300a** is found in immune cells from both myeloid and lymphoid lineages. It is detected on the surface of natural killer (NK) cells and in T and B lymphocytes[1,2,20,39–43]. Regarding the myeloid lineage, CD300a is expressed on the surface of dendritic cells (DC), monocytes, macrophages, neutrophils, eosinophils, basophils and mast cells [1,2,21,22,44–50]. Transcripts encoding mouse CD300a are also found in both myeloid and lymphoid cells. It is expressed on macrophages, mast cells, DCs and granulocytes. On lymphocytes, mouse CD300a is found on some B cell subpopulations, but is not detected on the surface of resting NK and T cells[6,51–53].

NK cells were one of the first cell types where CD300a was described[54]. Human CD300a molecule is found in all human NK cells[20,54,55], whereas in mice, CD300a expression was detected only in IL-12 stimulated NK cells[52]. The capacity of CD300a to transmit inhibitory signals was described in NK cells for the first time. Previous studies have reported that CD300a diminished NK cell killing activity after its engagement by agonist mAbs and when NK cells were confronted to PS-expressing tumor cells[56]. Another lymphocyte subset displaying CD300a receptor on the cell surface is T cells. Human CD300a receptor is known to be differentially expressed among CD4+ and CD8+ T cell subpopulations. Indeed, naïve CD4+ T cells display low expression levels of CD300a, while effector/memory CD4+ T cells can be divided into CD300a+ and CD300a- subsets and regulatory T (Treg) cells are negative for the receptor[39,41]. Regarding CD4+ T cells, CD300a expression has been related to higher proliferation and polyfunctional capacity[39–41], while in human CD8+ T cells its expression is coupled to a more cytotoxic molecular signature, which is characteristic of effector and memory CD8+ T cells but not naïve cells[43]. Cell surface expression of CD300a is modulated by several stimuli. For instance, stimulation with anti-CD3 plus anti-CD28 monoclonal antibodies (mAbs) and culture conditions for differentiation into Th1 phenotype increases CD300a expression, whereas transforming grow factor (TGF)-β diminishes it[39,41]. Moreover, CD300a is able to regulate CD4+ T cell function. In fact, crosslinking of CD300a with mAbs decreases T cell receptor (TCR)-mediated signaling[4,40]. The differential expression of CD300a was also observed among human B cell subsets: while naïve cells express low levels of CD300a, memory B cells and plasma cells show variable levels and germinal center B cells are CD300a-. In memory

B cells, CD300a expression is known to be up-regulated after TLR9 stimulation. In contrast, IL-4 and TGFβ are negative regulators of CD300a expression. In addition, the inhibitory function of CD300a was also demonstrated in B lymphocytes. The crosslinking of CD300a with mAbs induced a decrease in B cell receptor (BCR)-mediated signaling and the down-regulation of CD300a expression by means of small interference RNA (siRNA) increased the proliferation rate in response to the BCR stimulation[42].

Regarding the myeloid lineage, CD300a molecule is found in several cell types, including monocytes, macrophages and DCs. The expression of CD300a receptor on these cell types is modulated by different stimuli. For instance, LPS, interferon (IFN)γ and hypoxia upregulate CD300a expression on monocytes[57–59], but IFNα production by plasmacytoid DCs (pDCs) after TLR7 and TLR9 stimulation downregulates its expression[60]. As in lymphocytes, CD300a molecule has the capacity to modulate multiple functions of myeloid cells. In fact, the mAb-mediated crosslinking of CD300a reduces IL-8 secretion by myelomonocytic cell lines in response to LPS and the TLR9 agonist CpG, downregulates the uptake of apoptotic cells by macrophages and modulates type I IFN and tumor necrosis factor (TNF) secretion by pDCs after TLR7 and TLR9 stimulation[10,60]. CD300a is also detected on the surface of basophils, mast cells and eosinophils, three relevant cell types in the pathogenesis of allergic diseases[61–63]. It has been reported that CD300a expression is downregulated by eosinophil-derived major basic protein and eosinophil-derived neurotoxin in cord blood-derived mast cells[46]. Moreover, the expression of the receptor is upregulated by hypoxia and granulocyte-macrophage colony-stimulating factor (GM-CSF) in human peripheral blood eosinophils[50]. Importantly, CD300a receptor suppresses eosinophil survival, migration and inflammatory mediators production triggered by eotaxin, IL-5 and GM-CSF[49]. Additionally, CD300a is able to decrease mast cell activation, differentiation and survival, and to inhibit IgE-mediated mast cell and basophil degranulation[44,46,48,52,64,65]. As a matter of fact, PS-containing liposomes inhibit IgE-induced basophil degranulation in a CD300a-dependent manner[66]. Interestingly, CD300a is also known to interact with externalized PS in cis during mast cell degranulation regulating IgE-mediated degranulation in an autonomous manner[67]. Lastly, CD300a is also present in neutrophils. In this cell type, LPS and GM-CSF stimulation causes a rapid translocation of a pre-existing intracellular pool of CD300a, explaining the fast increased expression

in response to those stimuli. Furthermore, crosslinking of CD300a by specific mAb also inhibited neutrophil activity. Specifically, it reduces the production of FcgRIIa-triggered reactive oxygen species and Ca^{2+} mobilization[45].

CD300f is a receptor with the capacity to transmit both inhibitory and activating signals. Human CD300f is found on the surface of myeloid cells including monocytes, DCs, granulocytes, monocyte-derived DCs and basophils[11,68–70]. Mouse CD300f transcripts however, are found in myeloid cells but also in B cells[6,71]. Although CD300f is not expressed on T lymphocytes, it has the ability to modulate their function. For example, it has been shown that mouse CD300f negatively regulates DC-induced T cell proliferation in vitro and DC-initiated antigen-specific T cell responses both in vivo and in vitro. Furthermore, it inhibits antigen-specific Th1 and cytotoxic T cell responses in vivo[71].

Also, it has been described that CD300f has the capacity to modulate multiple immune processes by transmitting inhibitory or activating signals in several myeloid cells. On THP-1 cells, a monocytic cell line, CD300f inhibits TLR2, TLR3, TLR4, TLR9 and B cell activation factor (BAFF)-mediated[8–10]. In addition, other findings reported the implication of the receptor in Wallerian degeneration and nerve regeneration by regulating the influx and phenotype of macrophages[72]. The role of CD300f in macrophage functions was also demonstrated by Moshkovits et al. in a study describing CD300f as an IL-4-induced molecule in macrophages. It is remarkable that IL-4- and aeroallergen-treated CD300f knockout mice displayed reduced IgE production, chemokine production and inflammatory cell recruitment[19]. Regarding mast cells, crosslinking of CD300f is known to enhance IL-6 production by bone marrow-derived mast cells (BMMCs) after the stimulation with LPS[7]. In contrast, the interaction between CD300f with its ligands sphingomyelin and ceramide inhibits FcεRI-mediated activation of mast cells[33] and mAb-mediated CD300f crosslinking reduces FcεRI-mediated degranulation in the rat basophilic leukemia (RBL) RBL-2H3[12].

1.1.3.2. Non-ITIM-bearing receptors

As human CD300a, transcripts encoding **CD300c** are found in the majority of leukocytes[1]. In the lymphoid lineage, human CD300c is expressed on NK cells, only on the $CD56^{Bright}$ subset. Stimulation with IL-2 and IL-15 induces the overexpression of

CD300c exclusively on this $CD56^{Bright}$ NK cell subset, while IL-4 inhibits its expression. Furthermore, the receptor is able to enhance the degranulation and cytokine and chemokine production by $CD56^{Bright}$ NK cells through the crosslinking with specific mAb[20]. It has been described that CD300c molecule displays some homology in its amino acid sequence with B7 family members, and even if CD300c is known to be absent in T lymphocytes, an unidentified ligand, independent of PS and PE, is expressed on both CD4+ and CD8+ T cells. Following this, Cui et al have shown that a soluble CD300c-IgG2a Fc fusion protein inhibits the proliferation, activation and cytokine production by CD4+ and CD8+ T cells in vitro, and its administration reduces graft versus host disease in vivo in mouse model[73].

CD300c receptor is also expressed in the myeloid lineage, including monocytes, macrophages, DCs, mast cells and basophils[1,70]. Previous studies have reported that the expression of CD300c is modulated by several TLR ligands such as LPS and flagellin in monocytes[74], or CpG in pDCs[60]. Its expression is also induced, by the treatment with IL-3, in human basophils[70]. The capacity of CD300c to transmit activating signals has been previously demonstrated in various cell types. In monocytes, the crosslinking of the receptor with a specific mAb induces Ca^{2+} mobilization and up-regulation of the costimulatory molecule CD86, and also increases LPS-independent and LPS-mediated production of inflammatory cytokines[74]. Moreover, CD300c crosslinking also causes cytokine and chemokine production of both monocytes and mast cells, and generates activating signals in RBL-2H3 cells, having the FcRγ a key role in these processes[17,22]. Lastly, we have recently described that co-activation trough CD300c increases IgE-mediated degranulation, cytokine production, intracellular Ca^{2+} mobilization and phosphorylation of signaling intermediates in human basophils, which demonstrates the costimulatory role of CD300c during FcεRI-dependent basophil activation[70].

CD300e is found on the cell surface of monocytes and myeloid DCs (mDCs) in vivo, and is expressed at low levels in monocyte-derived macrophages and DCs in vitro[75–77]. mAb-mediated CD300e activation is known to induce several functions in monocytes and DCs, such as Ca^{2+} mobilization, release of reactive oxygen species, expression of activation markers and proinflammatory cytokine production. The crosslinking of CD300e also promotes survival in those cell types. Moreover, CD300e-mediated activation promotes the capacity of mDCs to stimulate allogeneic naïve T cells[75,76]. Lastly, murine CD300e has been recently described to be also an immune activating

receptor. Murine CD300e engagement induces cytokine production in BMMCs. Notably, the binding of sphingomyelin to mouse CD300e regulates the function of non-classical and intermediate monocytes and BMMCs, in a FcRγ and DAP12-dependent manner[34].

1.2. Disease relevance

The importance of CD300 molecules in several pathological conditions has been highlighted by multiple studies, describing the role of this family of receptors in allergic disorders (asthma, allergic airway inflammation, allergic rhinitis, food allergies, etc.), autoimmune and inflammatory diseases (psoriasis, colitis, diabetes, multiple sclerosis, etc.), cancer (acute lymphoblastic leukemia, acute myeloid leukemia, B cell lymphoma, lung cancer) and infections caused by different bacteria and viruses.

1.2.1. Allergies

Mast cells, eosinophils and basophils have an essential role in the initiation, regulation and effector phases of allergic responses[61–63]. **CD300a** inhibitory receptor is expressed in all these cell types and it is known to have a relevant role in immune processes during allergies[1,2,70,78]. One example of the important role of CD300a in allergic inflammation and its resolution was described in a CD300a$^{-/-}$ mouse model of allergic peritonitis. CD300a-deficient mice displayed a rapid augment in inflammatory cells infiltrates and tryptase content in the peritoneal cavity, and their resolution process was delayed, in comparison with wild type (WT) mice[79]. In humans, a decreased basal expression of CD300a was observed on basophils from birch pollen allergic patients in comparison with the ones from healthy donors[44]. Remarkably, Sabato V. et al. have demonstrated that basophil anaphylactic degranulation is suppressed after interaction with PS exposed on apoptotic cells via the CD300a molecule[66]. The expression of CD300a in cell types involved in allergies and its inhibitory capacity had led to the design of bispecific Ab fragments targeting CD300a, in order to downregulate the function of these cells during allergic diseases in mouse models. For example, bispecific Ab fragments for CD300a and c-Kit abolish stem cell factor (SCF)-induced mast cell degranulation[64], and other

bispecific Ab targeting CD300a and IgE abrogate allergic and inflammatory responses in acute experimental asthma and IgE-dependent passive cutaneous anaphylaxis[65]. Lastly, an upregulation of CD300a was observed in inflamed atopic dermatitis biopsies from lesional skin, being this overexpression feasibly regulated by mediators secreted by skin infiltrating inflammatory cells as eosinophils or mast cells, by Th2 and Th7 cytokines, and/or hypoxic milieu[80].

CD300f is another member of the studied receptor family that is known to be involved in allergic processes. IL-4- and aeroallergen-treated mice lacking CD300f exhibited less IgE production, chemokine expression and inflammatory cell recruitment than WT mice[19]. Moreover, elevated CD300f levels in eosinophils from patients with allergic rhinitis have been previously observed[19,81]. Importantly, the absence of CD300f in mice does not affect mast cell development. However, it led to exacerbated allergic responses by mast cells, including increased FcεRI-mediated anaphylaxis, passive cutaneous anaphylaxis, mast cell-dependent airway inflammation and atopic dermatitis[32]. Furthermore, it has been recently reported that in mouse models of OVA-induced food allergy, the interaction of CD300f with ceramide inhibited IgE-mediated activation of intestinal mast cells and that this inhibition of mast cell activation is induced by the skewing of Th2/Treg balance toward Th2[82]. Another recent study published by Izawa K et al showed, in a mouse model, that mast cells with a single amino acid substitution (D117A) in the IgV-like extracellular domain of CD300f failed to suppress passive cutaneous anaphylaxis responses. This is due to the importance of the residue D117 in the interaction between murine CD300f and its ligands, which modulates IgE-mediated mast cell activation[83].

Last but not least, our group has recently described that **CD300c** receptor has the ability to co-stimulate IgE-mediated basophil activation. Importantly, the potential use of CD300c as a biomarker for the diagnosis of allergic patients was exposed since an overexpression of CD300c on basophils was observed in cow's milk allergic children and the intensity of the expression was associated to the severity of the hypersensitivity symptoms[70]. Additionally, our group also demonstrated that surface CD300c expression was up-regulated in basophils from allergic individuals to dust mites and grass pollen, two IgE-dependent allergies[78]. Altogether, these studies suggested that the measurement of CD300c expression could help in the diagnosis of IgE-dependent allergies and that a

higher CD300c expression levels diminish the IgE-dependent activation threshold of basophils in allergic patients.

1.2.2. Autoimmune diseases and chronic inflammation

Alteration and impairment of **CD300a** expression has been also associated to the development and progression of different autoimmune and chronic inflammatory diseases[2]. First, a nonsynonymous polymorphism in the extracellular domain of CD300a was related to a higher susceptibility to psoriasis[84] and a lower CD300a expression was observed on CD4+ T cells from psoriatic patients than in healthy donors[39]. Interestingly, the mentioned CD300a nonsynonymous polymorphism exhibited lower binding ability to its ligands PS and PE[21]. On the other hand, mice with a high-fat diet lacking CD300a develop chronic intestinal inflammation[85], and in a model of Ag-induced arthritis, the absence of CD300a increases joint inflammation by regulating the activity of cell types involved in this process such as neutrophils and tissue resident macrophages[86].

Regarding **CD300e** activating receptor little is known about its implication in different diseases. Nevertheless, it has been described a higher titer of anti-CD300e Ab in patients with fulminant type 1 diabetes in acute phase in comparison with the titers of patients with autoimmune type 1 diabetes, type 2 diabetes, autoimmune thyroid disease and healthy controls[87].

Several publications have described the role of **CD300f** in autoimmune diseases and chronic inflammation conditions in mouse models, mainly by reporting the consequences of CD300f deficiency in this kind of pathologies. Firstly, in a model of multiple sclerosis, the treatment with CD300f-Fc fusion protein or the lack of CD300f led to a more severe disease, characterized by an increased release of inflammatory mediators and demyelination[88]. In this line, a missense mutation was found in CD300f in a group of patients with multiple sclerosis and the expression of the receptor was barely detectable on monocytes from these patients[16]. In addition, CD300f-deficient mice displayed a higher predisposition to the development of autoimmune diseases such as systemic lupus erythematosus-like disease than WT mice[89]. CD300f deficiency has also been described to exacerbate dextran sodium sulfate (DSS)-induced colonic

inflammation. This effect was a consequence of defects in the regulation of mast cell activation and DC function associated to an abnormal accumulation of apoptotic cells in the gut[90,91]. It is remarkable that the disruption of ceramide-CD300f interaction aggravated the DSS-mediated colitis, whereas the disease was suppressed after the treatment with ceramide liposomes[90]. On the contrary, Moshkovits et al. reported an attenuated DSS-treated active ulcerative colitis in CD300f$^{-/-}$ mice, which was accompanied with decreased inflammatory cell infiltration and pro-inflammatory cytokine production by eosinophils[92]. The reason for the apparently opposite results from these studies on DSS-mediated colitis is not known. It has been also demonstrated the importance of CD300f in the maintenance of immune homeostasis promoting self-antigens clearance by macrophages and inhibiting DC uptake and self-antigen presentation[89,93]. In fact, CD300f deficiency exacerbated efferocytosis by DCs and promoted susceptibility to develop autoimmune diseases in mice in a mechanism involving enhanced antigen processing and T cell priming by CD300f-/- DCs[93]. On the other hand, because of the impaired phagocytosis of apoptotic cells by macrophages from CD300f-/- mice, they are also predisposed to the development of autoimmune disease[89]. Lastly, other effect of the absence of CD300f is the enhancement of LPS-induced skin inflammation, including an augment in skin edema and neutrophil recruitment in mice. Importantly, the interaction between CD300f with its ligand ceramide suppressed chemical mediators release by mast cells and neutrophils in response to LPS. Thus, the administration of ceramide-containing vesicles inhibits LPS-induced skin inflammation in mice[94].

1.2.3. Cancer

Several studies have revealed the potential use of **CD300a** receptor as a new therapeutic target in cancer[2]. From one part, because CD300a is differentially expressed in acute lymphoblastic leukaemia (ALL) compared with normal B cells progenitors, it has been proposed, along with other molecules, as a marker for the detection of minimal residual disease (MRD) in ALL[95]. Very recently, it has also been proposed as a marker for monitoring of MRD in acute myeloid leukemia (AML)[96]. It has been also described that pre-B cell-derived ALL displays high levels of the receptor and that its overexpression at the time of diagnosis is associated with a shorter overall and relapse-free survival[97]. Also, it has been demonstrated that the interaction of CD300a with its ligand PS on

tumor cells decreases NK cell-mediated tumor cell killing[56]. Furthermore, recent findings have shown correlations between alterations on CD300a expression with the development of different cancers such as diffuse large B-cell lymphoma (DLBCL), AML and non-small-cell lung cancer. A decreased CD300a expression promoted apoptosis and inhibited proliferation of tumor cells in the three mentioned cancers[98,99]. Remarkably, utilizing a xenograft animal model, Jiang L. et al. showed that the knocked down of the CD300a expression levels in DLBCL cell lines inhibited tumor formation in vivo[98].

Apart from CD300a, **CD300f** has also been described as another receptor with a potential utility in tumor immunotherapy. As a matter of fact, in a mouse model of cancer, the downregulation of CD300f in DCs by silencing with specific siRNA enhances their antitumor effect[71]. In addition, the use of antihuman CD300f Abs induces complement- and antibody-dependent cytotoxicity, causing a delay in tumor growth in an AML xenograft model and significantly reducing the engraftment of primary human AML cells[68]. Lastly, a recent work proposed CD300f as a new target for antibody-based therapies against AML with monocytic differentiation. The authors distinguished different CD300f extracellular isoforms including alternatively spliced variants of the exon 4-encoded sequence, which were differentially expressed on AML cells and exhibited a different CD300f-specific antibodies binding pattern[100].

1.2.4. Infections

The **CD300a** receptor has been also related to bacterial infections and sepsis[2]. In a mouse model of cecal ligation and puncture (CLP) peritonitis, CD300a-deficient peritoneal mast cells displayed a higher production of chemoattractants, inducing neutrophil recruitment and a more effective bacterial clearance. In consequence, the absence of CD300a promotes prolonged survival in mice after CLP peritonitis[101,102]. In addition, **CD300f** molecule has also been described to promote disease progression in sepsis. CD300f-deficient mice with a CLP septic peritonitis were protected from death, since the absence of CD300f also enhances neutrophil accumulation by increasing chemoattractant production and consequently, promotes the efficient elimination of *Escherichia coli*[103]. Importantly, the disruption of the interaction between CD300a and CD300f with its ligands PS and ceramide, respectively, prolonged survival after CLP in

mice[23,103]. In line with this findings, Maehara et al. have recently reported that the blockade of CD300f-ceramide interaction leads to recovery from Gram-negative bacterial skin infections, also through the stimulation of neutrophil accumulation and hence, resulting in an efficient clearance of the bacteria[104]. In addition to bacterial infections, CD300f has been also related to other infections as cerebral malaria. Actually, it has been recently described that microglia cells from the brain of B6.WLA-Berr2 mice, that displayed high resistance to cerebral malaria, showed a higher CD300f expression comparing with susceptible mice[105].

1.3. CD300 receptors and viral infections

Over the last years, accumulating evidence have demonstrated the relevant role of CD300 molecules in several viral infections including human immunodeficiency virus (HIV), Dengue virus (DENV), murine norovirus (MNV) and porcine pseudorabies virus (PRV), among others. Some viral infections are known to alter CD300 expression and induce immune responses that could be somehow regulated by CD300 receptors. Additionally, some members of the receptor family are involved in viral mechanisms to bind and enter host cells. The role of CD300 molecules during viral infections is described in a review recently published by our group[3].

1.3.1. CD300 expression and function in immune responses during viral infections

Even though little is known, recent publications have reported the modulation of CD300 expression during viral infections. For example, a profound downregulation of CD300b gene expression has been observed in cells infected with rabies virus[106]. Regarding T cells, the frequency of CD300a+ cells increased in people seropositive for cytomegalovirus (CMV)[107]. In addition to T lymphocytes, CD300a was also overexpressed on NK cells, but in this case, both CMV infection and age, induced significant changes in the expression of the receptor[108]. Another virus that alters CD300a expression is the HIV. It is known that the expression of CD300a inhibitory receptor was significantly downregulated on B cells from HIV-1 infected patients,

indicating that CD300a might be involved in the hyperactivation and dysfunction of B cells characteristic of HIV infection[42].

As it has been described above, CD300 receptors have the capacity to regulate multiple immune responses in both normal and pathological conditions. One of the strategies of viruses to evade immune system attack is to promote the binding between inhibitory receptors found on different immune cells and their ligands exposed on infected cells, leading to the downregulation of antiviral responses[109]. In this line, Grawet K. et al. have described that CD300a protects the alpha-herpesvirus PRV from NK cell-mediated killing. In fact, they described that PRV induces the exposure of PS and PE on infected cells, promoting the binding of CD300a found on NK cells with these phospholipids from infected cells, leading to the inhibition of NK cell-mediated cytotoxicity (Figure 2)[110]. CD300a expression has been also related to the exhaustion of HIV-specific CD8+ T lymphocytes. T cell exhaustion is characterized by a reversible decrease of effector functions and proliferative capacity, caused by a persistent antigen exposure, for instance, in chronic HIV-1 infection[111–113]. The inhibitory receptor PD-1 has been identified as a major regulator of T cell exhaustion during chronic HIV infection and it is known to induce the expression of basic leucine transcription factor ATF-like (BATF)[112,114,115]. Interestingly, a positive correlation between CD300a mRNA levels and BATF levels was observed on HIV-specific CD8+ T lymphocytes[114], suggesting that this receptor might be involved in T cell exhaustion in the context of HIV infection.

1.3.2. CD300 molecules in viral binding and entry

One of the strategies that viruses utilize in order to bind and infect host cells is the so-called viral apoptotic mimicry. This process consists in hijacking cell's apoptotic recognition and clearance mechanisms for their own means[116]. When cells enter in apoptosis, the asymmetry of the plasma membrane is lost, resulting in the translocation of PS and PE to the outer leaflet of the cell membrane[26–28]. Enveloped viruses such as DENV deploy apoptotic mimicry by enclosing themselves in a lipid bilayer obtained from host cells and concentrating PS and PE in their envelope, thereby mimicking apoptotic cells [116,117]. Thus, viruses containing PS in their envelope interact with PS-binding receptors from host cell surface, such as TIM-1, -3 and -4, promoting viral binding and entry [118–121].

CD300a is other surface receptor known to directly recognize PS and PE, and has been proposed to be involved in viral entry mechanisms (Figure 2). For instance, pseudotyped lentiviral vectors with envelopes from Sindibis virus, Ross River virus, baculovirus and vesicular stomatitis-G virus, bound human CD300a expressed on 293T cells. Unlike TIM-1 and -4, CD300a did not increase viral transduction[119,120]. In contrast, it has been also reported that CD300a not only bind viral particles, but also promote the infection of DENV, Yellow Fever, West Nile and Chikungunya viruses[122]. Concerning DENV, Carnec X. et al. discovered that CD300a-mediated viral entry was dependent of clathrin and independent of the cytoplasmic tail of CD300a. They also demonstrated that the binding of DENV particles to CD300a from host cells depended on PS and mainly PE. Remarkably, CD300a blockade with specific Abs in monocyte-derived macrophages induced a decrease in the number of DENV infected cells. However, other CD300a expressing cell types as monocytes and mast cells were insensitive to the infection[122]. Thus, CD300a acts as an attachment factor of DENV and other mosquito-borne viruses, although it may induce viral internalization only in some cell types. In conclusion, CD300a has an important role in the binding of viruses that utilize apoptotic mimicry as a mechanism to enter host cells.

In addition to PS- and PE-dependent enhancement of viral infection, CD300 molecules have been reported to facilitate the entry of viruses in a lipid-independent manner (Figure 2). In a large-scale protein interaction screen, an interaction of human adenovirus-D47 E3/49K protein with CD300a and CD300c receptors was identified[38]. Furthermore, two groups recently identified mouse CD300f as a crucial receptor for MNV. The authors reported that depletion or blockade of CD300f caused resistance to MNV infection. They also showed that anti-CD300f polyclonal Abs inhibited MNV-induced cytopathic effects and MNV progeny. Importantly, after the pretreatment with soluble CD300f (sCD300f), mice challenged with MNV displayed a superior survival and CD300f$^{-/-}$ mice were resistant to MNV infection. Moreover, mouse CD300f receptor determines MNV host tropism, since cells from different species were only infected when they ectopically expressed the murine, but not human CD300f. As a matter of fact, structural and mutational experiments have identified the sites of the IgV-like extracellular domain of mouse CD300f that were important for viral binding and entry and the sites that conferred species specificity[123,124]. Additionally, serine palmitoyltransferase complex, which is required for sphingolipid biosynthesis, has been

described as an important factor in MNV binding and entry. Interestingly, serine palmitoyltransferase activity is crucial for an adequate conformation of CD300f, which allows viral binding[125]. Recently, it was also discovered that sCD300f binds to the topside of the MNV protruding domain and that bile acids act as cofactors enhancing viral infection[126,127]. Actually, Nelson et al described using a cryo-EM-derived model of MNV, that virions have the capacity to make multiple interactions with CD300f, including with bile acids[126].

Lastly, murine CD300d was also described as another member of the receptor family involved in viral entry mechanisms. Similar to CD300f, mouse CD300d has the ability to promote MNV infection in vitro. Nevertheless, it does not show a critical role in vivo[123,124,127]. Interestingly, surface plasmon resonance analyses revealed that mouse CD300d and CD300h and human CD300f also binds to the protruding domain of MNV, but murine sCD300f was the only one that displayed a strong binding[126]. Regarding CD300h, although it has been proposed as a possible MNV receptor, its expression on host cells did not show any effect on MNV infection[124].

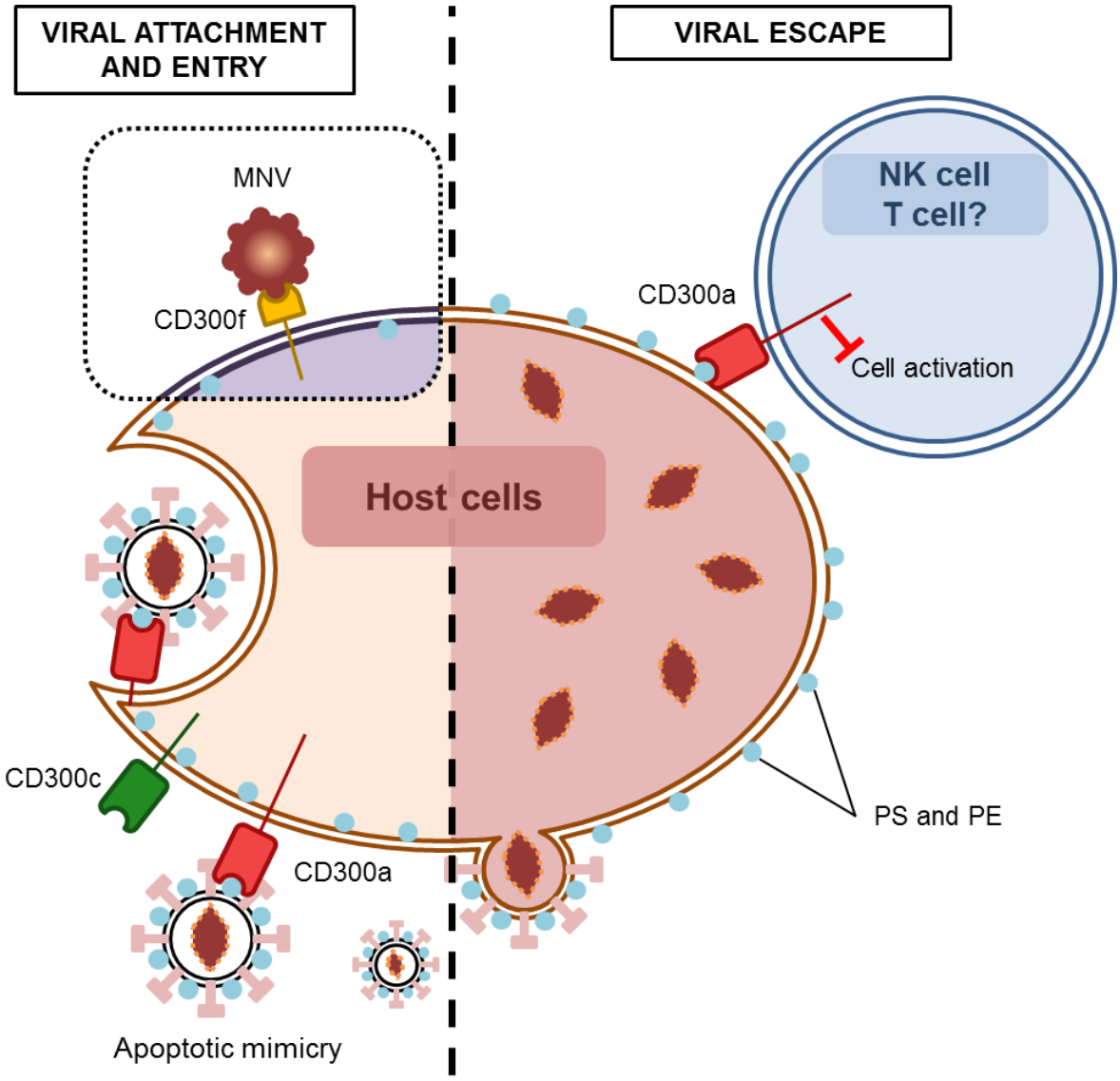


Figure 2. CD300 molecules in viral attachment, entry, and escape. When a host cell is infected by certain viruses, the asymmetrical distribution of phospholipids in the plasma membrane is lost, causing the externalization of phosphatidylserine (PS) and phosphatidylethanolamine (PE). The CD300a inhibitory receptor expressed on NK cells is able to recognize the PS and PE from the surface of pseudorabies virus infected cells, decreasing NK cell-mediated cytotoxicity and, in consequence, avoiding viral clearance. A similar process may occur regarding CD8+ T lymphocytes, since they also express CD300a and its expression have been associated to CD8+ T-cell exhaustion during HIV-1 infection (Viral Escape). On the other hand, through apoptotic mimicry, viruses enclose their capsids in a lipid bilayer obtained from the plasma membrane of host cells, leading to the incorporation of PS and PE to the viral envelope. Thus, surface receptors, such as CD300a, expressed on host cells bind PS and PE-containing viral particles and could also promote viral uptake. Other PS- and PE-binding CD300 molecules, such as CD300c, have the potential to bind viruses expressing these phospholipids in their envelopes. Lastly, mouse CD300f receptor binds murine norovirus particles and promotes the infection, in a PS- and PE-independent manner (Viral Attachment and Entry). Vitallé J *et al., Eur. J. Immunol.* 2019;49:364–374 (Annex 1).

1.4. HIV infection

HIV is a lentivirus that causes acquired immunodeficiency syndrome (AIDS), a chronic disease of pandemic proportions[128]. HIV infection is considered a global problem for public health. According to the latest published reports by World Health Organization (WHO), around 38 million people worldwide were infected with HIV and 1.7 million of new HIV infections were reported in 2018 (WHO. http://www.who.int/hiv/data/en/). It is well known that HIV infection induces an immune deficiency caused by the progressive loss and dysregulation of CD4+ T lymphocytes. Apart from CD4+ T cells, macrophages and monocytes are also a main target for HIV[129]. When it is not treated, HIV infection could be divided into three phases. First, there is an acute phase, a short period of time (weeks) in which patients display high viral load in blood and CD4+ T cell counts decrease. Afterwards, patients suffer a chronic phase that lasts years, in which they experiment a chronic inflammation and activation of the immune system, and hence, the viremia remain stable and the CD4+ T cell counts diminish slowly. Lastly, in most of patients if they are not treated, HIV infection causes AIDS, which is characterized by high viremia and lower than 200-250 CD4+ T cells/mm^3. In this last phase, infected patients suffer opportunistic infections and tumors that could lead to death[130–132].

The introduction of antiretroviral therapy (ART) and the development of potent antiretroviral drug combinations over the last years not only reduced dramatically the morbidity and mortality of HIV infected people, but also have considerably improved the quality of life of these patients[128,133]. Currently, there are multiple antiretroviral drugs that act targeting different steps of HIV life cycle: binding inhibitors, fusion inhibitors, reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors (Figure 3)[134]. The first step of HIV cycle is viral binding and fusion. Here, HIV envelope glycoprotein gp120 binds CD4 receptor and CCR5 and/or CXCR4 co-receptors from the surface of CD4+ cells, followed by the fusion of viral and host cell membranes, allowing the viral proteins to enter in the cell. Maraviroc is an example of CCR5 antagonists that avoids HIV binding and enfuvirtide is an inhibitor of HIV-cell fusion. Subsequently, as HIV is a retrovirus, the viral RNA is retro-transcribed into a double-stranded viral DNA by the reverse transcriptase enzyme. Non-nucleoside reverse transcriptase inhibitors (e.g. neviparine) and nucleoside reverse transcriptase inhibitors (e.g. tenofovir) intercept HIV multiplication through the inhibition of this enzyme.

Then, the produced viral DNA is transported to the nucleus and in the presence of integrase enzyme the DNA is integrated into the host genome and proviruses are formed. Raltegravir, among others, is one of the integrase inhibitors used for HIV infection treatment. Afterwards, the immature virions are produced and bud from the cell surface. Finally, the proviral polyproteins are cleaved and mature infectious virions are generated through the activity of protease enzyme. An example of a drug utilized for inhibiting protease enzyme is lopinavir[134,135].

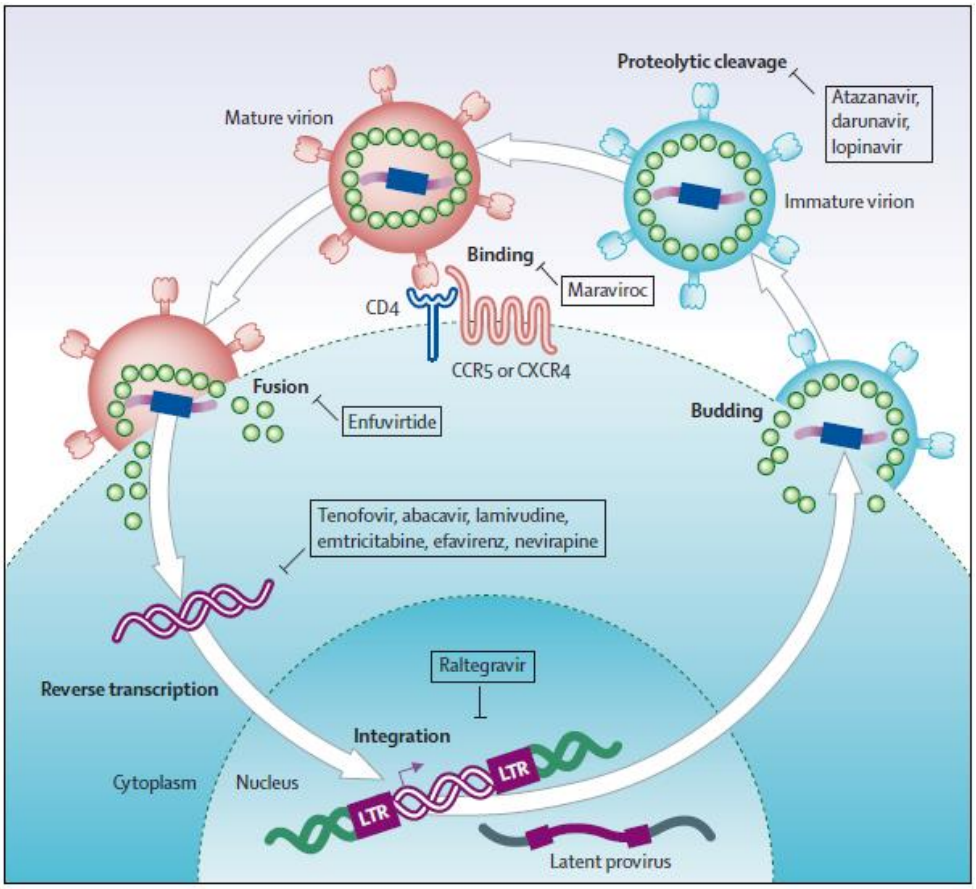


Figure 3. HIV life cycle and antiretroviral drugs. Most commonly used antiretroviral drugs targeting different steps from HIV life cycle are shown. In order to enter host cell, HIV follows three steps: attachment to CD4 receptor, binding to CCR5 and/or CXCR4 co-receptors and membrane fusion. Maraviroc and enfurvitide block the binding with CCR5 and fusion respectively. Nucleoside analogues and non-nucleoside reverse transcriptase inhibitors avoid the transcription of HIV RNA to double-stranded DNA. Raltegravir, among other integrase inhibitors, block the incorporation of HIV DNA into host genome. Lastly, the cleavage of polypeptide chains is inhibited and in consequence the maturation of the HIV is avoided by protease inhibitors. (Adapted from Volberding 2010, Lancet. 2010; 376:49-62).

In spite of the considerable progress in the therapy against HIV infection, numerous scientific observations have reported that this disease causes major abnormalities in the immune system and that ART is not able to eradicate HIV infection. In fact, the existence of long-lived latently HIV infected CD4+ T lymphocytes and/or the presence of residual viral replication that replenishes viral reservoir despite effective ART, made the eradication of HIV infection a very difficult task[136–138]. Therefore, in the last years, a great interest has emerged in the scientific community in the development of immunological approaches with the aim of eliminating the HIV reservoir and/or enhancing the magnitude of HIV-specific cellular and humoral immune responses. Multiple of new strategies have been developed against HIV infection, including stem cell therapy, interfering RNAs, CRISPR/Cas9, nanothechnology etc.[139], in addition to therapies targeting viral reservoir as chimeric antigen receptor (CAR) T cells, bispecific antibodies or bispecific T cell receptors[140]. Another strategy in which extensive efforts are ongoing is the one based on broadly neutralizing antibodies (bnAbs). These are antibodies with the capacity to neutralize multiple HIV viral strains by targeting conserved epitopes of the virus. Promising results have been observed with bnAbs and they have considerably contributed to HIV vaccine development. Nevertheless, there are several challenges in the development of these antibodies, indicating that advancement of the methodology for antibody generation and isolation and improvement of target delivery and specificity is required[141]. Importantly, numerous clinical trials and observational studies have been done and are also currently carrying out with different therapeutic vaccines (e.g. modified vaccinia Ankara-based vaccines or MVA) and/or immunotherapy-based strategies with the purpose of eradicating HIV infection (see http://www.treatmentactiongroup.org/cure/trials), but in spite of the promising results, further research is required in order to achieve a cure for HIV infection.

Therefore, the need of new strategies for the improvement of HIV-specific immune response and for the complete elimination of HIV reservoir highlights the importance of searching new biomarkers and therapeutic targets. As explained above, we have little information about the role of CD300 receptor family during HIV infection, which includes the overexpression of CD300a on B cells from HIV-infected patients[42] and the potential association of CD300a expression with HIV-specific CD8+ T cell exhaustion[114]. Given the relevance of CD300 molecules in viral entry mechanisms and in viral strategies to escape from immune system attack in the context of other viral

infections such as PRV or DENV (Figure 2)[3], it is reasonable to think that these molecules might help in the search of new biomarkers and/or therapeutic targets against HIV infection.

2. HYPOTHESIS & OBJECTIVES

The members of the CD300 family of receptors are expressed on cells from the myeloid and lymphoid lineages. We hypothesize that the expression pattern of these receptors is different depending on the age. In addition, we also predict that certain stimuli and pathological conditions, including HIV-1 infection, modulate the expression of CD300 molecules, leading to an alteration in the immune response. Finally, given the role of CD300a receptor in the pathogenesis of several viral infections, we postulate that the expression of this molecule may affect the susceptibility of CD4+ T cells to HIV-1 infection.

Therefore, the main purpose of this project was to investigate the expression, regulation of the expression and function of CD300 receptors in several immune cell types from healthy individuals, and to study how this is altered in the context of HIV-1 infection. For that, we have established the following objectives:

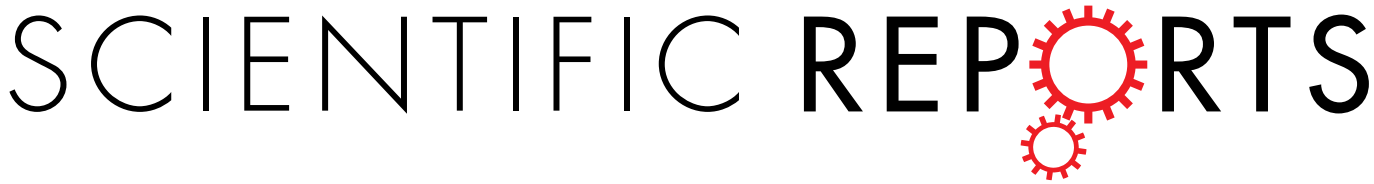
1- To study the expression, regulation and function of CD300 molecules in immune cell types from healthy adults and neonates.

2- To investigate the expression of CD300 receptors on monocytes and its association with monocyte cytokine production in cART HIV-1 infected patients before and after vaccination with a modified vaccinia Ankara-based HIV-1 vaccine (MVA-B).

3- To study the expression of CD300a on NK cell subsets and to investigate its capacity to inhibit CD16-mediated NK cell effector functions in HIV-1 infected patients.

4- To analyze the expression of CD300a on CD4+ T cell subsets from HIV-1 infected patients and to investigate its association with disease progression markers.

5- To study the susceptibility to HIV-1 infection of CD300a expressing CD4+ T lymphocytes.



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The expression and function of human CD300 receptors on blood circulating mononuclear cells are distinct in neonates and adults

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Neonates are more susceptible to infections than adults. This susceptibility is thought to reflect neonates' qualitative and quantitative defects in the adaptive and innate immune responses. Differential expression of cell surface receptors may result in altered thresholds of neonatal immune cell activation. We determined whether the expression and function of the lipid-binding CD300 family of receptors are different on neonatal immune cells compared to adult immune cells. A multiparametric flow cytometry analysis was performed to determine the expression of CD300 receptors on adult peripheral blood mononuclear cells and neonatal cord blood mononuclear cells. The expression of the CD300a inhibitory receptor was significantly reduced on cells from the newborn adaptive immune system, and neonatal antigen presenting cells exhibited a different CD300 receptors expression pattern. We also found differential LPS-mediated regulation of CD300 receptors expression on adult monocytes compared to cord blood monocytes, and that CD300c and CD300e-mediated activation was quantitatively different in neonatal monocytes. This is the first complete study examining the expression of CD300 receptors on human neonatal immune cells compared with adult immune cells. Significant differences in the expression and function of CD300 receptors may help to explain the peculiarities and distinctness of the neonatal immune responses.

It is well known that neonates are more susceptible to infectious agents than adults[1–3]. This increased susceptibility to infection is, at least in part, due to immaturity and naiveté of their immune system, affecting both the innate and adaptive immune responses[4–6]. For instance, it has been described that neonatal antigen presenting cells (APCs) are low in numbers, express lower major histocompatibility complex class II molecules (MHC-II), CD80 and CD86, differently respond to toll-like receptor (TLR) agonists, have a decreased ability to generate T helper 1 (Th1) responses and a marked decrease in the production of pro-inflammatory cytokines such as type 1 interferon (IFN) or tumour necrosis factor alpha (TNF-α)[5,7–13]. The lymphoid compartment in the newborn also exhibits qualitative and quantitative differences[5,14]. For example, it has been described that neonatal natural killer (NK) cells display an increased expression of the inhibitory receptor CD94/NKG2A and less cytotoxic activity than adult NK cells[15–17]. These differences in the newborn immune system could be crucial for protection during the transition from a sterile environment, the womb, to the outside world that is saturated with antigens, and thus avoid exuberant immune responses with the consequent danger that this would entail. In spite of the numerous findings already described, still we have an incomplete picture of the differences between neonatal and adult immune systems.

In order to preserve the identity and integrity of the host and at the same time being effective against insults, a balance between stimulating and inhibitory signals is required to adjust the activation status of the immune

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system. Among several other mechanisms that achieve this task, the balance is accomplished by signals that originate from cell surface receptors with activating and inhibitory capabilities[18–20]. The human CD300 family consists of 8 receptors encoded in chromosome 17 and they are expressed in both myeloid and lymphoid lineages, except CD300g that is expressed on endothelial cells. The CD300 molecules are type I transmembrane proteins with a single immunoglobulin (Ig)V-like extracellular domain. CD300a and CD300f receptors have a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which are required for the inhibitory signalling; while other members (CD300b, CD300c, CD300d, CD300e and CD300h), have a short cytoplasmic tail and a charged amino acid residue that allows their association with immunoreceptor tyrosine-based activating motifs (ITAM)-bearing adaptors which transduce activation signals. The biological and clinical significance of CD300 molecules and their participation in the pathogenesis of numerous diseases such as allergies, psoriasis, leukaemia, sepsis, etc. have been documented over the last years[21–24].

The knowledge about the expression and signalling-mediated abilities of the CD300 receptors in human newborn immune cells is nearly non-existent. Here, we have performed a comprehensive comparative analysis of the expression of this family of receptors on adult peripheral blood mononuclear cells (PBMCs) versus neonatal cord blood mononuclear cells (CBMCs). In addition, we have studied the regulation of the expression of certain CD300 members on monocytes and their functional capabilities. Our results reveal significant differences in the expression and function of these receptors that may help to explain the idiosyncrasies of the neonatal immune system.

Results

CD300 molecules are differentially expressed on peripheral blood adult immune system cell populations and subpopulations. Currently, a methodical study describing CD300 receptors expression in human mononuclear cells is absent. Therefore, we systematically analysed the expression pattern of CD300a, CD300c, CD300e and CD300f on adult PBMCs. We have chosen these receptor family members because they are expressed on the cell surface of immune cells and there are available mAbs for their detection by flow cytometric analysis. Even though there are commercially available specific anti-CD300c, anti-CD300e and anti-CD300f mAbs, to our knowledge, there is not a specific anti-CD300a fluorochrome conjugated mAb. In this study, we have used the clone E59.126, which recognizes both CD300a and CD300c, side by side with the specific anti-CD300c clone TX45 with the objective to distinguish the cell surface expression of the two receptors[25].

It is known that human T lymphocytes from healthy adult's peripheral blood express CD300a[26–29]. On $CD4^+$ T cells, the expression of CD300a is associated with Th1 cells that are more polyfunctional, and on $CD8^+$ T cells, CD300a expression is mostly associated with effector functions[26,28]. However, these studies were performed with the clone E59.126 without excluding the possibility that T cells also express CD300c. Here, by using the specific anti-CD300c clone TX45, we have determined that human T cells do not express CD300c (data not shown), therefore indicating that clone E59.126 is only detecting CD300a on T cells. Next, we have analysed the surface expression of CD300a in naïve ($CD45RA^+CD27^+$), memory ($CD45RA^-CD27^+$), effector/memory ($CD45RA^-CD27^-$), and terminal effector/memory (TEM) ($CD45RA^+CD27^-$) T cells (Fig. 1a and Supplementary Figure 1a). $CD4^+$ naïve T cells could be divided into two groups based upon the expression of CD300a, the $CD300a^{neg}$ and the $CD300a^{low}$ subsets. On the other hand, in $CD4^+$ memory, effector/memory and TEM cells two subsets were also distinguished, i.e. $CD300a^{neg}$ and $CD300a^{high}$. It is important to note that the majority ($>$90%) of TEM $CD4^+$ T cells were $CD300a^{high}$. On $CD8^+$ T cells, all subpopulations expressed uniform levels of CD300a, but the expression in memory, effector/memory and TEM subsets was 5–6 folds higher when compared to the expression levels of $CD8^+$ naïve T cells (Fig. 1a).

In order to identify mature and immature circulating human $CD19^+$ B cell subsets, we used a staining strategy based on the expression of CD10, CD20, CD21 and CD27 (Supplementary Figure 1b). Immature B cells are characterized by the expression of CD10, while mature B cells are $CD10^-$. Five mature B cell subpopulations are distinguished: naïve cells ($CD21^+CD27^-$), resting memory cells ($CD21^+CD27^+$), activated memory cells ($CD21^-CD27^+CD20^+$), plasmablasts ($CD21^-CD27^+CD20^-$) and tissue-like memory (TLM) B cells ($CD21^-CD27^-$). We observed that naïve B cells were mostly negative for CD300a expression, whereas the rest of B cell subsets could be divided into $CD300a^+$ and $CD300a^-$ populations (Fig. 1b). The lack of expression of CD300c on B cells as measured by the binding of specific anti-CD300c clone TX45, except for $\leq$5% of the TLM and activated memory B cells, indicate that clone E59.126 is mostly detecting CD300a on the cell surface of B cells. When we analysed $CD10^+$ immature circulating B cells, we observed that there is a small subset of $CD300a^+$ cells (Fig. 1b).

Human NK cells are phenotypically characterized by the expression of CD56 and the lack of CD3 on their cell surface. Examining the surface density of CD56 expression, NK cells are divided into two distinct subsets, $CD56^{bright}$ and $CD56^{dim}$ (Supplementary Figure 1c). In the periphery, approximately 90% of human NK cells are $CD56^{dim}$ expressing high levels of CD16 (FcγRIII) and are predominantly cytotoxic in function. In contrast, only 5–10% of NK cells are $CD56^{bright}$ and $CD16^{dim/neg}$ with a predilection for secreting pro-inflammatory cytokines[30]. Focusing on the cell surface expression of CD300 molecules, both $CD56^{dim}$ and $CD56^{bright}$ subsets expressed CD300a, with the latter subset having a tendency to express higher levels. On the other hand, only $CD56^{bright}$ NK cells expressed very low or negative levels of CD300c, while the $CD56^{dim}$ subset did not show any expression of this receptor (Fig. 1c), indicating that clone E59.126 only is detecting CD300a. NK cells, like the other lymphocyte populations analysed, did not express CD300e neither CD300f (data not shown).

Three subsets of monocytes are distinguished based on the expression of CD14 and CD16, namely classical monocytes ($CD14^{++}CD16^-$), intermediate monocytes ($CD14^{++}CD16^+$) and nonclassical monocytes ($CD14^+CD16^+$) (Supplementary Figure 1d)[31]. Previously, it has been published that monocytes from adult peripheral blood express CD300a, c, e and f[25,32–34], although the expression of these receptors was not analysed in each monocyte subset. Therefore, we determined the expression of CD300 receptors and found that intermediate

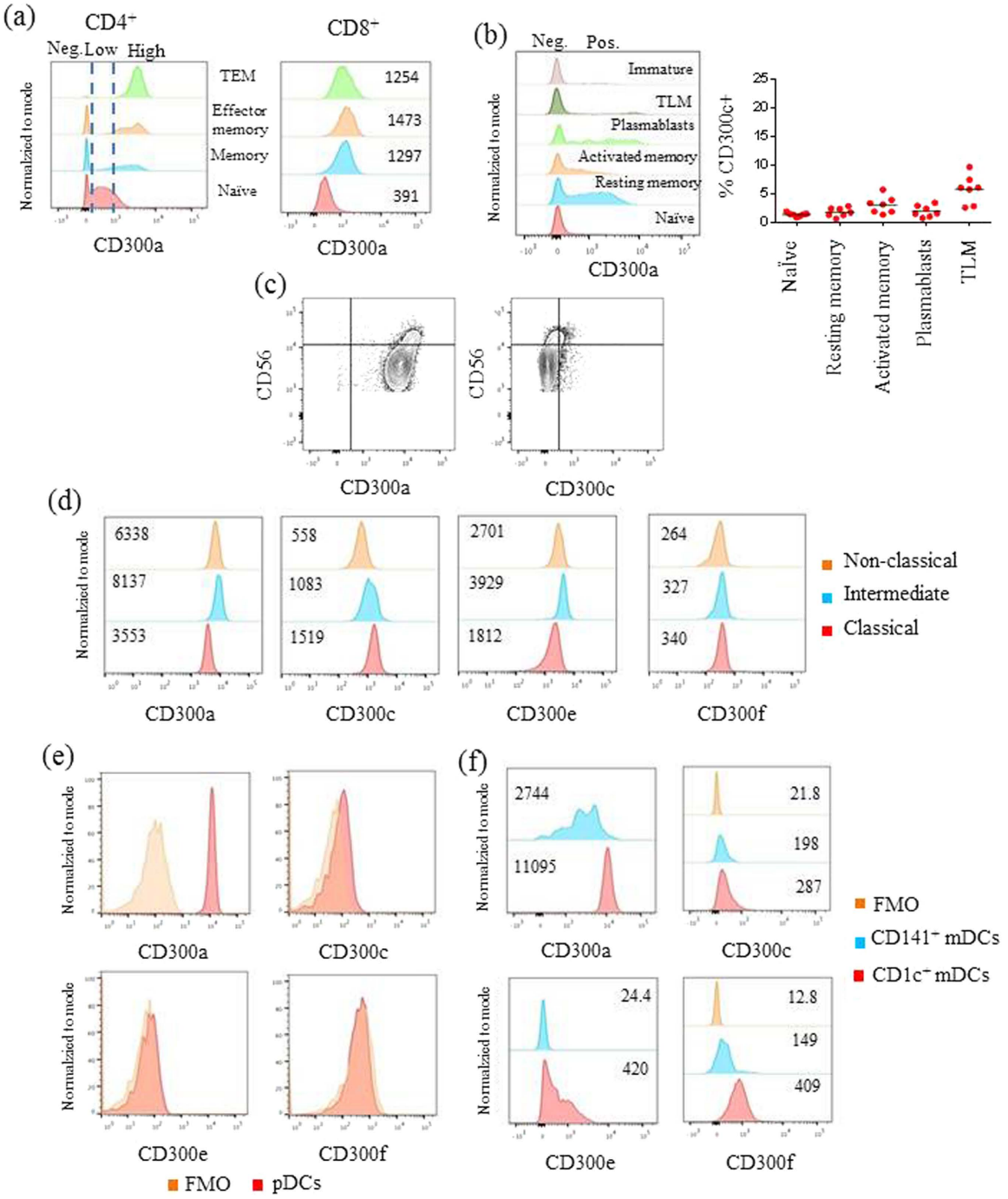

Figure 1. CD300 molecules expression on adult peripheral blood mononuclear cells. (**a**) The expression of CD300a was assessed on $CD4^+$ and $CD8^+$ naïve (red), memory (blue), effector/memory (orange) and TEM (green) T cells. Numbers represent the median fluorescence intensity (MFI) of CD300a expression. **(b)** The expression of CD300a was assessed on naïve (red), resting memory (blue), activated memory (orange), plasmablasts (green), TLM (dark green) and immature (pink) B cells. The dot graph represents the percentage of $CD300c^+$ cells in each B cell subset. **(c)** Contour plots representing the expression of CD300a and CD300c on $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets. **(d)** The expression of CD300a, CD300c, CD300e and CD300f were assessed on classical (red), intermediate (blue) and non-classical (orange) monocytes. Numbers represent the MFI of CD300 molecules expression. **(e)** The expression of CD300a, CD300c, CD300e and CD300f were assessed on pDCs. Red histograms represent the binding of anti-CD300 mAbs, and the orange histograms represent the FMO control. **(f)** The expression of CD300a, CD300c, CD300e and CD300f were assessed on $CD1c^+$ (red) and $CD141^+$ (blue) mDCs. The red and blue histograms represent the binding of anti-CD300 mAbs; and the orange histograms represent the FMO control. For the detection of the CD300 molecules, the following mAbs were used: clone E59.126 for CD300a (**c**), clone TX45 for CD300c, clone UP-H2 for CD300e and clone UP-D2 for CD300f. Histograms and contour plots are representative of data obtained from 7–9 healthy donors.

and nonclassical monocytes exhibited higher cell surface expression levels of CD300a and CD300e than classical monocytes, while the latter showed the highest expression of CD300c. In relation to CD300f expression, the three monocytes subpopulations showed similar levels, with the nonclassical monocytes displaying a tendency to express less CD300f (Fig. 1d).

According to Ziegler-Heitbrock *et al.*, there are three types of human dendritic cells (DC) in blood, that is, plasmacytoid DC (pDC) and two types of myeloid DCs (mDCs) (Supplementary Figure 1e)[31]. DCs are identified for being lineage marker negative cells and HLA-DR positive, and in the case of pDCs they are specifically recognized by the expression of CD303 (Supplementary Figure 1f). Cell surface expression analysis of CD300 receptor family members showed that pDCs express CD300a. They exhibited none or negligible expression of other CD300 molecules (Fig. 1e). As mentioned before, two types of blood mDCs are distinguished, one subset expresses the marker CD1c and the other is identified by the expression of CD141. The $CD1c^+$ mDCs, which are the vast majority, expressed the four CD300 receptor family members analysed in this study, including the expression of very high levels of the CD300a inhibitory receptor (Fig. 1f). On the other hand, the expression levels of CD300 molecules were significantly lower in the $CD141^+$ mDCs compared to the $CD1c^+$ mDCs (Fig. 1f).

The expression pattern of CD300 surface receptors is different in neonatal and adult mononuclear cells. Next, we were interested in determining if a different expression pattern of CD300 surface molecules on neonatal immune cells would help to explain the differences between the newborn and adult immune responses. Therefore, we decided to systematically analyse the expression of CD300 receptors on CBMCs in comparison with adult PBMCs.

We found that almost all $CD4^+$ and $CD8^+$ cord blood T lymphocytes were naïve, 97% and 90% respectively, while the rest of T cells had a memory phenotype. We did not find significant amounts of memory/effector cells or TEM cells in cord blood (Supplementary Figure 2a). The vast majority (>95%) of the naïve $CD4^+$ T cells from cord blood were negative for CD300a expression, while >50% of adult circulating naïve $CD4^+$ T cells expressed CD300a at low levels (Fig. 2a). On the other hand, although we observed a fraction of cord blood memory $CD4^+$ T cells that were positive for CD300a, the frequency of $CD300a^+$ of neonatal memory $CD4^+$ T cells was significantly lower when compared with adult memory $CD4^+$ T cells ($28.9 \pm 5.7\%$ of $CD300a^+$ cells in CBMCs vs. 62.0 ± 4.7 of $CD300a^+$ in adult PBMCs) (Fig. 2a). Lastly, CD300a expression was absent or very low on both naïve and memory $CD8^+$ T cell from cord blood (Fig. 2a).

As in T cells, naïve B lymphocytes represented the majority of mature B cells from cord blood, while there is a small subset (<5%) of resting memory and TLM B cells. Activated memory B cells and plasmablats are almost absent from cord blood, and the frequency of $CD10^+$ immature B cells was significantly higher in cord blood than in adult peripheral blood (Supplementary Figure 2b). As in adults, naïve B cells from neonates express none or very low levels of CD300a, while the expression of CD300a in memory B cells from cord blood was significantly lower than in the adult (Fig. 2b). Very interestingly, the frequency of $CD300c^+$ resting memory B cells was significantly higher in CBMCs than in adult PBMCs (Fig. 2b). Finally, $CD10^+$ immature B cells from cord blood exhibited significantly lower frequencies of $CD300a^+$ cells than $CD10^+$ immature B cells from adult blood (Fig. 2b).

Regarding NK cells, we did not observe any significant difference between the frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells from cord blood and adult blood (Supplementary Figure 2c). Similarly, the cell surface expression of CD300a and CD300c was very similar on NK cells from both neonates and adults (Fig. 2c). Cord blood NK cells, T cells and B cells did not express CD300e neither CD300f (data not shown).

The neonatal monocyte population is thought to be immature, but not much is known regarding the different monocyte subsets[5,14]. We examined the frequencies of monocyte subsets as well as the cell surface expression of CD300 molecules. We observed that the percentages of classical monocytes are slightly higher, although not statistically significant, in CBMCs than in adult PBMCs ($95.7 \pm 3.8\%$ of cord blood monocytes vs. $91.7 \pm 3.2\%$ of monocytes in adults) and the percentage of intermediate monocytes were similar in both samples. However, we observed that cord blood exhibited significantly lower frequencies of nonclassical monocytes than adults (Supplementary Figure 2d). Analysis of cell surface expression of CD300 molecules on monocytes from cord blood showed that the expression of CD300c was significantly lower on all monocytes subsets from neonates compared with adult monocytes (Fig. 3a). On the other hand, we did not find differences in the expression of CD300a between adult and neonate monocytes. Intermediate and nonclassical monocytes from cord blood have a tendency to express higher levels of CD300e compared with intermediate and nonclassical monocytes from adult blood, and regarding CD300f expression we did not find any significant differences between adult and cord blood monocytes (Fig. 3a).

DCs were found in lower frequencies in cord blood than in adult peripheral blood (Supplementary Figure 2e). Like in adult PBMCs, pDCs in CBMCs only expressed CD300a, but they showed significantly lower levels than adult peripheral blood pDCs (CD300a MFI: 5979 ± 368 in CBMC vs. 8619 ± 466 in adult PBMC) (Fig. 3b). Regarding mDCs, we only observed differences in the expression levels of CD300c on the $CD1^+$ mDC subset, which were higher in neonates than in adults (CD300c MFI: 486.3 ± 92.3 in neonates vs. 218.7 ± 41.1 in adults) (Fig. 3c). We did not find differences on CD300a expression between adult and neonate mDCs. CD300e and CD300f expression were similar on neonates and adults mDCs (data not shown).

Altogether, our results show that the expression of CD300 molecules is different between CBMCs and adult PBMCs. Cells from the neonatal adaptive immune system significantly expressed lower levels of the CD300a inhibitory receptor compared with cells from the adult immune system. CD300 receptors expression on monocytes from cord blood also exhibited significant differences when compared with adult monocytes. Finally, the expression levels of the CD300c activating receptor differed between adult PBMCs and CBMCs depending on the cell type, while we observed few differences on the expression of CD300e and CD300f receptors between adult and neonatal monocytes and DCs.

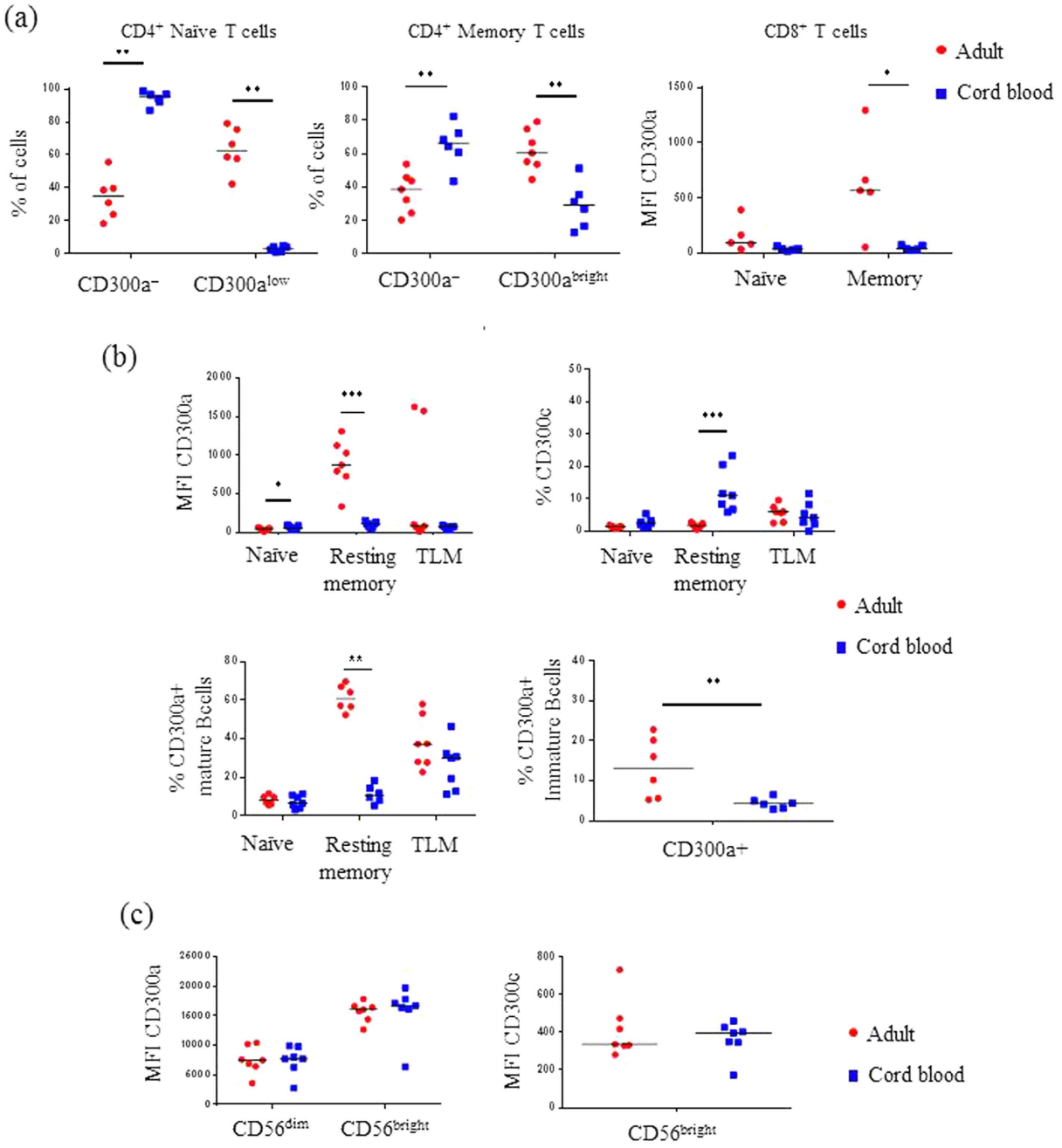

Figure 2. The expression pattern of CD300 surface receptors on lymphocytes is different in neonates from adults. (a) Dot graphs representing the percentage of CD300a^{-} and CD300^{low} cells in CD4^{+} naïve cells (left), the percentage of CD300a^{-} and CD300a^{bright} on CD4^{+} memory T cells (middle), and the MFI of CD300a expression on CD8^{+} naïve and memory T cells (right). **(b)** Dot graphs showing the MFI (upper left) of CD300a expression and the percentage (lower left) of CD300a^{+} cells in naïve, resting memory and TLM mature B cell subsets, the percentage of CD300c^{+} cells in naïve, resting memory and TLM mature B cell subsets (upper right), and the percentage of CD300a^{+} immature B cells (lower right). **(c)** Dot graphs representing the MFI of CD300a (left) and CD300c (right) expression on CD56^{dim} and CD56^{bright} NK cells is represented. Each dot represents a different donor, and the medians are represented. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

LPS-mediated regulation of CD300 molecules on monocytes differs in adults and neonates. Next, we were interested in understanding how CD300 receptors expression is regulated on neonatal immune cells. We chose to work with monocytes because they express the four CD300 molecules tested in this study. Also, it is known that the TLR4 ligand LPS modulates the expression of CD300c on monocytes[25]. Therefore, we investigated CD300 receptors expression on adult monocytes after 24 h of LPS treatment and we observed a significant increase on CD300c and CD300e expression when compared with untreated monocytes, while the expression

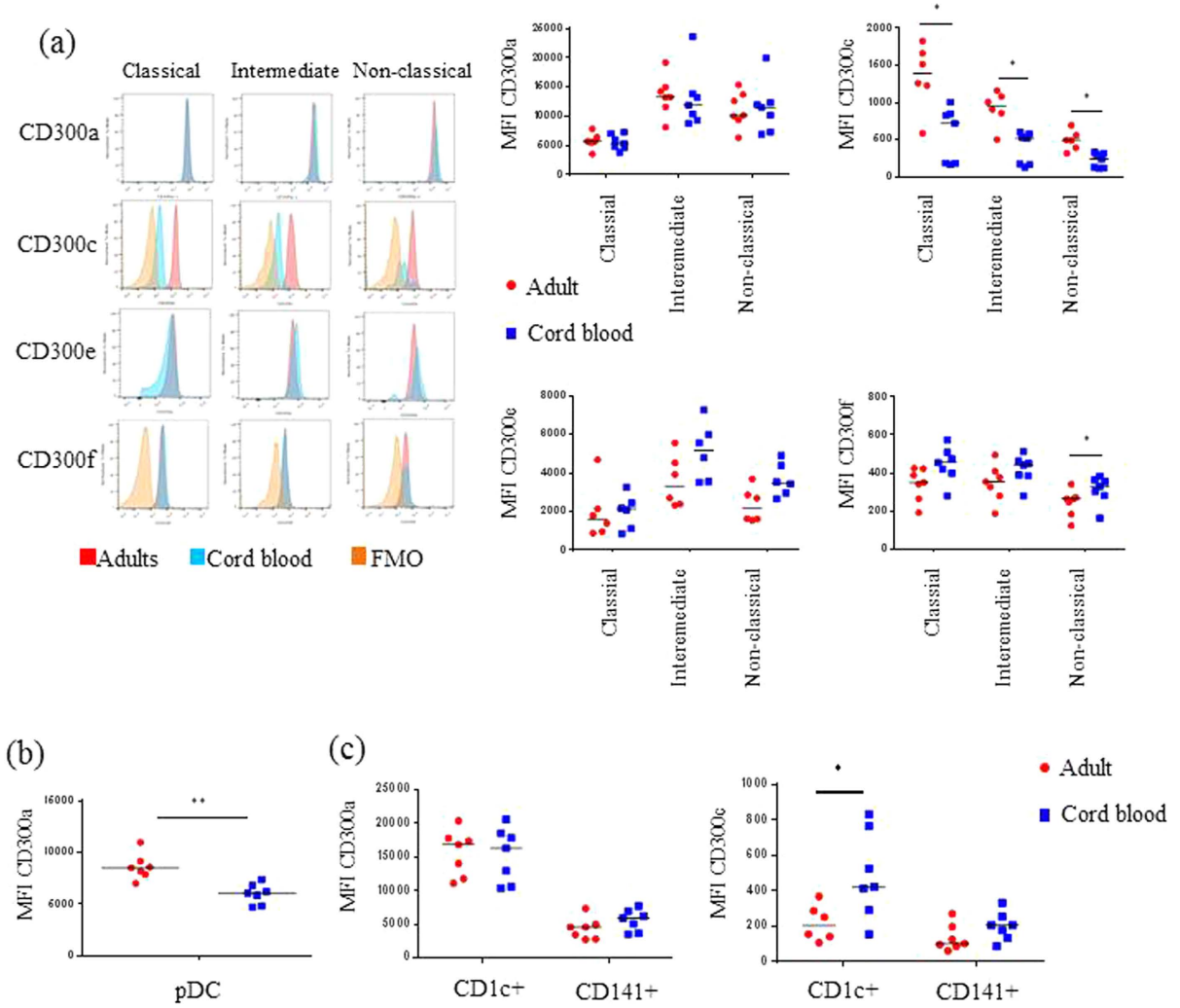

Figure 3. The expression pattern of CD300 surface receptors on myeloid cells is different in neonates from adults. (a) The expression of CD300 molecules was assessed on monocyte subsets. On the left, representative histograms are shown. On the right, dot graphs representing the MFI of CD300a, CD300c, CD300e and CD300f expression in classical, intermediate and nonclassical monocytes are shown. **(b)** Dot graphs showing the MFI of CD300a expression on pDCs. **(c)** Dot graphs representing the MFI of CD300a and CD300c in $CD1c^+$ and $CD141^+$ mDCs are shown. Each dot represents a different donor, and the medians are represented. *$p < 0.05$, ** $p < 0.01$.

of CD300a and CD300f decreased (Fig. 4a). It is well accepted that cord blood monocytes are somehow less responsive to LPS[12,35]. For example, they exhibit impaired TNF-α production in response to LPS and bacterial lipopeptides[12]. Hence, we analysed CD300 molecules in response to LPS on monocytes from CBMCs. Our results showed that, opposite to adult monocytes, CBMCs monocytes did not upregulate CD300c cell surface expression and CD300e up-regulation was significantly lower when compared with adult monocytes (Fig. 4b). On the other hand, CD300a and CD300f expression were unaltered after LPS treatment on cord blood monocytes, while it was down-regulated on adult monocytes (Fig. 4b). Altogether, our results showed that LPS differentially regulates CD300 cell surface expression on monocytes from adults and neonates.

CD300c and CD300e mediated activation is quantitatively different in neonatal monocytes. It is known that CD300c and CD300e are functional activating receptors in adult monocytes[25,33,34] and we have shown that there are significant differences in the expression of CD300c, and to a minor extent, in CD300e expression, between monocytes from adults and newborns (Fig. 3a). Therefore, we decided to characterize the function of these two activating receptors in neonatal monocytes. We studied if cross-linking of CD300c and CD300e with specific mAbs was able to induce similar or quantitatively different activation signals in monocytes from cord blood. We first investigated their ability to induce intracellular calcium mobilization as an early event in the signalling cascade mediated by these two receptors. As expected, engagement of CD300c and CD300e with soluble anti-CD300c or anti-CD300e mAbs respectively, followed by cross-linking with anti-mouse IgG $F(ab')_2$, induced transient and rapid increase in intracellular calcium in adult monocytes, which was not observed when cells were stimulated with isotype matched control. However, when we carried out the same assay in monocytes

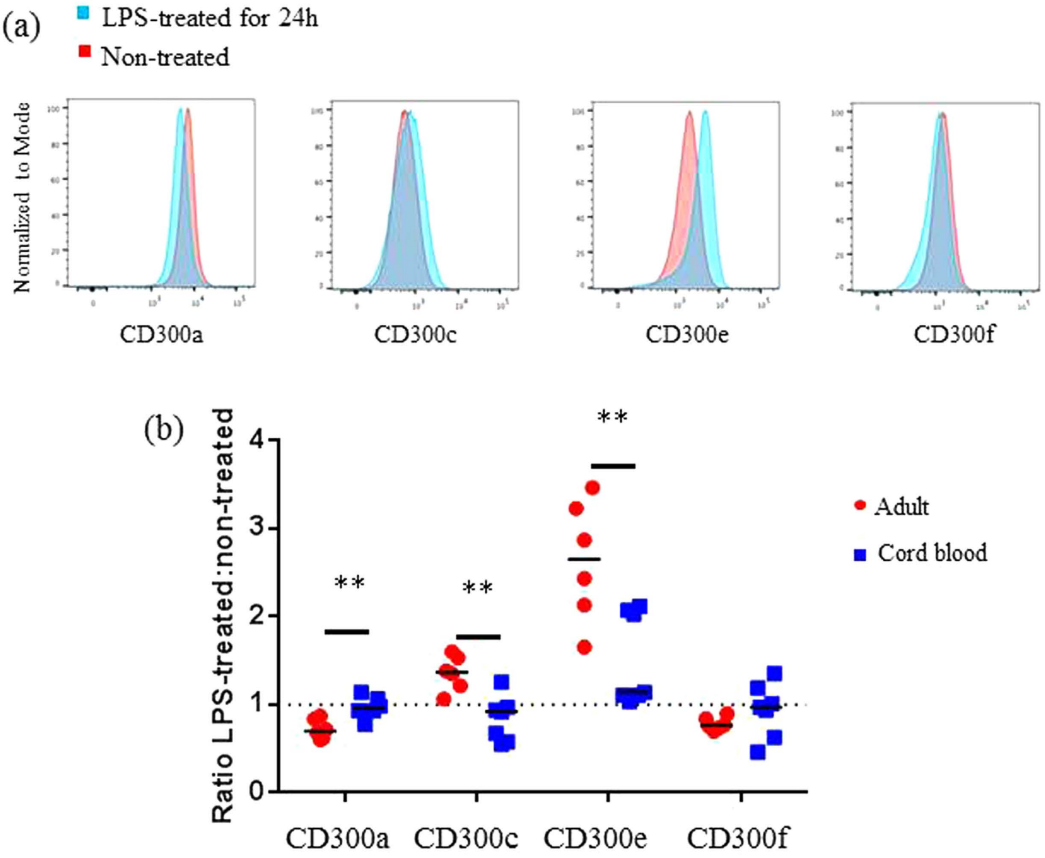

Figure 4. LPS-mediated regulation of CD300 molecules on neonatal and adult monocytes. Monocytes were either left untreated or treated with 10 ng/ml of LPS for 24 h. Then, cells were harvested and checked for CD300a, CD300c, CD300e and CD300f cell surface expression. **(a)** Representative histograms showing CD300 receptors modulation in adult monocytes in response to LPS. The red histograms represent the untreated condition and the blue histograms represent the LPS-treated condition. Results are illustrative of data from 7 adult healthy donors. **(b)** Dot plot graph showing CD300 receptors modulation on monocytes from adults and newborns after LPS treatment. Data are represented as the ratio of MFI of each CD300 molecule between LPS-treated and untreated cells. The horizontal dotted line (value 1) represents no change on CD300 molecules expression after LPS treatment. Each dot represents a different donor, and the medians are represented. $^{**}p < 0.01$.

from cord blood we observed significantly less CD300c and CD300e mediated calcium mobilization, indicating that early activation signals through these activating receptors are dampened in neonatal monocytes (Fig. 5).

Then, we determined the ability of CD300c and CD300e to modulate costimulatory molecules' cell surface expression and cytokine production. In spite of significant differences in calcium mobilization, we only observed a non-significant statistical tendency of neonatal monocytes to exhibit a smaller increase on CD86 expression after CD300c engagement with specific mAbs when compared with adult monocytes (data not shown). We and others have before demonstrated that engagement of CD300c upregulates cytokine secretion from LPS treated monocytes and cross-linking of CD300e induces TNF-α secretion by adult monocytes[25,33]. Therefore, we wanted to investigate the role of these two CD300 molecules in the production of cytokines by neonatal monocytes. Freshly isolated classical adult and cord blood monocytes were cultured either with plate-bound anti-CD300c mAb, with anti-CD300e mAb or with isotype-matched control. In contrast to the results obtained with adult monocytes, engagement of CD300c and/or CD300e on neonatal monocytes did not increase LPS-mediated cytokine production, except TNF-α secretion after the cross-linking of CD300c (Fig. 6 and Supplementary Figure 3). Altogether, our results indicate that CD300c and CD300e mediated monocyte activation is significantly reduced in neonatal monocytes, suggesting that it may play a role in the differences between newborn and adult immune responses.

Discussion

The main objective of this study was to carry out a comparative analysis of the expression of CD300 receptors in neonatal and adult immune cells, along with their regulation and function in monocytes that could help to explain the immaturity of the neonatal immune system. Previous studies have demonstrated that CD300 molecules have a very important role in the responses during viral infections, sepsis, cancer, allergies, autoimmunity and inflammation[36–50], indicating that this family of receptors is very important for regulating the immune response. Except for a study describing the expression of CD300f on neonatal monocytes[14], ours is the first comprehensive work

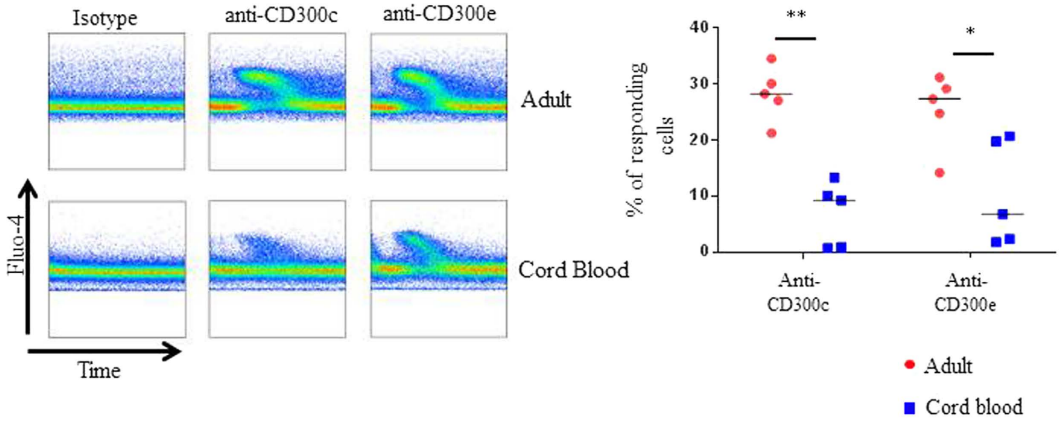

Figure 5. CD300c and CD300e induced calcium mobilization is quantitatively different in neonatal monocytes compared with adult monocytes. Adult PBMCs and CBMCs were loaded with Fluo-4. To establish a baseline, electronically gated monocytes were first acquired for 30 s at which point the primary antibodies, anti-CD300c, anti-CD300e or isotype-matched control, were added. Then at 60 s, the primary antibodies were cross-linked with goat anti-mouse IgE $F(ab')_2$ and fluorescence was measured. Ca^{++} mobilization is represented as an elevation in the fluorescence intensity of the Fluo-4. The pseudocolor representation (left) is a representative experiment. The percentages of responding cells in each experiment were represented in the dot plot graph (right). Each dot represents a different donor, and the medians are shown. *$p < 0.05$, **$p < 0.01$.

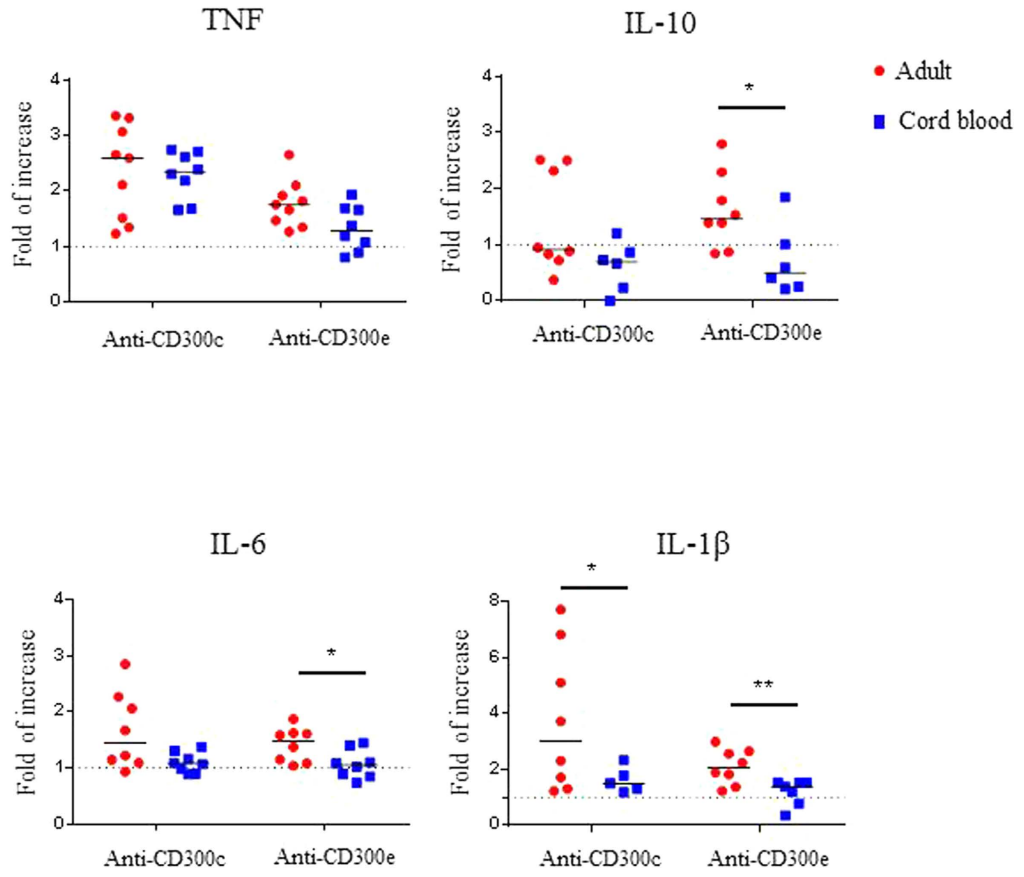

Figure 6. Differential cytokine production after the engagement of CD300c and CD300e in adult and cord blood classical monocytes. Enriched classical monocytes from healthy adult and cord blood were either stimulated with plate bound isotype-matched control antibody, anti-CD300c mAb or anti-CD300e mAb in presence of LPS for 18 h. Culture supernatants were harvested and tested for the secretion of human inflammatory cytokines using flow-cytometric bead analysis. The values on the y-axis correspond to fold of increase over the isotype-matched control antibody for the production of cytokines TNF-α, IL-10, IL-6 and IL1-β. Each dot represents a different donor, and the medians are represented. *$p < 0.05$, ** $p < 0.01$.

examining the expression of CD300 cell surface receptors on human adult PBMCs and CBMCs. Our results show that cells from the neonatal adaptive immune system express low levels of the CD300a inhibitory receptor when compared with adult T and B cells. Specifically, immature B cells and the minor subset of resting memory B cells in cord blood are mostly CD300a negative. Related to T cells, both $CD4^+$ T cells and memory $CD8^+$ T cells from cord blood exhibited significant lower levels of CD300a when compared with adult T cells. Also, CD300 receptors expression on monocytes from cord blood displayed significant differences when compared with adult monocytes. Furthermore, we also show that LPS differentially regulated CD300 receptors expression on monocytes from adults and neonates, and that CD300c and CD300e mediated activation is quantitatively different in neonatal monocytes compared with adult monocytes.

First, we systematically studied the expression of CD300a, CD300c, CD300e and CD300f on human adult PBMCs. It has been described that transcripts encoding CD300a were present in myeloid and lymphoid cells. Nevertheless, the cell surface expression of CD300a was somewhat difficult to determine due to a lack of specific fluorochrome-conjugated mAbs able to distinguish between CD300a and CD300c. By the simultaneous usage of two fluorochrome-conjugated mAbs that help to differentiate between CD300a and CD300c[25], we can conclude that human blood T cells only express the CD300a inhibitory receptor on their cell surface. B cells, except for less than 5% of $CD300c^+$ memory and TLM B cells, almost exclusively express the CD300a inhibitory receptor. It is important to point out that resting memory B cells conforms a very small subset in cord blood, and the differences between adult and neonates in the expression of CD300 molecules could be due to a different number of both switched and unswitched resting memory B cells on adult and cord blood. More studies are required to address this possibility. On the other hand, $CD56^{dim}$ NK cells express only CD300a, while $CD56^{bright}$ NK cells express CD300a and very low or negative levels of CD300c. We have recently demonstrated that activation with IL-2 and IL-15 of $CD56^{bright}$ adult NK cells, but not $CD56^{dim}$ NK cells, induces the expression of CD300c, while the levels of CD300a were unchanged[51]. Hence, we can conclude that freshly isolated human blood lymphocytes only express the CD300a inhibitory receptor, except for a very small population, less than 5%, of memory and TLM B cells and the very low expression levels of CD300c on the surface of $CD56^{bright}$ NK cells. Both CD300e and CD300f are not expressed on lymphocytes.

On the other hand, the circulating myeloid cells that we have analysed in this report express the four CD300 receptors. However, their expression levels significantly vary between the different monocyte and DC subsets. CD300a and CD300e expression is higher on intermediate and non-classical monocytes, while CD300c is higher in classical monocytes. $CD1c^+$ mDCs have higher expression levels of the four CD300 receptors when compared with $CD141^+$ mDCs, and pDCs only express CD300a. A previous report analysing the effect of an anti-CD300a/c mAb regulating type I IFN and TNF-α secretion by pDCs[52] can be ascribed to solely CD300a mediated signals, since we have demonstrated that pDCs only express this inhibitory receptor but not the highly homologous CD300c activating receptor. Until now, the described ligands for human CD300 family of receptors, specifically CD300a and CD300c, are phosphatidylserine (PS) and phosphatidylethanolamine (PE)[34,51,53,54]. PS and ceramide have also been described as ligands for mouse CD300f[41,55], while the ligand of CD300e is unknown. The differential expression of the CD300 receptors combined with the lipid binding ability of some of them suggest that, for example, certain cell types could be more susceptible to infection by viruses that use CD300a to enhance infections[36] and to protect infected cells from immune cell attack[40].

The circumstances that naïve $CD4^+$ T cells in cord blood do not express CD300a and that there are significantly lower frequencies of $CD300a^+$ memory $CD4^+$ T cells in CBMCs, may indicate that neonate $CD4^+$ T cells are exposed to different signals, that are important for the expression of this inhibitory receptor, than adult $CD4^+$ T cells. It is well known that the cytokine milieu is different in foetus and newborns compared with that in adults [10,56–58]. For example, transforming growth factor (TGF)-β1, a cytokine previously demonstrated to inhibit CD300a expression on adult $CD4^+$ T cells[28], may have an important role in regulating the expression of CD300a on cord blood $CD4^+$ T cells. In effect, it has been described that cord blood from healthy newborns after normal spontaneous vaginal delivery displayed high levels of TGF-β1[58] and that the expression of TGF-β1 is increased in fetal lymph nodes compared with that in adults' lymph nodes[57]. Also, we have previously shown that *in vitro* cultures with $CD4^+$ naïve T cells revealed that Th1 polarization in the presence of IL-12 results in the generation of mostly $CD300a^+$ cells and the majority of the IFN-γ producing cells in these cultures were $CD300a^+$[28]. Furthermore, expression of CD300a on memory $CD4^+$ T cells was associated with Th1 IFN-γ producing cells isolated *ex vivo*[28]. The low frequency of $CD300a^+$ memory $CD4^+$ T cells in the newborn is consistent with the well-known described defective development of Th1 responses in neonates[56], which partly is a consequence of a decreased IL-12 production by neonatal APCs[5,10]. Similarly, the lower CD300a expression levels on neonate $CD8^+$ T cells may be due to the same factors that control its expression on $CD4^+$ T cells. The differential expression of CD300 molecules on adult versus neonatal B cells could also be a consequence of the different cytokine environment. High expression of TGF-β1 and a T helper 2 (Th2) skewed response in the neonates[5,56] may be responsible for the significantly low CD300a expression on resting memory B cells. In effect, we have previously shown that the Th2 cytokine IL-4 and TGF-β1 act as negative regulators of CD300a expression on memory B cells[59]. Finally, other factors such as the strength of antigen receptor-mediated signals, TLR-mediated signals and other cytokines, etc., may well have an important and significant role in regulating CD300a and CD300c expression on neonatal circulating lymphocytes.

We have also observed significant differences in the expression pattern of CD300 molecules between monocytes from adults and neonates; specifically in the expression of CD300c, which is significantly less expressed on neonatal monocytes. This lower expression could be the consequence of less exposure and less responsiveness of neonate cells to TLR ligands, such as LPS[12,35]. Indeed, our results showing that CD300 molecules expression is significantly less modulated by *in vitro* treatment with LPS supports this conclusion, although we cannot exclude other factors involved in CD300 expression independently of TLR expression. For example, it has been shown that plasma from newborns has the ability to diminish LPS mediated TNF-α release by adult monocytes,

suggesting either that newborn plasma is deficient in a factor that enhances ligand-induced TLR activation or that it contains an inhibitor[12]. We have also shown that CD300c and CD300e mediated signals, as shown by intracellular calcium mobilization, are significantly down-regulated in neonatal monocytes when compared with adult monocytes. In the instance of CD300c, this could be easily explained by the reduced expression of this receptor on monocytes from neonates. However, CD300e mediated intracellular calcium mobilization was also significantly down-regulated in neonatal monocytes, while CD300e expression was similar, in classical monocytes, or somehow higher, in intermediate and non-classical monocytes, in newborns compared with adults. This indicates that other factors, such as lower levels of adaptor proteins and/or of signalling intermediates, differences in the agonistic effects of the antibodies, among others, could be responsible for the reduced signalling ability of these two activating receptors on neonatal monocytes. Receptor levels are just one component of the effect of this family of receptors on neonate immunity. Clearly, more studies are required to address this issue.

It is well known that the innate immune system provides the first contact between invading microbes and the host's defence response. Monocytes are recruited to sites of inflammation where they get activated and secrete a variety of cytokines[60,61]. Our results show that while the cross-linking of CD300c and CD300e enhanced LPS-mediated cytokine production by adult monocytes, there was a smaller enhancement (TNF-α) or no enhancement (IL-1β, IL-6 and IL-10), by monocytes from CBMCs. These results are not only explained by the previously described distinct neonatal monocyte responses to LPS, but also by the decreased ability of CD300c and CD300e to signal on newborn monocytes as we have shown here. Therefore, we would like to suggest that the diminished LPS-mediated modulation of CD300 molecules coupled with reduced CD300-mediated signals could help to partially explain the differences on monocyte responses between adults and neonates.

We conclude that neonatal innate and adaptive immune cells exhibit a distinctive pattern of CD300 receptor family expression from adults. We also demonstrate that CD300c and CD300e-mediated activation signals are quantitatively different between adult blood and cord blood monocytes. Therefore, this study adds to our understanding about the function of the neonatal immune system.

Methods

Cell isolation and enrichment. Adult and cord blood samples from healthy donors were collected through the Basque Biobank (http://www.biobancovasco.org) under an institutional review board-approved protocol by the Basque Committee of Ethics and Clinical Research. The methods were carried out in accordance with the approved guidelines. The Basque Biobank complies with the quality management, traceability and biosecurity, set out in the Spanish Law 14/2007 of Biomedical Research and in the Royal Decree 1716/2011. All study subjects provided written informed consent. Adult PBMCs and CBMCs from cord blood were obtained by ficoll density centrifugation. Monocyte enrichment was carried out by a negative selection method using the Human Monocyte Isolation Kit II from Miltenyi Biotec.

Flow cytometry analysis. The following fluorochrome conjugated anti-human monoclonal antibodies (mAbs) were used for the flow-cytometric analysis: PE-Cy7 anti-CD3 (clone SK7), BV421 anti-CD4 (clone RPA-T4), FITC anti-CD8 (clone RPA-T8), PerCP-Cy5.5 anti-CD10 (clone HI10a), PE-Cy7 anti-CD14 (clone MφP9), BV510 anti-CD19 (clone SJ25C1), PE-Cy7 anti-CD20 (clone 2H7), BV510 anti-CD45RA (clone HI100), BV421 anti-CD66b (clone G10F5), BV510 anti-CD141 (clone 1A4), PerCP-Cy5.5 anti-HLA-DR (clone G46-6) and FITC Lineage cocktail 3 from BD Biosciences; FITC anti-CD21 (clone BL13) and PE anti-CD300a-c (clone E59.126) from Beckman-Coulter; PE-Cy7 anti-CD1c (clone L161), FITC anti-CD16 (clone B73.1) and APC anti-CD56 (clone MEM-188) from BioLegend; APC anti-CD300e (clone UP-H2) and PE anti-CD300f (clone UP-D2) from Miltenyi Biotec; APC-eFluor780 anti-CD27 (clone O323), PE and eFluor660 anti-CD300c (clone TX45) and PerCP-eFluor710 anti-HLA-DR (clone L243) from eBioscience. Adult PBMCs, CBMCs and enriched monocytes were washed with staining buffer containing 1X phosphate-buffered saline (PBS) and 1% of fetal bovine serum (FBS) to block Fc receptors and stained with the respective fluorochrome conjugated antibodies for 30 min on ice. Then, cells were washed to remove unbound antibodies and further acquired in a FACSCanto II Flow cytometer (BD Biosciences). We used fluorescence minus one (FMO) control, which contains all the fluorochrome conjugated mAbs in a panel, except for the one that is being measured. FMO control is used to identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel. Flow cytometry data were analysed by using FlowJo software, version 10.0.7 (TreeStar).

Stimulation of monocytes with Lipopolysaccharide (LPS). Adult PBMCs and CBMCs were cultured (2×10^6 cells/ml) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 1% GlutaMax and stimulated with 10 ng/mL of LPS (Sigma-Aldrich) in a 24-well plate for 24 h. Then, cells were harvested and analysed for CD300 receptors surface expression by flow cytometric analysis. Monocytes were electronically gated according to forward and side scatter parameters and by the expression of CD14.

Calcium mobilization assay. Freshly isolated adult PBMCs and CBMCs were washed and resuspended in Rosswell Park Memorial Institute (RPMI) 1640 Medium containing 10% FBS (5×10^6 cells/ml). Next, cells were labelled with Fluo-4 (2 μg/ml) from Life Technologies, for 30 min at 30 °C protected from light. Then, cells were washed twice and resuspended at 2×10^6 cell/ml. Aliquots of 1 ml were warmed at 37 °C for 5 min, followed by acquisition in a FACSCanto II flow cytometer. To establish a baseline, cells were first acquired for 30 s without stimuli and then they were cross-linked either with 5 μg of anti-CD300c (clone TX45), anti-CD300e (clone UP-H2) or isotype control (clone MOPC-21) for 30 s, followed by the addition of 8.5 μg of goat anti-mouse IgG $F(ab)_2$. Cells were further acquired for 6 more min. Monocytes were electronically gated based on their forward and side scatters properties. The percentage of responding cells was determined by electronically gating monocytes

that had higher fluorescence intensity than the baseline between 60 s (after cross-linking of receptors) and 300 s, once cytoplasmic Ca^{++} has reached the basal levels. Data were analysed by using FlowJo software (Treestar).

Cross-Linking of CD300c and CD300e on monocytes and measurement of cytokine production. Culture plates (48 wells) were coated with 2.5 μg of either anti-human CD300c (clone TX45), anti-human CD300e (clone UP-H2), or isotype control (clone MOPC-21) for 2–3 h at 37 °C. Enriched monocytes from adult PBMCs and CBMCs were then added to the antibody-coated plates (1×10^6 cell/ml) in IMDM medium supplemented with 10% FBS. Monocytes were also stimulated with 1 ng/ml of LPS. After 18 h of incubation, supernatants were harvested and stored at −80 °C. Human Inflammatory Cytokine CBA kit (BD Biosciences) was used for the measurement of cytokine production, following the manufacturer's protocol. Samples were acquired on FACSCanto II Flow cytometer and analysed with BD FCAP array software (BD Biosciences).

Statistical Analysis and graphical representation. Data were analysed using GraphPad Prism software. The data were plotted as individual dot graphs showing medians and unpaired nonparametric Mann-Whitney U test was applied.

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Author Contributions

O.Z. and F.B. conceived and coordinated the study. O.Z., J.V. and F.B. designed and analysed the experiments. O.Z. and J.V. performed the experiments. O.Z. and F.B. wrote the paper. S.G.-O., I.A., C.E., S.S. and V.R.S. critically revised the paper. All the authors analysed the results and approved the final version of the manuscript.

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Supplementary Information

The expression and function of human CD300 receptors on human blood circulating mononuclear cells are distinct in neonates and adults.

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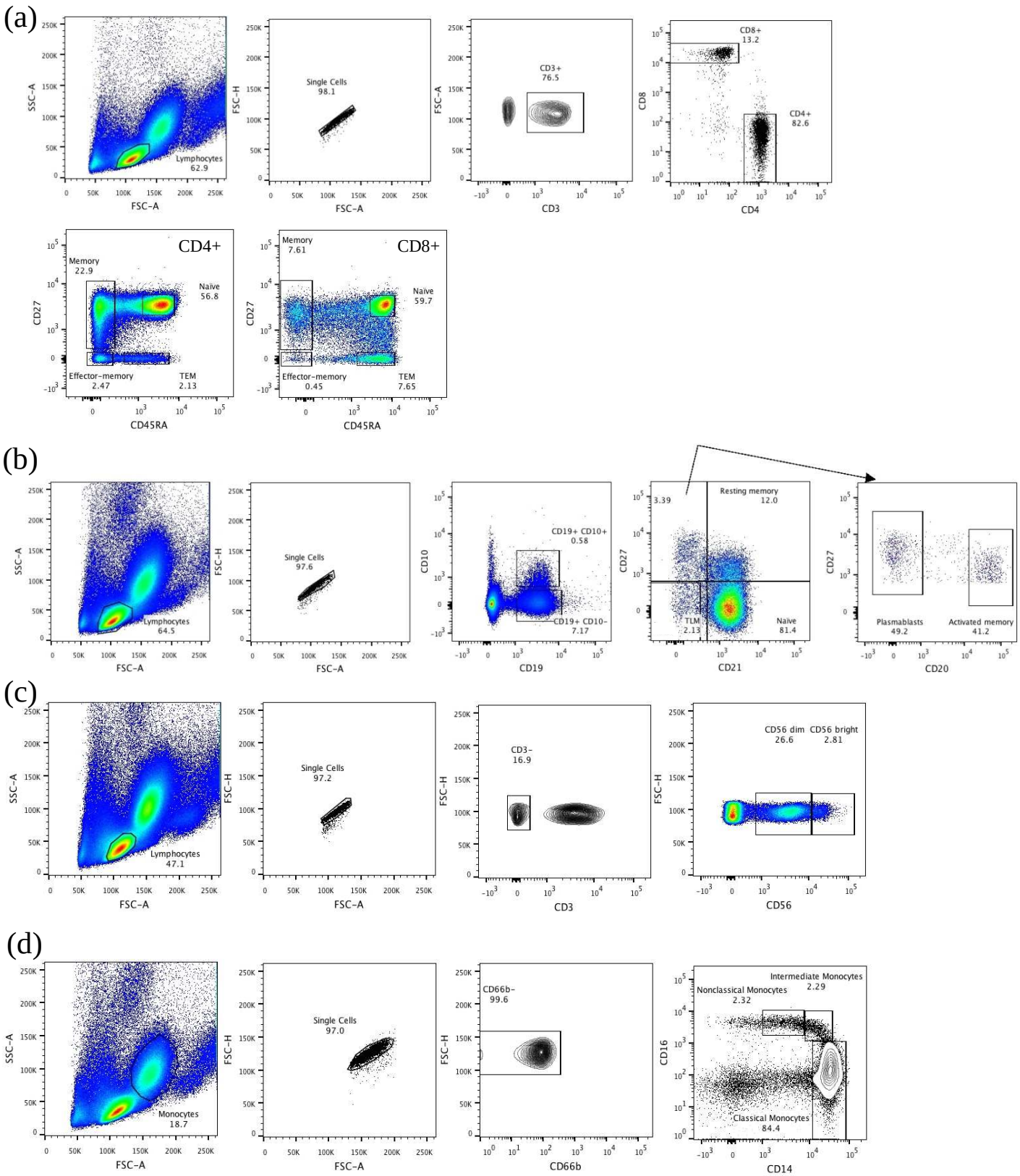
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FSC-A
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FSC-H
Single Cells
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FSC-A
FSC-A
CD3+
76.5
FSC-A
CD3
CD8
CD8+
13.2
CD4+
82.6
CD4
CD4+
Memory
22.9
Naïve
56.8
Effector-memory
2.47
TEM
2.13
CD27
CD45RA
CD8+
Memory
7.61
Naïve
59.7
Effector-memory
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TEM
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CD27
CD45RA
(b)
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FSC-A
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FSC-H
Single Cells
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FSC-A
CD10
CD19+ CD10+
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CD19+ CD10-
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CD19
CD27
3.39
Resting memory
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TLM
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Naïve
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CD21
CD27
Plasmablasts
49.2
Activated memory
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CD20
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FSC-A
Lymphocytes
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FSC-H
Single Cells
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FSC-H
CD3-
16.9
CD3
FSC-H
CD56 dim
26.6
CD56 bright
2.81
CD56
(d)
SSC-A
FSC-A
Monocytes
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97.0
FSC-A
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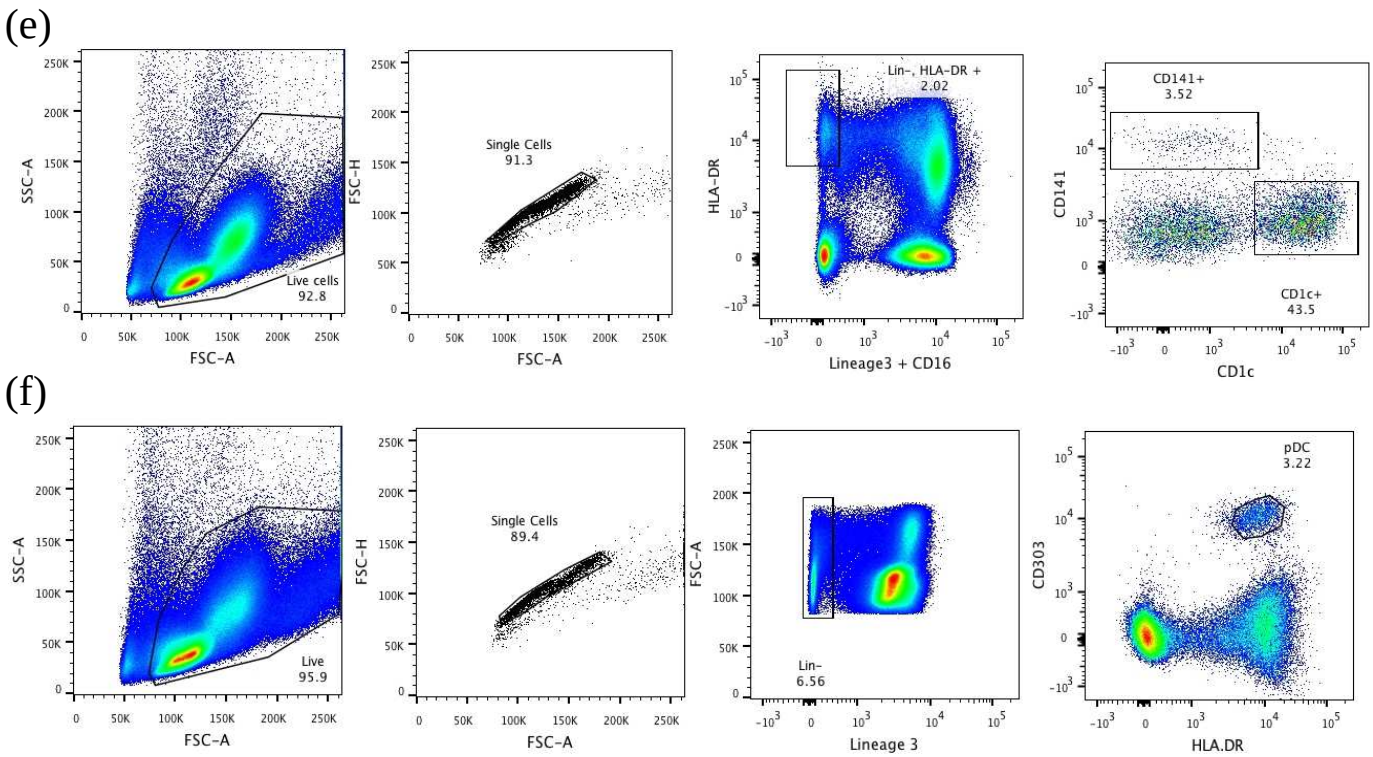
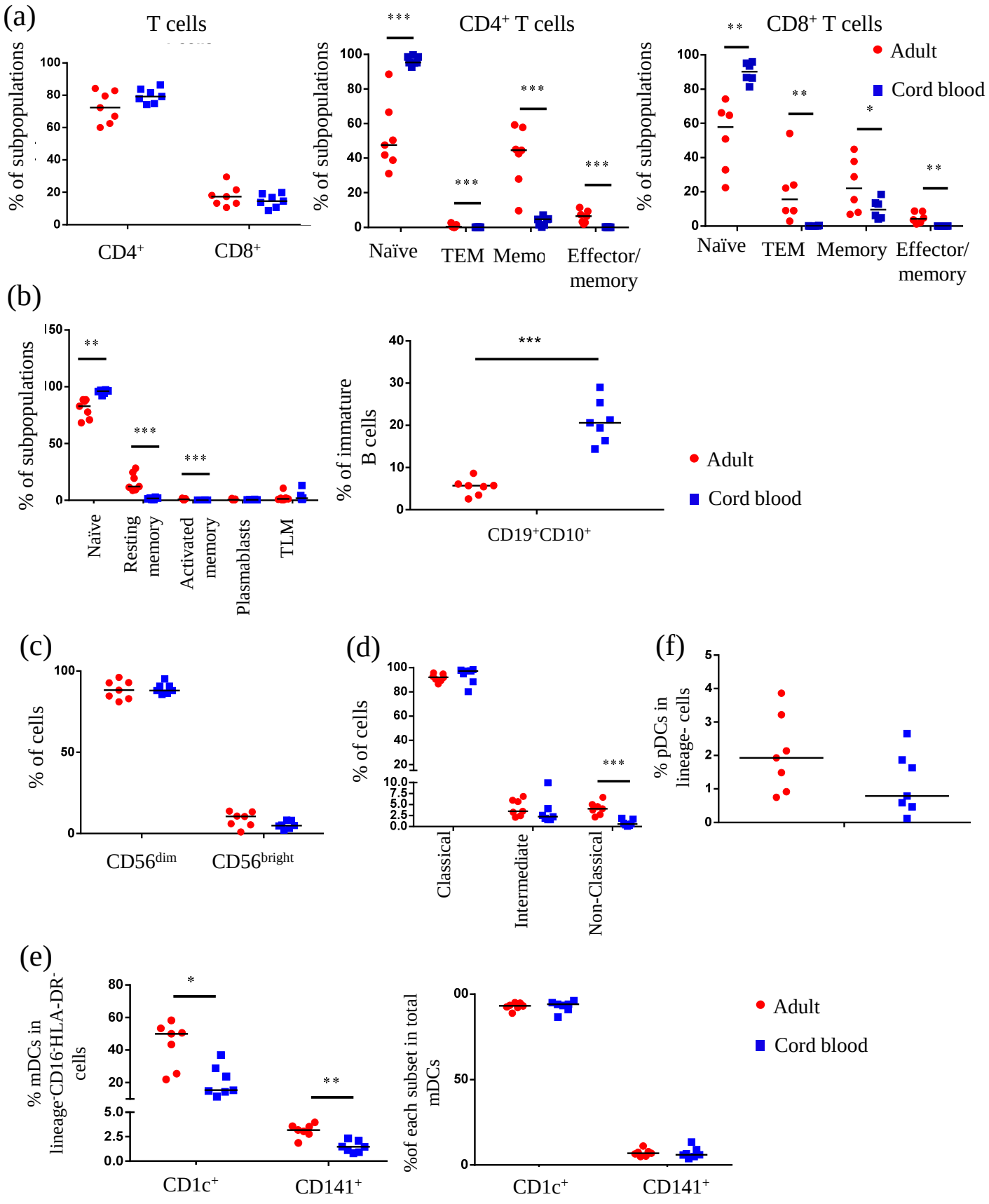
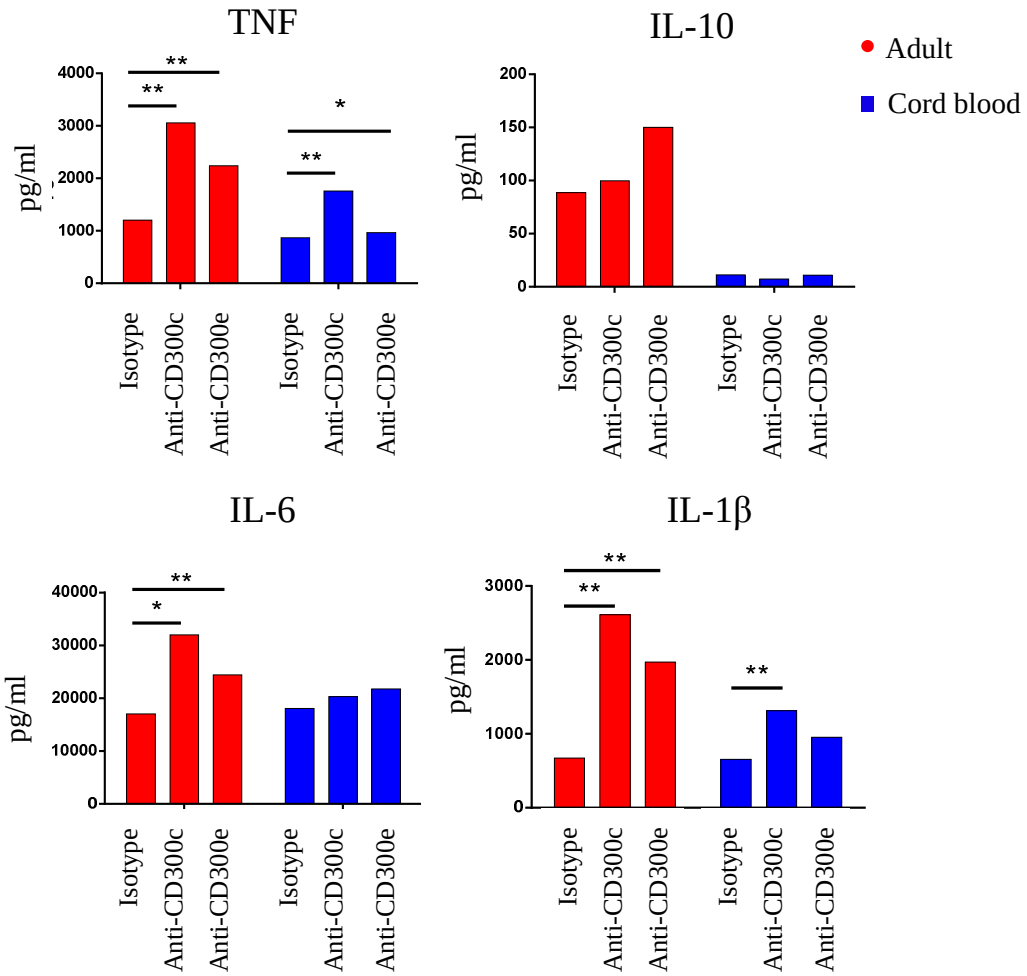


Supplementary Figure 1. Gating strategy used for the identification of cell subsets from adult PBMCs and CBMCs. First, cells were electronically gated according to forward and side scatter parameters. Then, single cells were gated according to their area and height. (a) T cells: CD3+ cells were gated and CD4+ and CD8+ subsets were distinguished within the CD3+ T cells. Naïve (CD45RA+CD27+), memory (CD45RA-CD27+), effector/memory (CD45RA-CD27-), and terminal effector/memory (TEM) (CD45RA+CD27-) T cells were distinguished within the CD3+CD4+ and CD3+CD8+ subsets defined by the expression of CD45RA and CD27. (b) B cells: CD19+CD10- (mature) and CD19+CD10+ (immature) B cells were distinguished. Five mature B cell subpopulations were distinguished defined by the expression of CD21, CD27 and CD20: naïve cells (CD21+CD27-), resting memory cells (CD21+CD27+), activated memory cells (CD21-CD27+CD20+), plasmablasts (CD21-CD27+CD20-) and tissue-like memory (TLM) B cells (CD21-CD27-). (c) NK cells: CD3- cells were gated and NK cells were identified as the CD3-CD56dim and CD3-CD56bright populations. (d) Monocytes: CD66b- cells were gated and classical (CD14++CD16-), intermediate (CD14++CD16+) and nonclassical (CD14+CD16+) monocytes were defined by the expression of CD14 and CD16. (e) mDCs: lin-CD16-HLA-DR+ cells were gated, and CD141+ and CD1c+ mDCs were distinguished within them. (f) pDCs: lin- cells were gated, and pDCs were identified within them defined by the expression of HLA-DR and CD303.



Supplementary Figure 2

Supplementary Figure 2. Adult and cord blood immune cell populations and subpopulations. The percentages of each cell populations and subpopulations in adult PBMCs and cord blood CBMCs were represented. Each dot represents a different donor, and the medians are represented. Adults are represented in red and cord blood in blue. (a) T lymphocytes. (b) B lymphocytes. (c) NK cells. (d) Monocytes. (e) mDCs. (f) pDCs. *$p<0.05$, ** $p<0.01$, ***$p<0.001$.



Supplementary Figure 3. Differential cytokine production after the engagement of CD300c and CD300e in adult and cord blood monocytes. Enriched monocytes from healthy adult and cord blood were either stimulated with plate bound isotype-matched control antibody, anti-CD300c mAb or anti-CD300e mAb in presence of LPS for 18 h. Culture supernatants were harvested and tested for the secretion of human inflammatory cytokines using flow-cytometric bead analysis. The values on the y-axis correspond to the concentration of cytokines TNF-α, IL-10, IL-6 and IL1-β. Each bar represents the medians. Adults are representing in red and cord blood in blue. *p<0.05, ** p<0.01.

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Monocytes Phenotype and Cytokine Production in Human Immunodeficiency Virus-1 Infected Patients Receiving a Modified Vaccinia Ankara-Based HIV-1 Vaccine: Relationship to CD300 Molecules Expression

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A modified vaccinia Ankara-based HIV-1 vaccine clade B (MVA-B) has been tested for safety and immunogenicity in low-risk human immunodeficiency virus (HIV)-uninfected individuals and as a therapeutic vaccine in HIV-1-infected individuals on combined antiretroviral therapy (cART). As a therapeutic vaccine, MVA-B was safe and broadly immunogenic; however, patients still showed a viral rebound upon treatment interruption. Monocytes are an important part of the viral reservoir and several studies suggest that they are partly responsible for the chronic inflammation observed in cART-treated HIV-infected people. The CD300 family of receptors has an important role in several diseases, including viral infections. Monocytes express CD300a, c, e, and f molecules and lipopolysaccharide (LPS) and other stimuli regulate their expression. However, the expression and function of CD300 receptors on monocytes in HIV infection is still unknown. In this work, we investigated for the first time the expression of CD300 molecules and the cytokine production in response to LPS on monocytes from HIV-1-infected patients before and after vaccination with MVA-B. Our results showed that CD300 receptors expression on monocytes from HIV-1-infected patients correlates with markers of HIV infection progression and immune inflammation. Specifically, we observed a positive correlation between the expression of CD300e and CD300f receptors on monocytes with the number of CD4+ T cells of HIV-1-infected patients before vaccination. We also saw a positive correlation between the expression of the inhibitory receptor CD300f and the expression of CD163 on monocytes from HIV-1-infected individuals before and after vaccination. In addition, monocytes exhibited a higher cytokine production in response to LPS after vaccination, almost at the same levels of monocytes from healthy donors.

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Furthermore, we also described a correlation in the expression of CD300e and CD300f receptors with TNF-α production in response to LPS, only in monocytes of HIV-1-infected patients before vaccination. Altogether, our results describe the impact of HIV-1 and of the MVA-B vaccine in cytokine production and monocytes phenotype.

Keywords: human immunodeficiency virus, monocytes, CD300, CD300c, CD300f, therapeutic vaccine, lipopolysaccharide, HIV-1 vaccine

INTRODUCTION

The development of combined antiretroviral therapy (cART) has significantly improved the clinical outcome in human immunodeficiency virus (HIV)-infected patients. However, long-term cART poses considerable side effects and costs, and stopping the treatment generally causes rapid viral rebounds, mostly due to the latent viral reservoirs (1, 2). For this reason, several strategies are being studied in order to achieve a permanent control of HIV replication inducing an effective antiviral T cell response. Among the most immunogenic approaches for inducing HIV-specific CD8+ T cell responses have been poxvirus vector boost vaccines (3, 4). Recently, a modified vaccinia Ankara vector expressing HIV-1 antigens clade B (MVA-B) was tested as a therapeutic vaccine. MVA-B was first tested with healthy volunteers (RISVAC02), which demonstrated that this vaccine was safe, well tolerated (5) and induced polyfunctional and durable T cell responses in most individuals (6). Importantly, it has also been tested as a therapeutic vaccine in a phase-I clinical trial in HIV-1-infected individuals on cART (RISVAC03), and the vaccination with MVA-B vaccine was also safe and broadly immunogenic. Nevertheless, HIV-1-infected patients still showed a viral rebound upon treatment interruption, and vaccination did not affect the viral reservoir even in combination with disulfiram, a drug able to reactivate latent HIV-1 (7, 8). The viral rebound after removal of cART has been linked to the fact that vaccination with MVA-B tips the balance between activation and regulation toward regulation of the response of HIV-specific CD8+ T cells (9). Nevertheless, in order to design more effective therapeutic vaccines, more studies are required to completely understand the effects on the host of the MVA-B vaccination.

Although latently infected CD4+ T cells comprise the majority of the HIV reservoir, monocytes (mainly CD16+ monocytes) provide an important part of this reservoir and also perpetuate HIV replication through ongoing cell-to-cell transfer of virions and efficient infection of CD4+ T cells, even in the presence of cART (10). In addition, recent studies suggest that monocytes are also responsible for the chronic inflammation in cART-treated HIV-infected people (11). In fact, it has been described that monocytes of chronically HIV-infected subjects differ from monocytes of healthy people in subsets distribution (12), expression of different markers (e.g., CD163) (13), and cytokine production (e.g., IL-6) (11). All these findings emphasize the importance of studying the mechanisms that regulate the activation of monocytes in HIV-infected patients.

The human CD300 molecules (a, b, c, d, e, f, g, h) are type I transmembrane proteins that, with the exception of CD300g which is expressed on endothelial cells, are found in both lymphoid and myeloid cell lineages. CD300a and CD300f are inhibitory receptors while CD300b, CD300c, CD300d, CD300e, and CD300h are activating receptors (14–16). Inhibitory receptors contain a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which are required for the inhibitory signaling. Activating receptors have a short cytoplasmic tail with a charged transmembrane amino acidic residue, that allows their association with adaptor proteins containing immunoreceptor tyrosine-based activating motifs and other activating motifs which induce activation signals (14, 16). CD300 molecules have an important role in several diseases, including viral infections (14, 16, 17). In the context of HIV infection, there are few publications describing the role of CD300 family. In HIV-infected patients, the expression of the CD300a inhibitory receptor is down-regulated on B lymphocytes, which may help to explain the hyperactivation and dysfunction of B cells observed in these individuals (18). Another important detail about CD300a involvement in the pathogenesis of HIV infection is given by the description of a positive correlation between mRNA levels of CD300a and the expression of BATF, a transcription factor that inhibit T cell function, in HIV-specific CD8+ T cells (19).

At least, monocytes express four members of this family: the CD300a and CD300f inhibitory receptors, and the CD300c and CD300e activating receptors. Among others, age and lipopolysaccharide (LPS) regulate the expression of these receptors (14, 16, 20). However, in HIV infection, the expression and function of CD300 receptors on monocytes is still unknown. In this work, we have analyzed the expression of CD300 molecules on monocytes from chronically HIV-1-infected patients and calculated the correlation with markers of HIV-1 infection progression (CD4+ T cell count) and immune inflammation (CD163 expression). Moreover, we investigated the effect of the vaccination with MVA-B in the cytokine production of monocytes stimulated with LPS in HIV-infected subjects and we studied the correlation with the CD300 family of molecules expression. Our results may contribute to a better knowledge of monocytes dysfunction in HIV-1 infection and the influence of the MVA-B therapeutic vaccine in these cells.

PATIENTS AND METHODS

Patients and Samples

Samples were obtained from HIV-1-infected patients enrolled in the RISVAC03 clinical trial (NCT01571466) (8). RISVAC03

is a double-blinded randomized phase-I trial in which cART-treated HIV-1-infected individuals received four intramuscular injections of MVA-B vaccine at weeks 0, 4, 16, and 36, combined with disulfiram for 3 months after the last dose of the vaccine. Specifically, in this study we have analyzed available frozen peripheral blood mononuclear cells (PBMCs) from eight HIV-1-infected patients before (week 0) and after last vaccination (week 48). Clinical data of HIV-1-infected patients are shown in **Table 1**. Frozen PBMCs from seven healthy donors (HD) available from the phase-I trial RISVAC02 (NCT00679497) (5) were also studied. Only cells from non-vaccinated healthy individuals were analyzed. The means of the percentages of viable cells after thawing were: 69.4 ± 4.55% (HD), 70.0 ± 3.33% (HIV-infected patients before vaccination), and 67.3 ± 3.59% (HIV-infected patients after vaccination). This study was approved by the Research Ethics Committee of Hospital Clìnic, Barcelona, Hospital Germans Trias i Pujol, Badalona and Hospital Gregorio Marañón, Madrid, Spain. All subjects that participated in RISVAC02 and RISVAC03 clinical trials provided written and signed informed consent (5, 8).

Flow Cytometry Analysis

The following anti-human fluorochrome conjugated antibodies were used for flow cytometric analysis: PE-Cy7 mouse anti-CD14 (clone MφP9), PerCP-Cy5.5 mouse anti-HLA-DR (clone G46-6), PE mouse anti-IL-1α (clone 364-3B3-14), and FITC rat anti-IL-6 (clone MQ2-13A5) from BD Biosciences; FITC mouse anti-CD16 (clone B73.1), BV421 mouse anti-CD163 (clone GHI/61), and APC mouse anti-TNFα (clone Mab11) from Biolegend; PE mouse anti-CD300a (clone E59.126) from Beckman Coulter; eFluor660 mouse anti-CD300c (clone TX45) from eBioscience; and APC mouse anti-CD300e (clone UP-H2) and PE mouse anti-CD300f (clone UP-D2) from Miltenyi Biotec. To test the viability of the cells, the 633–635 nm excitation LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies) was used. Frozen PBMCs from HD and HIV-1-patients were thawed, washed, and incubated at 37°C for 1–2 h in R10 (10% FBS and 1% Penicillin/Streptavidin in RPMI-1640 medium) medium with 10U of DNase (Sigma-Aldrich), in a concentration of 2×10^6 cells/ml. Afterward, cells were stained first with the LIVE/DEAD kit in order to detect dead cells, and then, they were incubated with different fluorochrome conjugated antibodies. Both steps were carried out for 30 min on ice protected from the light. PBMCs were fixed with 4% of paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C and washed two times with PBS. A FACSCanto II flow cytometer (BD Biosciences) was used for sample acquisition and data was analyzed with FlowJo 10.0.7 software (TreeStar).

LPS Stimulation and Intracellular Cytokine Staining (ICS)

Peripheral blood mononuclear cells from HD and HIV-1-infected patients were cultured (10^6 cells/ml) in R10 medium with 1 ng/ml of LPS (Sigma) for 5 h at 37°C, in the presence of GolgiStop protein transport inhibitor containing monensin, following manufacturer's indications (BD Biosciences). After the stimulation, PBMCs were stained with LIVE/DEAD kit, followed by incubation with different fluorochrome conjugated antibodies for extracellular staining. In order to accomplish the ICS, cells were first permeabilized with Cytofix/Cytoperm Plus Kit following the manufacturer's protocol (BD Biosciences) and then they were incubated with different fluorochrome conjugated antibodies for the detection of cytokines. Sample acquisition and data analysis were carried out as described before.

Data Representation and Statistical Analysis

GraphPad Prism software (version 6.01) was used for graphical representation and statistical analysis. Data were represented in dot plot graphs and bar graphs showing the mean with SEM, and pie chart graphs. Values obtained from different subject groups were compared with non-parametric tests; the comparison between HD and HIV-1-infected patients' data was made with the unpaired Mann–Whitney test; and differences between HIV-1-infected patients before and after vaccination were evaluated with the Wilcoxon matched-pairs signed rank test. Correlation analyses were done using the same software. In the case of cytokine production data, percentages of poly-functional, mono-functional, and non-functional cells were obtained by a Boolean gate analysis with FlowJo software and the representation of these data were done using GraphPad Prism software.

TABLE 1 | Clinical data of HIV-1-infected patients.

Patient	Undetectable VL (years)	CD4+ T cells Nadir (cells/mm^3)	CD4+ T cells before ART (cells/mm^3)	CD4+ T cells baseline (cells/mm^3)	Age	Sex	Weight (kg)	Coinfection hepatitis C virus	Time of known HIV infection (years)
101	9	179	368	541	49	M	74	No	14
103	1	290	489	530	50	M	69	No	10
107	2	274	274	866	41	M	68	No	12
108	2	396	396	823	33	M	73	No	12
109	4	645	688	1,179	39	M	65	No	6
110	12	376	376	1,238	40	F	56	No	15
111	3	296	396	632	44	M	78	No	6
112	2	507	680	794	39	M	60	No	3

VL, viral load; ART, antiretroviral therapy; HIV, human immunodeficiency virus.

RESULTS

CD300 Receptors Expression on Monocytes from HIV-1-Infected Patients Correlates with Markers of HIV Infection Progression and Immune Inflammation

We first determined the expression of CD300a, CD300c, CD300e, and CD300f molecules on monocytes from HD and chronically HIV-1-infected subjects that are receiving cART at baseline, i.e., just before starting the RISVAC03 clinical trial. Monocytes were electronically gated based on their forward and side scatter properties, and the expression of CD14 and CD16; concretely, classical (CD14++ CD16−), intermediate (CD14++ CD16+), and non-classical (CD14+ CD16++) monocytes were analyzed (Figure S1A in Supplementary Material). As it has been described before (10, 12), the percentages of intermediate and non-classical monocytes were slightly increased in HIV-1-infected patients in comparison with HD (Figure S2 in Supplementary Material). The expression of four members of the CD300 receptor family was tested: the inhibitory receptors CD300a and CD300f, and the activating receptors CD300e and CD300c. We did not observed significant differences in the expression of CD300 receptors on monocytes of HIV-1-infected patients compared with HD (**Figure 1A**), not even when we separately analyzed each monocyte subpopulation (Figure S3 in Supplementary Material). In spite of that, we observed a tendency, although not statistically significant, of CD300c expression to decrease on monocytes of HIV-1-infected subjects [HD median fluorescence intensity (MFI) = 2,717 ± 630.4 vs HIV MFI = 1,596 ± 465.5] (**Figure 1A**), especially in non-classical monocytes (data not shown).

Next, we investigated the association between CD300 receptors expression and patients' clinical features. Clinical data, which consists mainly of CD4+ T cell numbers, are shown in **Table 1**. CD300a and CD300c receptor expression on monocytes did not correlate with the number of CD4+ T cells at baseline (data not shown); however, the expression of CD300e ($p < 0.05$, $r = 0.7820$) and CD300f ($p < 0.05$, $r = 0.7592$) receptors was positively correlated with the CD4+ T cell numbers (**Figure 1B**).

Afterward, the expression of the CD163 receptor was analyzed and calculated the correlation with CD300 molecules expression in monocyte subpopulations. CD163 is a scavenger receptor, expressed exclusively on monocytes and macrophages, that has been investigated as a potential inflammation marker in different infectious diseases (13). In fact, sCD163 plasma levels are elevated in chronically HIV-1-infected patients and this has been related to a higher risk of comorbid disorders (11). We saw that CD163 expression of classical (HD MFI = 1,025 ± 106.7 vs HIV MFI = 1,744 ± 243.8) and intermediate (HD MFI = 1,079 ± 175.3 vs HIV MFI = 1,200 ± 158.2) monocytes was higher in HIV-1-infected subjects than in HD; unlike non-classical monocytes, which exhibited a very low expression in both groups (**Figure 1C**). Correlation analysis showed that in monocytes of HD, CD163 and CD300 receptors expression were not associated (data not shown). In contrast, there was a positive correlation between CD163 and CD300c expression ($p < 0.05$, $r = 0.7234$) on monocytes of HIV-1-infected subjects, and also between CD163 and CD300f expression ($p < 0.01$, $r = 0.9559$) in intermediate monocytes of HIV-1-patients (**Figure 1D**).

Effects of MVA-B Vaccination on Monocytes from HIV-Infected Subjects

The safety and immunogenicity of the MVA-B vaccine in chronically HIV-1-infected patients and healthy people has been previously tested (6–8). This vaccine improves the magnitude of HIV-specific T cell responses (6, 7), although it does also tilt the balance between activation and regulation of T cell specific responses toward regulation (9), somehow explaining the viral rebound after removal of cART in patients that has received the vaccine. However, the effects of vaccination in other immune cells have not been studied. Considering that monocytes play an important role in chronic inflammation characteristic of HIV-1-infected subjects (11), we studied the phenotype and cytokine production of monocytes in HIV-1-infected patients after vaccination with MVA-B and we compared them with monocytes from the same patients before vaccination.

First, the expression of CD163 and CD300 surface receptors was determined in HIV-1-infected patients before and after the vaccination with MVA-B. The percentages of monocyte subpopulations in vaccinated HIV-1-infected individuals were very similar to the percentages found before the vaccination (Figure S2 in Supplementary Material). The expression of CD300 molecules was determined and we observed that the expression pattern in monocytes of HIV-1-infected patients before and after vaccination was almost identical (**Figure 2A**, left panel). CD163 expression on monocytes was not significantly different when compared before and after vaccination. However, on intermediate monocytes (HIV before vaccination MFI = 1,103 ± 153.4 vs HIV after vaccination MFI = 793.6 ± 173.8), CD163 tended, although not statistically significant, to be down-regulated in patients after vaccination, while in classical and non-classical monocytes CD163 expression was very similar before and after vaccination (**Figure 2A**, middle panel). Lastly, we analyzed the correlation between the expression of CD300 receptors and CD163 receptor, and no significant values were observed in any case, except for a positive correlation between the levels of CD300f and CD163 ($p < 0.05$, $r = 0.9275$) on intermediate monocytes, as it was found before vaccination (**Figure 2A**, right panel).

Afterward, PBMCs from HD and HIV-1-infected patients, before and after vaccination, were stimulated with 1 ng/ml of LPS for 5 h, followed by ICS in order to study IL-6, IL-1α, and TNFα production in monocytes. These were gated according to their forward and side scatter properties, and they were defined as CD14++ HLA−DR+. In our hands, monocyte subpopulations were not distinguished due to the down-regulation of CD16 receptor after LPS stimulation (data not shown). Positive cells for each cytokine were determined based on non-stimulated cells. First, we checked the level of cytokine production by the stimulated cells by MFI of cytokine staining, a value known to be correlated with the amount of cytokine produced by cells (21). We observed that monocytes from HIV-1-infected subjects produced less IL-6 and TNFα than monocytes from HD in response

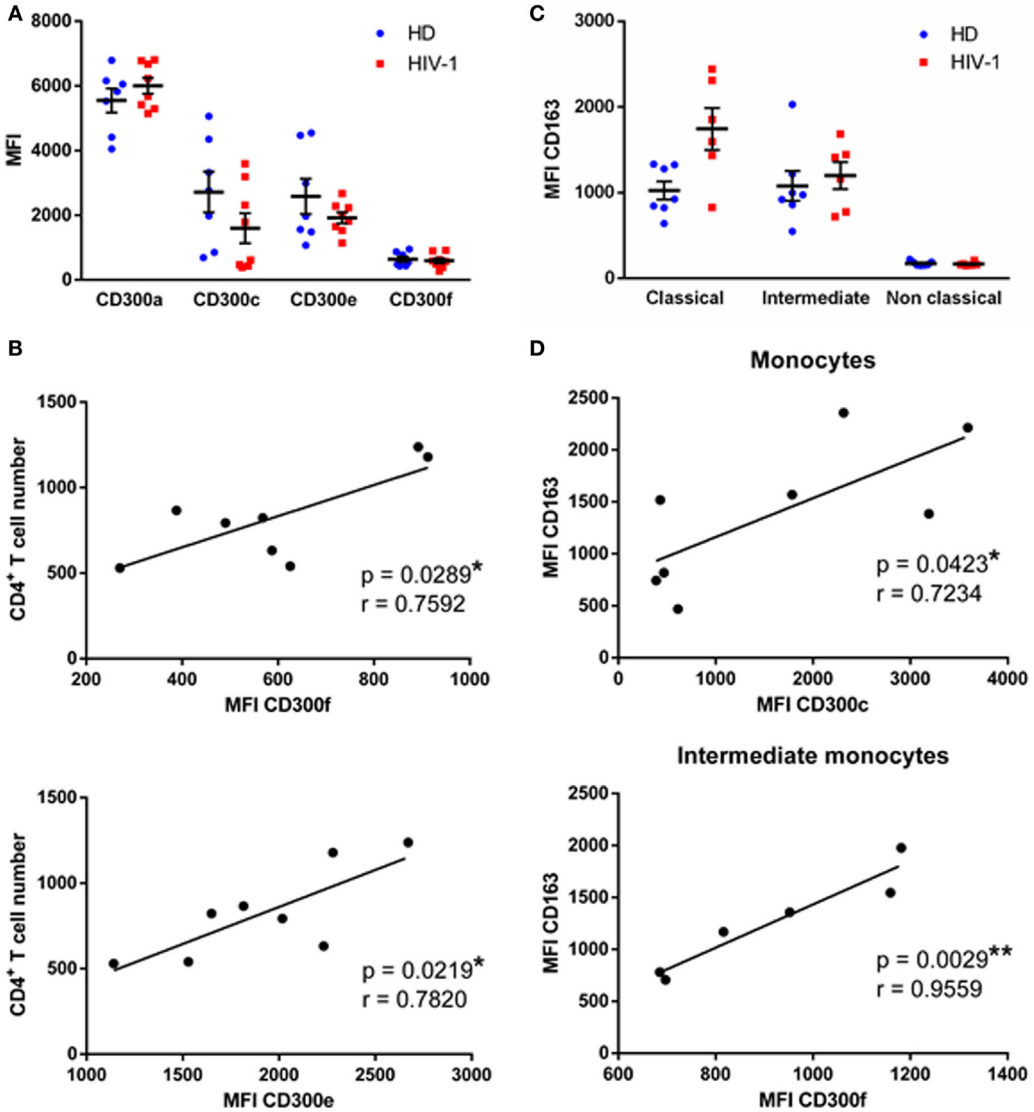

FIGURE 1 | CD300 receptors expression in human immunodeficiency virus (HIV)-1-infected patients. **(A)** Dot plot graph presenting the median fluorescence intensity (MFI) of CD300a, CD300c, CD300e, and CD300f receptors expression on monocytes from healthy donors (HD) and HIV-1-infected patients. Each dot corresponds to an individual and the mean with the standard error of the mean (SEM) is shown. **(B)** Correlation between CD4+ T cell number at baseline of the study and the MFI of CD300f and CD300e receptors expression on monocytes from HIV-1-infected patients is represented; the linear regression is shown. **(C)** Dot plot graph representing the MFI of CD163 receptor expression on classical, intermediate, and non-classical monocytes from HD and HIV-1-infected individuals. Each dot corresponds to an individual and the mean with SEM is shown. **(D)** Correlation between the MFI of CD163 and CD300c receptors expression on total monocytes and CD163 and CD300f receptors expression on intermediate monocytes from HIV-1-infected patients; the linear regression is shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

to LPS. Interestingly, monocytes of vaccinated HIV-1-infected patients produced higher levels of IL-6, IL-1α, and TNFα in response to LPS after vaccination. Although IL-6 levels in vaccinated patients remained lower than in HD, TNFα production in vaccinated subjects reached the same levels as those from HD (**Figure 2B**). Moreover, analysis showed that the percentage of triple positive (IL-6+IL-1α+TNFα+) monocytes in response to LPS was higher in vaccinated HIV-1-infected subjects compared with the percentage of triple positive monocytes from the same patients before vaccination. On the other hand, the percentage of only double positive (IL-6-IL-1α+TNFα+) monocytes was higher in patients before the vaccination. These results indicate that monocytes of HIV-1-infected subjects were more polyfunctional in response to LPS stimulation after vaccination than before vaccination. As expected, although differences were not significant, probably due to the small sample, it was observed a higher percentage of non-cytokine (IL-6−IL-1α−TNFα−) producing monocytes from patients before vaccination than in monocytes after vaccination and from HD (HD = 7.63% vs HIV no vaccinated = 9.23% vs HIV vaccinated = 6.45%) (**Figure 2C**).

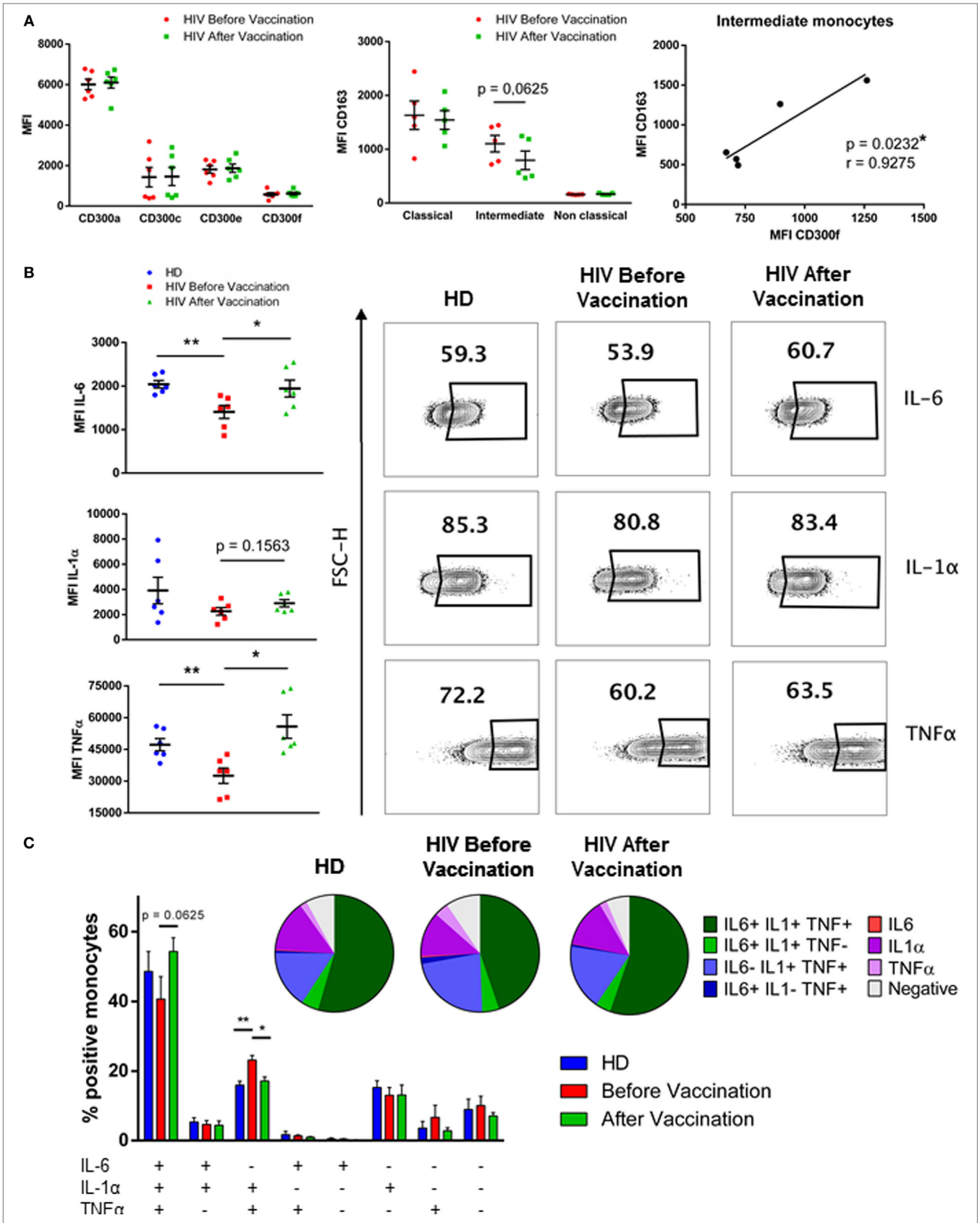

FIGURE 2 | Continued

FIGURE 2 | Continued
Phenotypical analysis and cytokine production of monocytes from HIV-1-infected patients after vaccination with MVA-B vaccine. **(A)** Dot plot graph (left panel) displaying the median fluorescence intensity (MFI) of CD300a, CD300c, CD300e, and CD300f receptors expression on monocytes from HIV-1-infected patients before (HIV before vaccination) and after (HIV after vaccination) vaccination. Each dot corresponds to an individual and the mean with SEM is shown. Dot plot graph (middle panel) representing the MFI of CD163 receptor expression on classical, intermediate, and non-classical monocytes from HIV-1-infected individuals before and after vaccination. Each dot corresponds to an individual and the mean with SEM is shown. The correlation between the MFI of CD163 receptor and the MFI of CD300f on intermediate monocytes of HIV-1-infected patients is represented (right panel); the linear regression is shown. **(B)** Dot plot graphs showing the MFI of positive monocytes for each cytokine; the mean with SEM is represented (left). Contour plots representing the percentage of positive monocytes for each cytokine after stimulation with lipopolysaccharide. Data from a representative healthy donor (HD) and an HIV-1-infected patient before and after vaccination are shown (right). **(C)** Boolean gate analysis representing the percentages of monocytes producing IL-6, IL-1α, and TNFα, in HD and HIV-1-infected patients before and after vaccination. Bar graphs showing the mean with SEM and pie charts are represented. $^{*}p < 0.05$, $^{**}p < 0.01$.

In conclusion, monocyte cytokine production in response to LPS in HIV-1-infected patients was higher after vaccination and resembled that observed in HD.

Relationship between CD300 Receptors Expression and Cytokine Production by Monocytes of HIV-1-Infected Patients Before and After Vaccination

The last step of the work was to investigate if the expression levels of CD300 molecules could have a correlation with the increased functionality found after the MVA-B vaccination in monocytes of HIV-1-infected individuals. We performed correlation analysis between CD300 receptors expression and cytokine production in response to LPS. The expression of CD300 molecules was not correlated with the percentage of IL-6+ monocytes in any case. In contrast, the expression of CD300e and CD300f correlated with IL-1α and TNFα production. The correlation with IL-1α production was only observed in monocytes from HD (data not shown); however, the expression of CD300e ($p < 0.05$, $r = 0.7505$) and CD300f ($p < 0.01$, $r = 0.8873$) was positively correlated with TNFα production in monocytes of HIV-1-infected patients before vaccination (**Figure 3B**). The percentages of TNFα+ monocytes of HD and vaccinated patients were not correlated with the MFI of CD300e and CD300f (**Figures 3A,C**). In fact, as it can be observed in the graphical representation (**Figure 3**), monocytes from HIV-1-infected patients are more similar to those from HD than to the monocytes from the same patients before vaccination. Taking altogether, we could propose that the monocyte phenotype and functional pattern in response to LPS stimulation of HIV-1-infected patients after vaccination with MVA-B are more similar to those found in monocytes from HD than from monocytes from HIV-1-infected subjects before vaccination.

DISCUSSION

Monocytes have been described as one of the cell types involved in the chronic inflammation characteristic of cART-treated HIV-1-infected people, which is currently the cause of death of the majority of HIV-1-patients (11). High numbers of circulating intermediate and non-classical monocytes have been associated with inflammation and immune activation during HIV infection (10). Furthermore, inflammatory mediators (e.g., IL-6) secreted by monocytes predict serious non-AIDS events in virologically suppressed HIV-infected subjects (11). Three main mechanisms have been proposed to explain the monocyte activation and consequently, the inflammation found in cART-treated HIV-infected patients: the microbial translocation, which augments LPS levels in plasma, the residual HIV viremia, and coinfection with human cytomegalovirus or some herpesviruses (11).

Since the CD300 family of receptors are able to modulate monocytes function (20, 22–24), our first objective was to investigate the CD300 receptors expression in monocytes from cART-treated chronically HIV-1-infected patients. Our results revealed that the expression pattern of CD300 molecules in monocytes from HD and in monocytes from HIV-1-infected people were not significantly different. However, we observed that the expression of CD300c tended, although not statistically significant, to be down-regulated in monocytes from HIV-1-infected patients, in comparison with monocytes from HD. This could be explained in part with the increase of the percentage of non-classical monocytes in HIV-1-infected patients, which express lower levels of CD300c than classical monocytes (Figure S3 in Supplementary Material) (20). It is important to keep in mind that many immunological abnormalities observed during the course of HIV infection can be reversed by cART, and therefore it is possible that the expression of CD300 molecules is altered in non-cART-treated patients with detectable viremia. More studies with blood samples from viremic patients are needed to obtain a more complete picture on the expression of the CD300 molecules during HIV infection. We did found a significant correlation between the expression of the activating receptor CD300e and the inhibitory receptor CD300f in monocytes with CD4+ T cell count in patients whose viremia is controlled by undergoing cART. These results may suggest that the levels of expression of CD300e and CD300f on monocytes could potentially be used as biomarkers of disease progression in combination with the well know predictive value of CD4+ T cell count (25, 26). Prospective studies with larger cohorts will confirm the predictive value of CD300e and CD300f expression on monocytes from HIV-infected patients.

We have not seen a significant increase in the expression of CD163 on monocytes from HIV-infected patients compared with monocytes from HD. Somehow, our results are different from those reported by others (13). We believe that this discrepancy is due to the low number of patients we have studied, since it is possible to observe a tendency, although not statistically significant, to increase CD163 cell surface expression on monocytes from HIV-infected individuals. Interestingly, there was a positive correlation between the expression of CD300f and CD163 in intermediate monocytes, a subset with a significant role in inflammation (27).

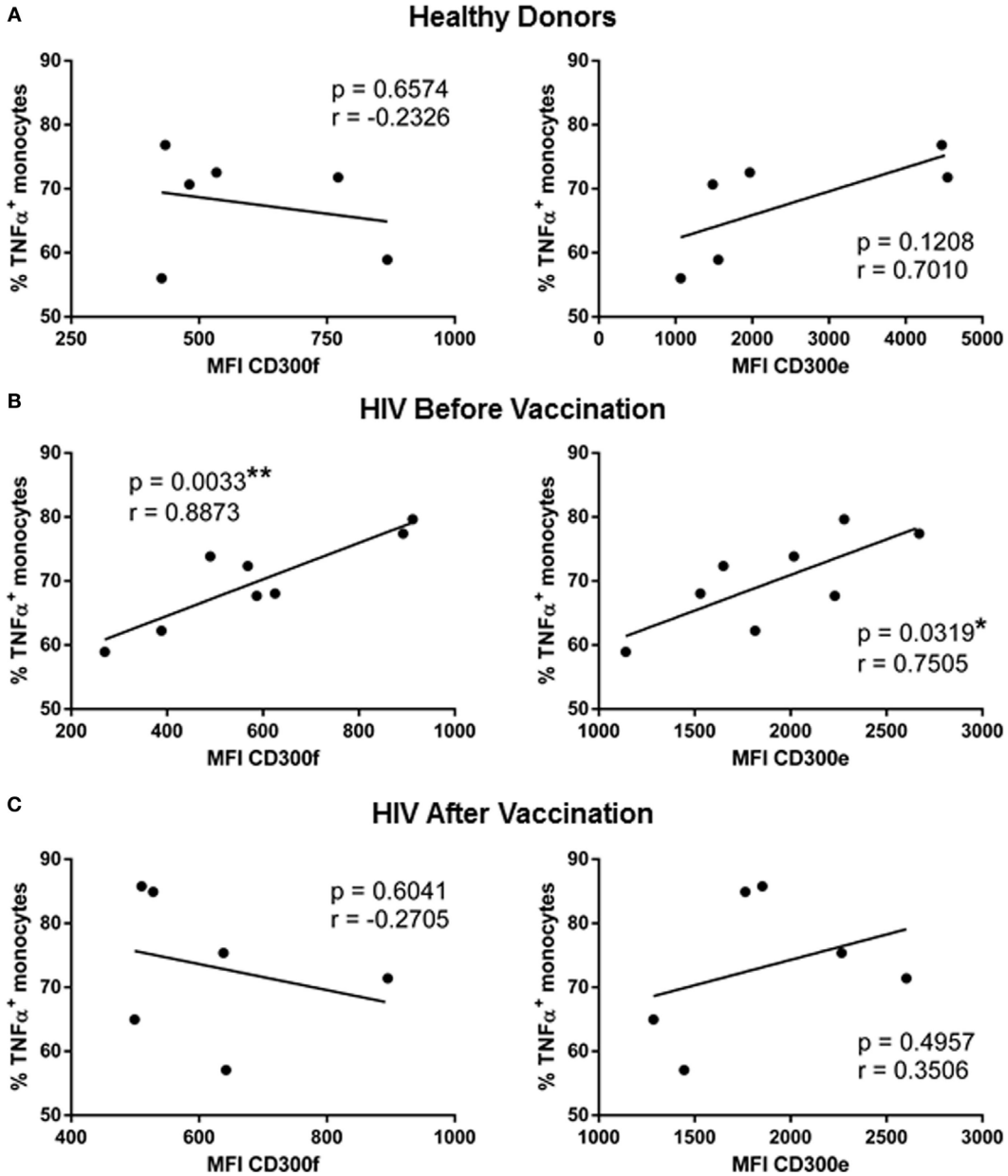

FIGURE 3 | Correlation analysis of TNFα production with the expression of CD300 receptors in human immunodeficiency virus (HIV)-1-infected patients before and after vaccination with MVA-B. Representation of the correlation between the percentage of TNFα positive monocytes and the median fluorescence intensity of CD300f and CD300e receptors expression, in healthy donors **(A)** and HIV-1-infected patients before **(B)** and after **(C)** vaccination with MVA-B; the linear regression is shown in each graph. *$p < 0.05$, **$p < 0.01$.

The positive correlation between the expression of CD300f and CD163 was maintained after vaccination. These results also suggest that the expression of CD300f, along with other markers, could be used as a biomarker of inflammation in HIV-infected patients. Human and mouse CD300f is commonly considered an inhibitory receptor because of the presence of ITIMs motifs in its intracellular tail (14). Several publications have shown its inhibitory role on monocyte cell lines (28–30). However, it has also been demonstrated that CD300f is able to deliver activating signals through motifs reported to bind the p85α regulatory subunit of PI3K (YxxM) (31–33). *In vivo* models in mice have shown that CD300f both inhibits and promotes the development of autoimmune diseases and allergic and inflammatory responses (34–39). This dual role of CD300f somehow may depend, not only on the cell type this intriguing receptor is expressed, but also on its described association with other receptors and adaptor proteins (33, 38, 40, 41). It would be of great interest to determine the signaling pathways of CD300f on monocytes during HIV infection, and determine if this receptor has different roles in monocytes from HD and HIV-1-infected patients.

Several therapeutic vaccines have been tested with the objective of controlling viral replication and to avoid viral rebound after treatment interruption in chronically HIV-1-infected patients (42, 43). MVA-B is an immunogenic vaccine which induces a T cell response in HIV-1-infected patients (7, 8). As expected, we did not observe any significant differences in the expression of CD300 molecules in monocytes of HIV-1-infected patients before and after vaccination. The most intriguing finding of this study was that the response of monocytes to LPS stimulation from patients after vaccination was different from the response before the vaccination, and at the same time similar to the response of monocytes from HD. Monocytes from non-vaccinated HIV-1-infected patients produced less cytokines in response to LPS than HD. This is in agreement with previous findings showing that HIV impairs TNFα production by human macrophages in response to Toll-like receptor 4 stimulation (44). Furthermore, this lower production of cytokines could also be due to the fact that monocytes when are chronically stimulated *in vivo* during chronic HIV infection become refractory to further stimulation with LPS *in vitro* (45), and it has been published that ART-treated infected patients exhibit higher levels of LPS in plasma than HD (46).

Vaccination with MVA-B induced higher levels of IL-6, IL-1α, and TNFα by monocytes in response to LPS. In fact, monocytes of vaccinated subjects exhibited a functional pattern more similar to the one of HD than to non-vaccinated HIV-1-infected patients. Furthermore, when we investigated if the expression of CD300 receptors might be correlated with the cytokine production levels, we also observed that the results were comparable between HD and HIV-1-infected patients after vaccination, and not between patients before and after vaccination. For example, the expression of CD300e and CD300f was positively correlated with TNFα levels in monocytes of HIV-1-infected subjects before vaccination, but not after vaccination or in monocytes of HD. We do not know the causes of this increase in the production of pro-inflammatory cytokines by monocytes in response to LPS after vaccination and if our results have some role in the lack of efficacy of the MVA-B vaccine as shown by a viral rebound after treatment interruption. It is possible that tipping the balance between activation and regulation toward regulation of the response of HIV-specific CD8+ T cells is not the only factor responsible for the lack of efficacy of the MVA-B vaccine. On the one hand, and considering our results showing lower CD163 expression on monocytes after vaccination, it seems that the administration of MVA-B vaccines may favor a less inflammatory environment. However, on the other hand, monocytes after vaccination have the potential to produce higher levels of pro-inflammatory cytokines and therefore could help to explain the lack of efficacy of the vaccine due to higher inflammation (10, 47–49). Also, it is important to remember that these patients have received disulfiram along with the MVA-B vaccine. Although the effect of disulfiram in monocytes of HIV-1-infected patients is unknown, several publications suggest that this drug have a role in decreasing the production of inflammatory mediators by monocytes. For example, it has been described that this compound diminishes the number of inflammatory cells and TNFα levels in the aqueous humor, in rats with endotoxin-induced uveitis (50). Furthermore, diethyldithiocarbamate, the active compound produced *in vivo* from disulfiram, impairs the release of oxygen metabolites and prostaglandins of human monocytes, two major pathways related to inflammatory processes (51). Undoubtedly, further research is required to delineate the role of monocytes in the efficacy of therapeutic vaccines.

In conclusion, our results have shown that vaccination with MVA-B, in addition to induce a specific T cell response, has also an effect on monocytes phenotype and their ability to produce cytokines after stimulation with LPS. We acknowledge that the number of patients included in this study is low and that it is very possible that a higher number of patients will provide more robust results. Clearly, more studies would be required to determine if the MVA-B mediated effect on monocytes favors the efficacy of the vaccine, or by the contrary is counterproductive. However, we believe that the results obtained with this work may form the basis of future studies to determine the functionality and phenotype of monocytes from patients enrolled in clinical trials testing therapeutic vaccines.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethical Committee of Hospital Clìnic, Barcelona, Hospital Germans Trias i Pujol, Badalona, and Hospital Gregorio Marañón, Madrid, Spain with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Hospital Clínic, Barcelona, Hospital Germans Trias i Pujol, Badalona, and Hospital Gregorio Marañón, Madrid, Spain.

AUTHOR CONTRIBUTIONS

JV designed the study, designed and performed experiments, analyzed, and interpreted the data, designed the figures, and wrote the manuscript. OZ performed experiments and interpreted the data. IT designed the figures. MP participated in the design of the study and interpreted the data. AG participated in analysis and interpretation of the data. LL recruited and followed the patients and was responsible of vaccinations and clinical monitoring. JP interpreted the data. FG recruited and followed the patients and was responsible of vaccinations and clinical monitoring. FB conceived and designed the study, interpreted the data, and wrote the manuscript. All the authors critically reviewed, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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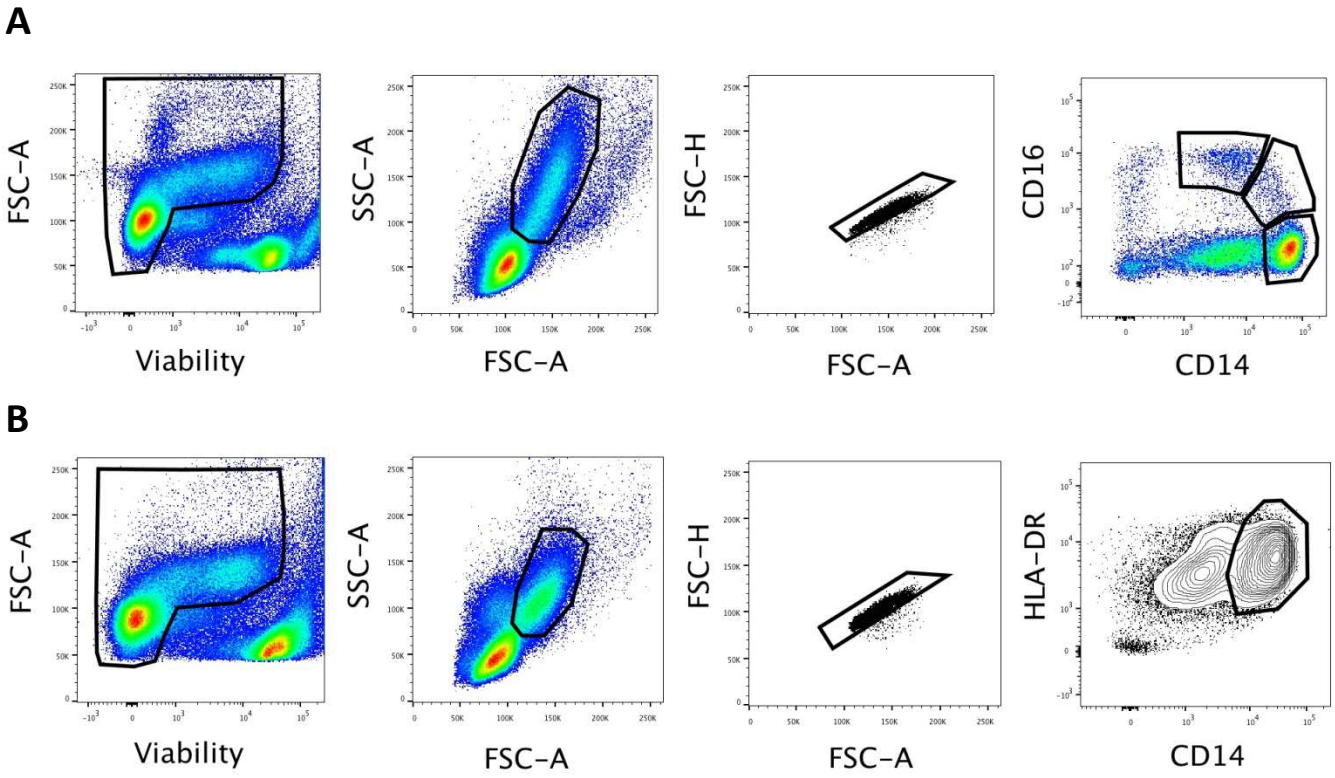
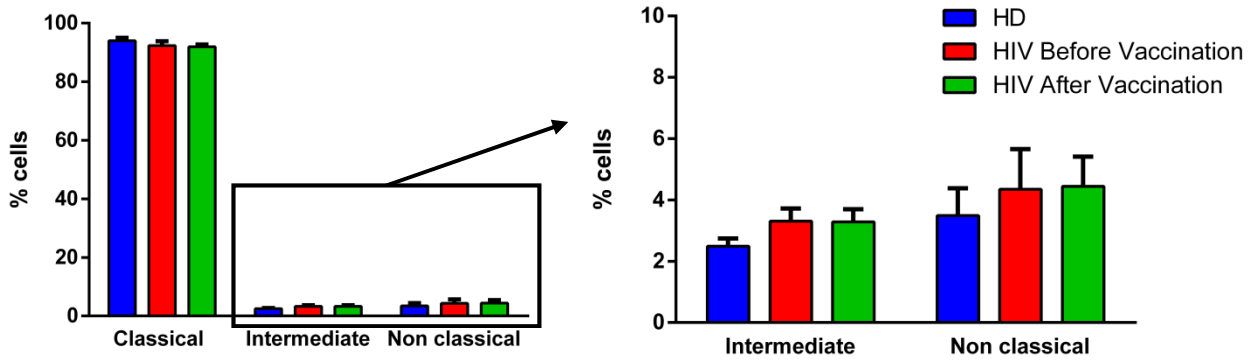
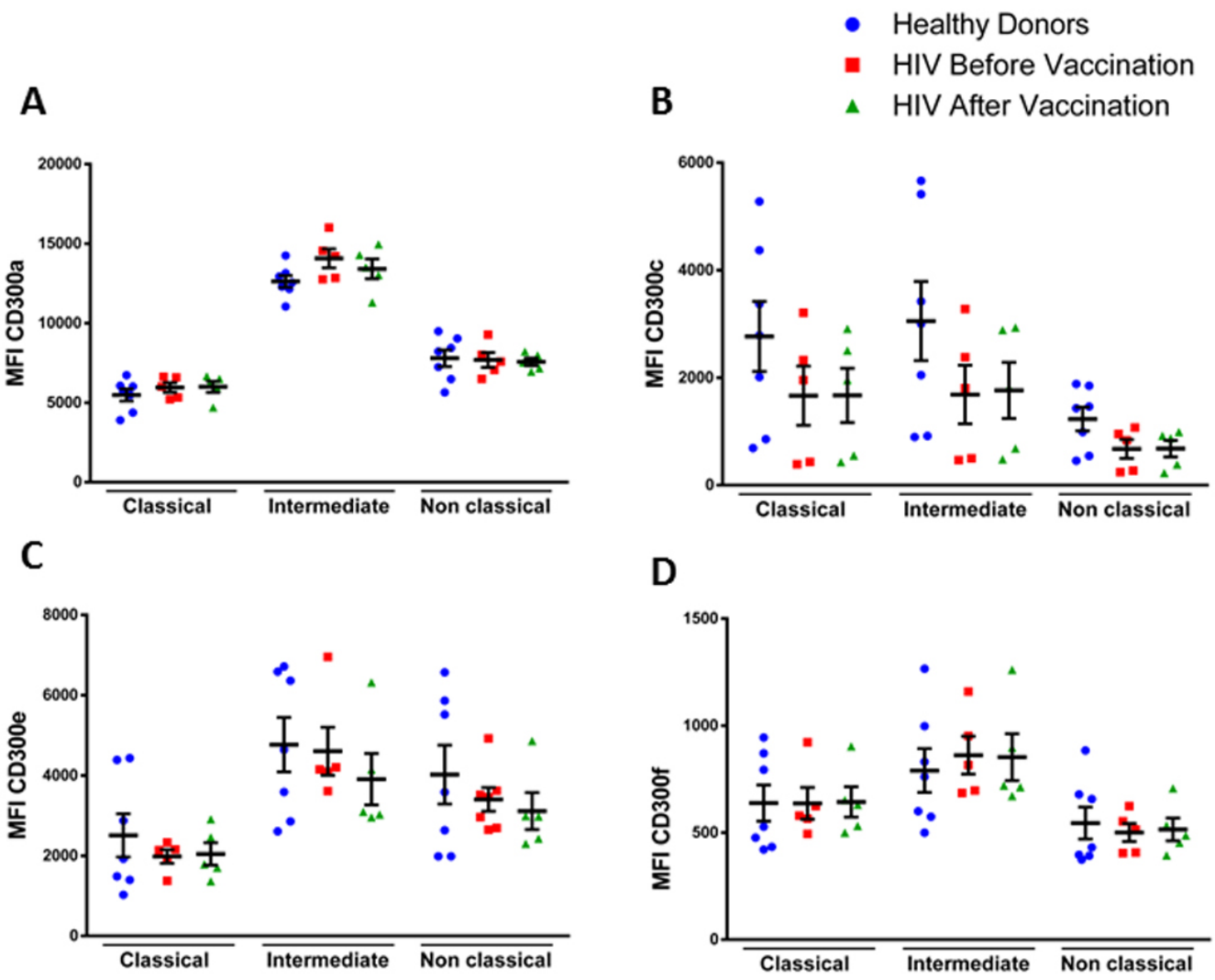
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



Supplementary figure 1. Gating strategy for phenotypic and functional analysis of monocytes and its subpopulations. (A) For phenotypic analysis, live PBMCs were selected and then cells were electronically gated based on their forward and side scatter parameters. Afterwards, doublets were excluded. The expression of CD14 and CD16 receptors were used to differentiate three monocyte subpopulations: classical (CD14++ CD16-), intermediate (CD14++ CD16+) and non-classical (CD14+ CD16++) monocytes. CD14+ CD16- cells were excluded. **(B)** To analyze the cytokine production of monocytes in response to LPS, first live PBMCs were selected and then cells were electronically gated based on their forward and side scatter parameters. Afterwards, doublets were excluded. Finally, monocytes were selected according to high expression of CD14 and HLA-DR.



Supplementary figure 2. Percentage of monocyte subpopulations from healthy donors and HIV-1-infected patients. Bar graphs represent the percentage of classical, intermediate and non-classical monocytes from healthy donors and HIV-1-infected patients before and after vaccination with MVA-B; the mean with SEM is shown.



Supplementary figure 3. CD300 receptors expression on monocyte subpopulations. Dot plot graphs representing the median fluorescence intensity (MFI) of CD300a **(A)**, CD300c **(B)**, CD300e **(C)** and CD300f **(D)** on classical (CD14++ CD16-), intermediate (CD14++ CD16+) and non-classical (CD14+ CD16++) monocytes from healthy donors and HIV-1-infected patients before and after vaccination with MVA-B. Each dot corresponds to an individual and the mean with the standard error of the mean (SEM) is shown.

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CD300a inhibits CD16-mediated NK cell effector functions in HIV-1-infected patients

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Natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) through CD16 plays a critical role in anti-human immunodeficiency virus (HIV) responses.[1–3] CD300a is a surface receptor highly expressed on NK cells that has the capacity to inhibit NK cell-mediated cytotoxicity in healthy donors.[4] The CD300a molecule has been related to several viral infections and is able to diminish the NK cell killing of pseudorabies-infected cells through interactions with its ligands phosphatidylserine and phosphatidylethanolamine.[5] In addition, CD300a expression on B and CD4+ T lymphocytes is altered during HIV-1 infection, and combined antiretroviral therapy (cART) does not restore nonpathological expression levels.[5,6] However, the expression and function of CD300a on NK cells during HIV-1 infection is still unknown. We have determined the surface expression of CD300a on different NK cell subsets and the capacity of this receptor to inhibit CD16-induced NK cell effector functions in healthy and HIV-1 infected individuals.

As HIV-1 infection modulates CD300a expression on some immune cells,[5,6] we first analyzed the expression of the CD300a receptor on different NK cell subpopulations from healthy donors, untreated HIV-1-infected subjects, and patients on cART by flow cytometry. The samples were provided by the HIV BioBank integrated in the Spanish AIDS Research Network (RIS) (see Supplementary Material). Clinical data from HIV-1-infected patients are shown in Table S1. Three different NK cell subsets were studied: $CD56^{bright}$ ($CD56^{++}NKp80^{+}$), $CD56^{dim}$ ($CD56^{+}NKp80^{+}$), and $CD56^{neg}$ ($CD56^{-}NKp80^{+}$) (Fig. S1). When we examined the CD300a expression, no significant differences were observed between the groups, with the exception of $CD56^{neg}$ NK cells, which displayed a higher frequency of CD300a+ cells in untreated HIV-1 infected patients (Fig. S2). Ongoing HIV replication induces the expansion of a dysfunctional $CD56^{neg}$ NK cell subset[1,7,8] (Fig. S1). Thus, we suggest that the overexpression of the CD300a inhibitory receptor on the $CD56^{neg}$ NK cell subset may contribute to the dysfunctionality observed in this expanded population in HIV-1 infected patients, which is partially restored with cART.

We also examined CD300a expression on different NK cell subsets selected according to the expression of NKG2A, NKG2C, CD57, and NKp46 (Fig. S3). These receptors are altered during HIV-1 infection,[7,8] and some of them are commonly used to distinguish NK cell maturation stages.[9] In general, we observed that different CD300a expression levels were associated with the expression of these markers in all subjects (Fig. S4). Specifically, higher CD300a expression was found on NKG2A+ $CD56^{dim}$ NK cells, while CD57+ cells displayed lower CD300a expression levels (Fig. S4), indicating that CD300a is more expressed on immature $CD56^{dim}$ NK cells, a cell subset that is significantly decreased in HIV-1 infected patients.[3,7]

NK cells express the FcγRIIIA (CD16) surface receptor, which is responsible for ADCC.[1,2] To investigate the capacity of CD300a to inhibit CD16-mediated NK cell activation in HIV-1-infected patients, we performed a redirected lysis assay (Fig. S5). We cocultured NK cells with the Fc receptor-bearing cell line P815. CD16 and CD300a from NK cells were triggered with specific mAbs, and the MOPC-21 isotype control was utilized as a negative control (Fig. S5). To study NK cell effector functions, we determined the percentage of NK cells positive for the degranulation marker CD107a, the cytokines interferon (IFN)γ and tumor necrosis factor (TNF), and the chemokine macrophage inflammatory protein (MIP)-1β utilizing flow cytometry-based procedures (see Supplementary Material).

In agreement with the literature,[1,3,7] the $CD56^{dim}$ NK cell subset displayed the highest response to CD16-mediated stimulation, and NK cell effector functions were significantly diminished in HIV-1 infected patients (Fig. S6). Very importantly, we observed that all NK cell subsets from the three groups exhibited lower effector functions after the CD16-mediated stimulation and cross-linking of CD300a with specific mAbs (Fig. 1a, b). Moreover, when we compared the degranulation and MIP-1β production by different NK cell subsets, we observed that $CD56^{bright}$ cells were the most inhibited subset after CD300a cross-linking in all donors (Fig. S7), consistent with the higher CD300a expression in this NK cell subpopulation (Fig. S2). Finally, we observed a higher CD300a-mediated inhibition of degranulation and MIP-1β production by $CD56^{bright}$ and $CD56^{dim}$ NK cells from HIV-1 infected patients, particularly from those who were under cART (Fig. 1c).

ADCC has been demonstrated as an important factor for the long-term control of HIV-1 infection that subsequently results in better disease prognosis.[1–3,7] Furthermore, the relevance of ADCC in new anti-HIV therapies has been emphasized with the introduction of broadly neutralizing antibodies.[2,3] Nevertheless, decreased HIV-specific effector antibody responses have been found in HIV-1-infected individuals, including those receiving cART.[7,10] Our results

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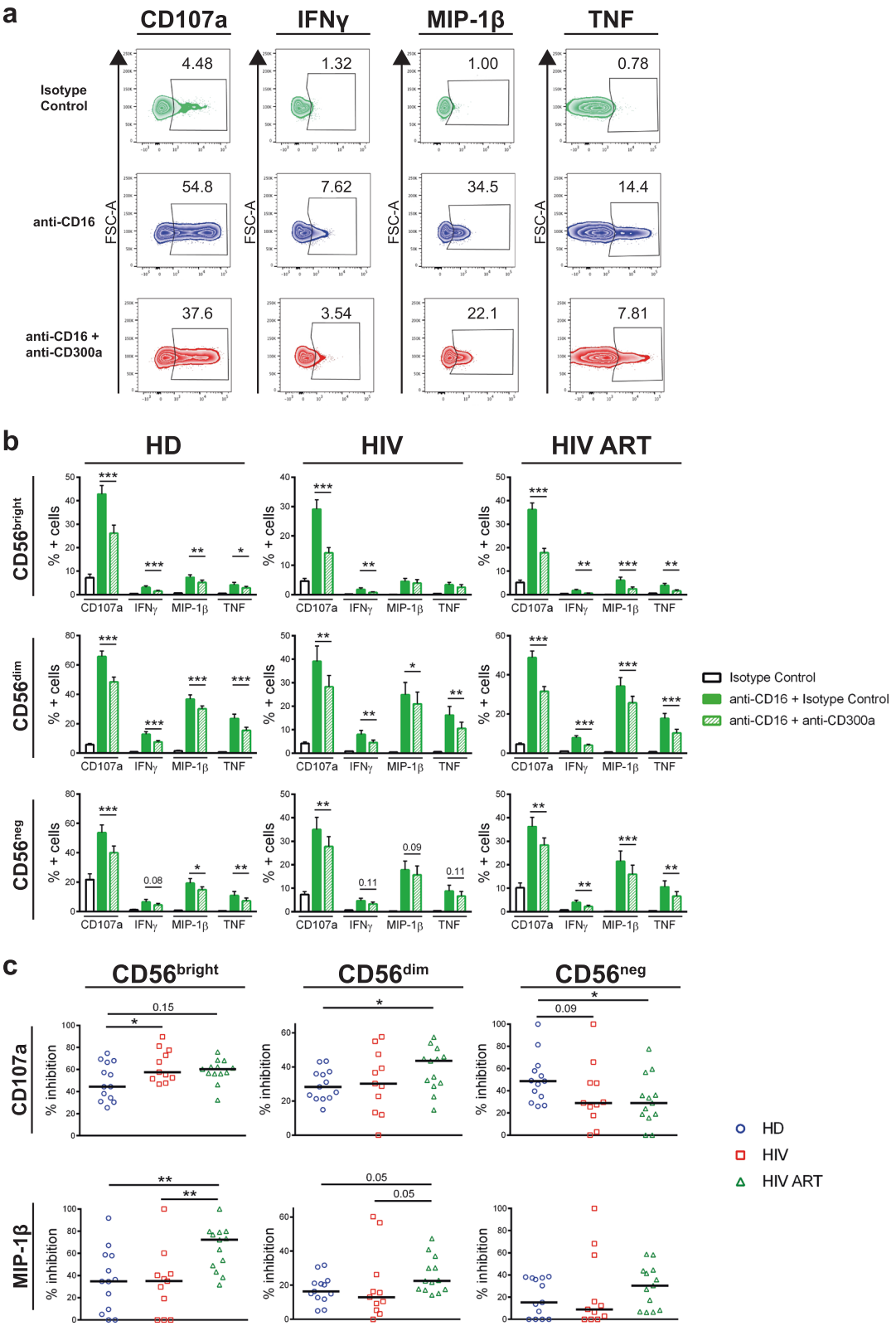

Fig. 1 Degranulation and cytokine production by NK cells in response to the cocrosslinking of CD16 and CD300a. **a** Zebra plots showing the percentage of $CD56^{dim}$ NK cells positive for CD107a, IFNγ, MIP-1β, and TNF from a representative untreated HIV-1 infected patient after stimulation with isotype control, anti-CD16 plus isotype control, or anti-CD16 plus anti-CD300a mAbs. **b** Bar graphs showing the percentage of $CD56^{bright}$, $CD56^{dim}$, and $CD56^{neg}$ NK cells positive for CD107a, IFNγ, MIP-1β, and TNF from healthy donors (HD), untreated HIV-1 infected subjects (HIV), and subjects under cART (HIV ART) after stimulation with isotype control, anti-CD16 plus isotype control, or anti-CD16 plus anti-CD300a mAbs. The mean with the SEM is represented. **c** Dot plots showing the percentage of CD300a-mediated inhibition of degranulation (CD107a) and MIP-1β production by $CD56^{bright}$, $CD56^{dim}$, and $CD56^{neg}$ NK cells, comparing HD, HIV, and HIV ART patients. Each dot represents a subject, and the median is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

suggest that CD300a might decrease ADCC-mediated NK cell killing of HIV-infected cells by inhibiting degranulation and cytokine and chemokine production by NK cells. Previous findings have indicated that successful cART is not enough to achieve effective antibody-mediated HIV-1 control,[7,10] highlighting the importance of our study in the search for new strategies to achieve more effective ADCC in HIV-1-infected patients. Therefore, similar to other therapeutic strategies in which NKG2A and KIR inhibitory receptors are targeted with monalizumab and lirilumab, respectively, targeting CD300a could represent a new strategy to improve NK cell functions. Nonetheless, further experiments are required to confirm our hypothesis.

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AUTHOR CONTRIBUTIONS

J.V. designed and performed the experiments, analyzed and interpreted the data, designed the figures, and wrote the manuscript. I.T. analyzed the results and made the figures. A.O. participated in the interpretation of the data. R.P.-G. determined the CMV serology and the levels of CRP and B2M. F.V., J.A.I., C.R., A.M.L.L., and E.B. clinically characterized the patients and participated in the interpretation of the data. O.Z. participated in the design of the study and interpreted the data. F.B. conceived and designed the study, interpreted the data, and wrote the manuscript. All the authors critically reviewed, edited, and approved the final manuscript.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41423-019-0275-4) contains supplementary material.

Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Information

CD300a inhibits CD16-mediated NK cell effector functions in HIV-1 infected patients

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Donors, Materials and Methods

Subjects and Samples

For this study, cryopreserved peripheral blood mononuclear cells (PBMCs) and plasma from healthy donors (n = 13), cART naïve HIV-1 infected subjects (n = 14) and patients under cART (n = 13) were kindly provided by the HIV BioBank integrated in the Spanish AIDS Research Network (RIS). Samples were processed following current procedures and frozen immediately after their reception. All subjects provided written and signed informed consent in accordance with the Declaration of Helsinki. Institutional and Ethical Review Board approvals were obtained from all healthy donors and patients. The study was approved by the Basque Ethics Committee for Clinical Research (PI2014017 and PI2013108).

All HIV-1 infected patients were asymptomatic when the sample was collected, were not co-infected with hepatitis C virus (HCV), had more than 200 CD4+ T cells/mm^3 and they had never been diagnosed with AIDS. Untreated HIV-1 infected subjects had detectable viremia (>10,000 HIV-RNA copies/ml) and they had never been treated with cART, while patients under cART had undetectable viremia and had been treated with cART at least for 6 months. Clinical data of HIV-1 infected patients were obtained from the RIS database, which are shown in Table S1 (see below).

Antibodies

To perform flow cytometry-based experiments, the following mouse anti-human mAbs were utilized: BV421 anti-CD56 (clone NCAM 16.2), BV510 anti-CD3 (clone UCHT1), BV510 anti-CD14 (clone MϕP9), FITC anti-MIP-1β (clone D21-1351),

PerCP-Cy5.5 anti-IFNγ (clone B27), BV421 anti-CD107a (clone H4A3) and unconjugated anti-CD16 (clone 3G8) from BD Biosciences; PE anti-CD56 (clone MEM-188), PerCP-Cy5.5 anti-CD57 (clone HNK-1), APC anti-NKp46 (clone 9E2), APC anti-TNFα (clone Mab11) and unconjugated IgG isotype control (clone MOPC-21) from Biolegend; PE anti-CD300a (clone E59.126) and APC anti-NKG2A (clone Z199) from Beckman Coulter; PE-Vio770 anti-NKp80 (clone 4A4.D10) from Miltenyi Biotec; PerCP anti-NKG2C (clone 134591) from R&D Systems and unconjugated anti-CD300a (clone MEM-260) from Invitrogen.

Extracellular staining

Frozen PBMCs from healthy donors and HIV-1 infected patients were thawed at 37℃ and washed two times with R10 medium by spinning down the cells at 1,500 rpm for 5 min at room temperature. R10 medium was composed of RPMI 1640 containing GlutaMAX (Thermo Fisher Scientific), 10% of Fetal Bovine Serum (Hyclone) and 1% of penicillin/streptomycin (Thermo Fisher Scientific). Then, PBMCs were incubated in a 6 well plate in R10 at 37℃ and 5% CO_2 overnight at a concentration of 2 x 10^6 cells/ml. After the resting period, cells were washed twice with PBS containing 2.5% of Bovine Serum Albumin (BSA) at 4℃. For the phenotypical characterization of NK cells, PBMCs were first stained with the 405 nm LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) following the manufacturer's protocol, for the detection of dead cells. Afterwards, the extracellular staining was carried out by adding fluorochrome-conjugated mAbs for the detection of NK cell subsets and NK cells surface receptors. In this step, cells were incubated with the mAbs at 4℃ during 30 min in the dark. Stained PBMCs were washed again with PBS 2.5% BSA and fixed with 200µl of 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at 4℃. Finally,

200µl of PBS were added and sample acquisition was done in a FACS Canto II flow cytometer (BD Biosciences).

Redirected lysis assay and intracellular cytokine staining

Cryopreserved PBMCs were thawed and maintained in resting conditions overnight as explained above. For the NK cell-mediated redirected lysis assay, PBMCs were cultured in R10 medium in a U-bottom 96 well plate along with the mouse cell line P815 at a Effector:Target (E:T) ratio of 1:1, in a final concentration of 1 x 10^6 cells/200µl per well. Afterwards, the anti-CD107a mAb and GolgiStop (monensin) and GolgiPlug (brefeldin A) protein transport inhibitors were added to the cell culture according to the manufacturer's protocol (BD Biosciences). Both PBMCs and P815 cells were incubated for 6 h at 37℃ in the presence of mAbs. Three different conditions were studied: a) 2.5µg/ml of MOPC-21 isotype control; b) 2.5µg/ml of anti-CD16 and 10µg/ml of MOPC-21 isotype control; and c) 2.5µg/ml of anti-CD16 and 10µg/ml of anti-CD300a (Figure S5). After the incubation period, the intracellular cytokine staining was carried out. For that, cells were stained with LIVE/DEAD reagent to detect dead cells and with fluorochrome-conjugated mAbs for the identification of NK cell subsets as explained above. Then, cells were fixed and permeabilized using Cytofix/Cytoperm Plus Kit (BD Biosciences) following manufacturer's protocol and were incubated for 30 min at 4℃ in the dark with several fluorochrome-conjugated mAbs for the detection of intracellular cytokines. Stained cells were washed again and were resuspended in 350 µl of PBS. Lastly, samples were acquired with the FACS Canto II flow cytometer.

Laboratory methods

Anti-CMV IgM and IgG levels from plasma belonged to HIV-1 infected patients were determined using a IMMUNITE/IMMUNITE 1000 and IMMUNITE/IMMUNITE 2000 assay (Siemens), following manufacturers protocol. Immunonephelometric assays using latex particles were carried out in order to measure plasma levels of C-reactive protein (CRP) and β2-microglobulin (B2M) from HIV-1 infected patients (N Latex CRP mono and N Latex-β_2-Microglobulina, from Siemens), also following the instructions from the manufacturer.

Statistics

Flow cytometry data were analyzed utilizing FlowJo software (version 10.0.7) and graphical representation and statistical analysis were done with GraphPad Prism software (version 6.01). In order to determine the distribution of the data, D'Agostino & Pearson normality test was performed. Our data were not normally distributed, and therefore non-parametric tests were applied for the statistical analysis. For the comparison between healthy donors, untreated HIV-1 infected subjects and patients under cART, the unpaired Mann-Whitney test was utilized. The Wilcoxon matched-pairs test was applied to study the differences between cell subsets and different stimulation conditions in the functional assays. To calculate the percentage of CD300a-mediated inhibition of NK cell effector functions, the following formula was used:

$$\%\ \text{inhibition} = \left(\frac{(\alpha CD16 - isotype)}{(\alpha CD16 - isotype)} - \frac{(\alpha CD16\ \&\ \alpha CD300a - isotype)}{(\alpha CD16\ - isotype)}\right) \times 100.$$

Table S1. Clinical data of naïve and under cART HIV-1 infected patients.

	Naïve HIV-1 patients		**HIV-1 patients on cART**	
	Median	**Range (min-max)**	**Median**	**Range (min-max)**
Sex	Male: n=14 Female: n=0	-	Male: n=12 Female: n=1	-
Age (years)	27.5	(22 – 49)	39	(25 – 47)
cART (years)	-	-	1	(1 – 3)
Viral load (RNA copies/ml)	29,600	(11,735 – 125,892)	<20	-
CD4+ T cells/mm^3	501.5	(33 – 915)	612	(344 – 1043)
CMV	Negative: n=0 Positive: n=14	-	Negative: n=3 Positive: n=10	-
B2M (mg/l)	2.2	(0.99 – 3.05)	2.2	(1.47– 3.58)
CRP (mg/l)	1.0	(0.15 – 7.22)	1.8	(0.17 – 8.43)

Supplementary Figures

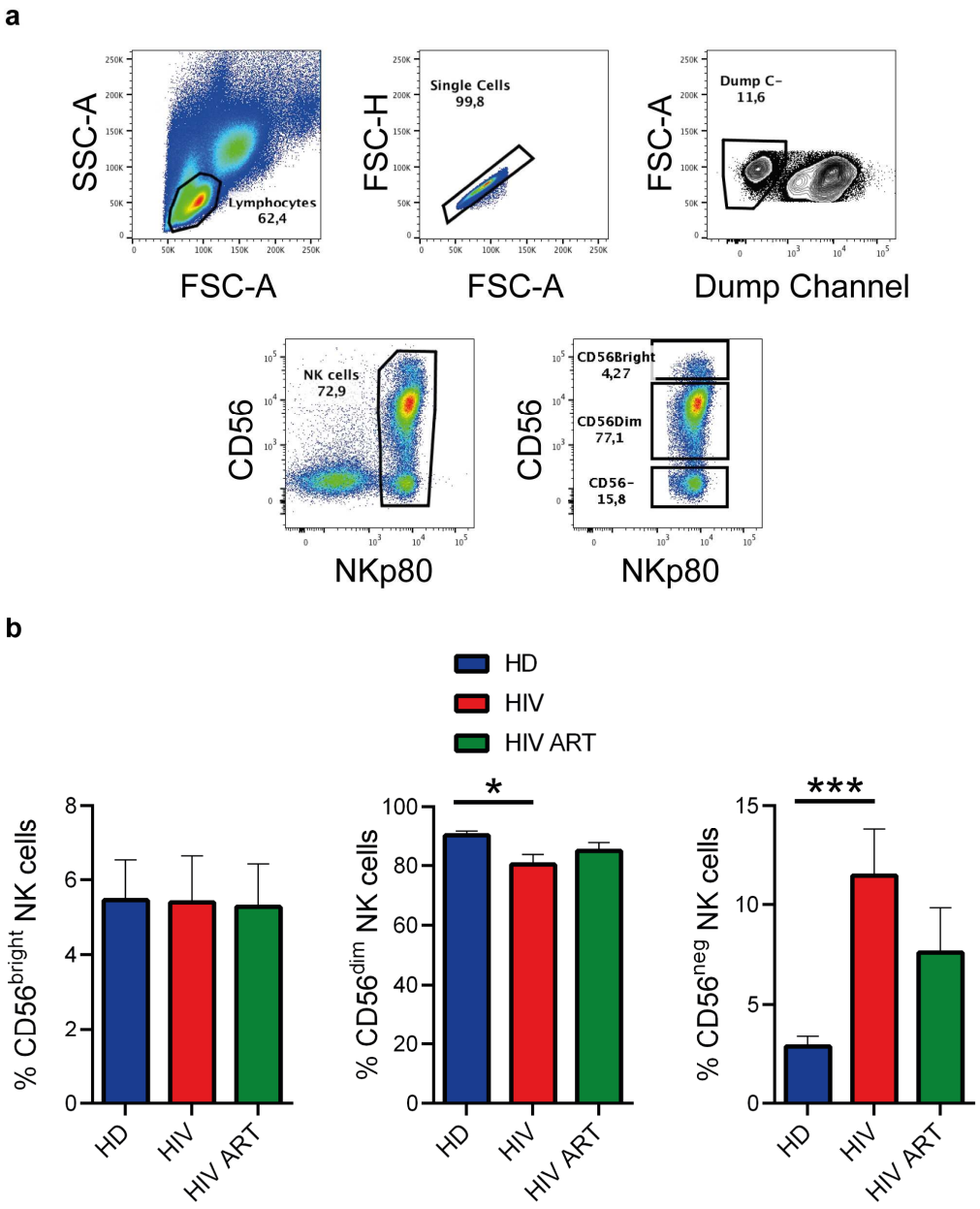

Fig. S1. NK cell subsets from healthy donors and HIV-1 infected patients. (**a**) Pseudocolor and contour plots representing the gating strategy utilized in this study. Data from a representative untreated HIV-1 infected patient is shown. Lymphocytes were electronically gated based on their forward and side scatter parameters and then single cells were selected. To identify NK cells, the population negative for the dump

channel (viability, CD3 and CD14) was selected and then three NK cell subsets were identified: $CD56^{bright}$ (CD56++NKp80+), $CD56^{dim}$ (CD56+NKp80+) and $CD56^{neg}$ (CD56-NKp80+). (**b**) Bar graphs showing the percentage of each NK cell subpopulation from healthy donors (HD), untreated HIV-1 infected subjects (HIV) and patients under cART (HIV ART). The mean with the standard error of the mean (SEM) is represented. *$p<0.05$, *** $p<0.001$.

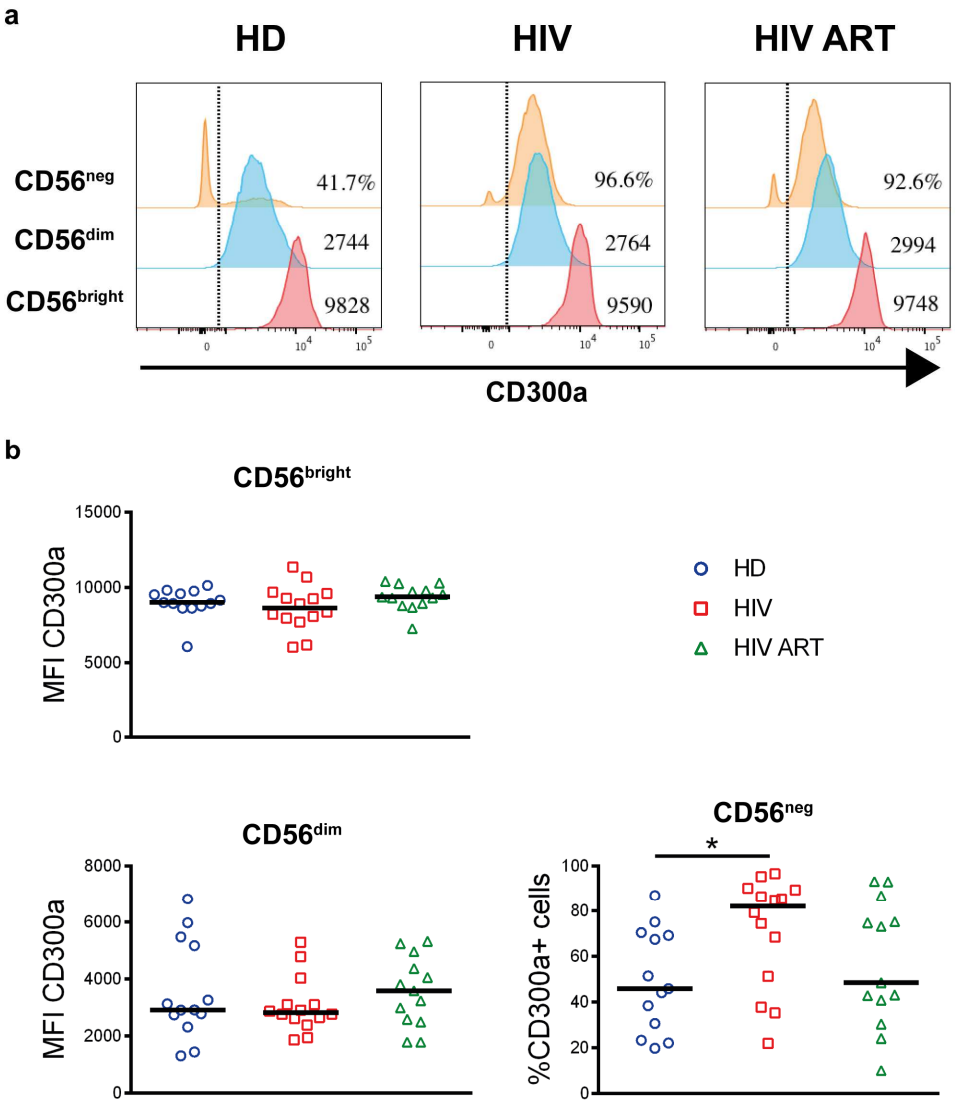

Fig. S2. CD300a expression on NK cell subpopulations from healthy donors and HIV-1 infected patients. (**a**) Histograms showing the percentage of CD300a+ cells on $CD56^{neg}$ NK cells and the median fluorescence intensity (MFI) of CD300a on $CD56^{bright}$ and $CD56^{dim}$ NK cells. Data from a representative a healthy donor (HD), an untreated HIV-1 infected subject (HIV) and a patient on cART (HIV ART) are shown. (**b**) Dot plots showing the MFI of CD300a on $CD56^{bright}$ and $CD56^{dim}$ NK cells, and the percentage of CD300a+ cells on $CD56^{neg}$ NK cells, from HD, HIV and HIV ART patients. Each dot represents a subject and the median is shown. *$p<0.05$.

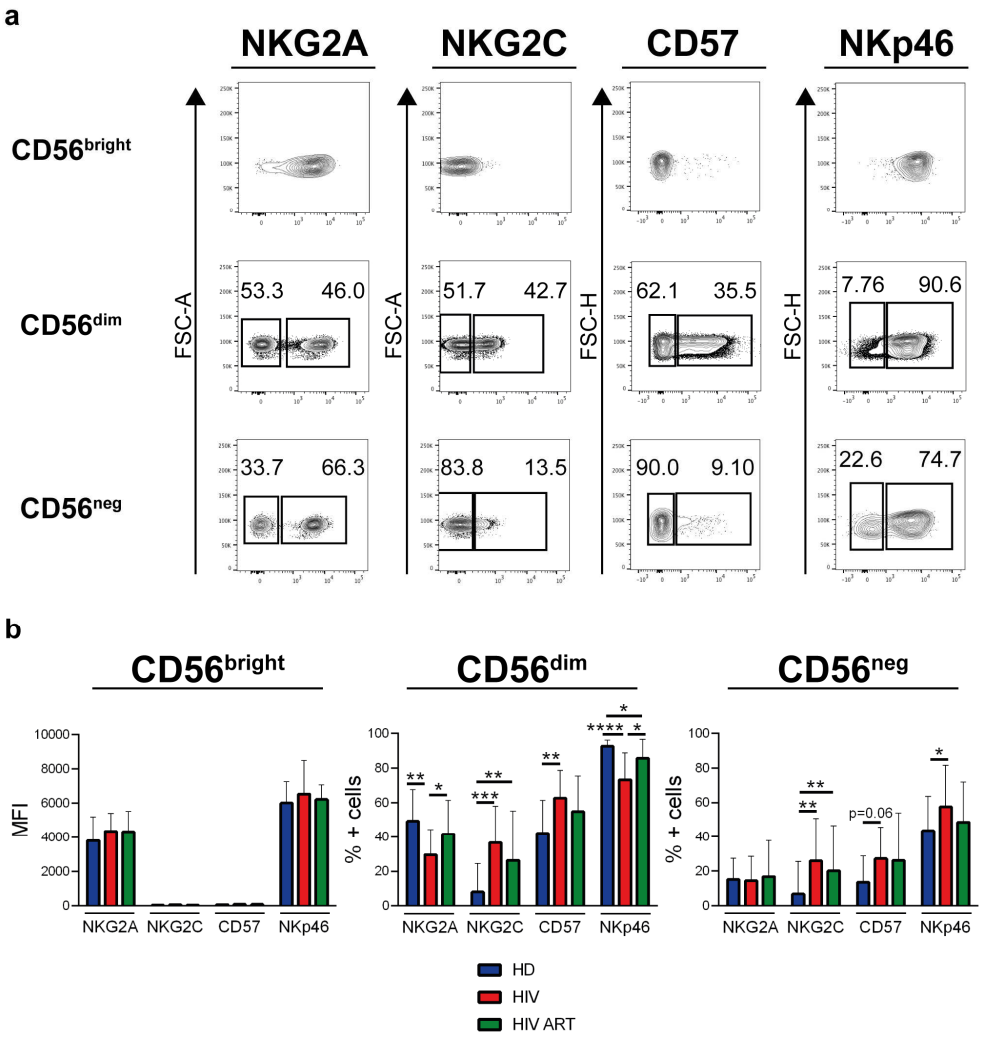

Fig. S3. Expression of NK cell surface receptors on NK cells from healthy donors and HIV-1 infected patients. (**a**) Contour plots representing the expression of NKG2A, NKG2C, CD57 and NKp46 on CD56^{bright} NK cells and the percentage of NKG2A, NKG2C, CD57 and NKp46 expressing cells within CD56^{dim} and CD56^{neg} NK cells from a representative untreated HIV-1 infected patient. (**b**) Bar graphs showing the MFI of NKG2A, NKG2C, CD57 and NKp46 on CD56^{bright} NK cells and the percentage of cells positive for NKG2A, NKG2C, CD57 and NKp46 within CD56^{dim} and CD56^{neg} NK cells from healthy donors (HD), untreated HIV-1 infected subjects (HIV) and patients under cART (HIV ART). The mean with the standard deviation (SD) is represented. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

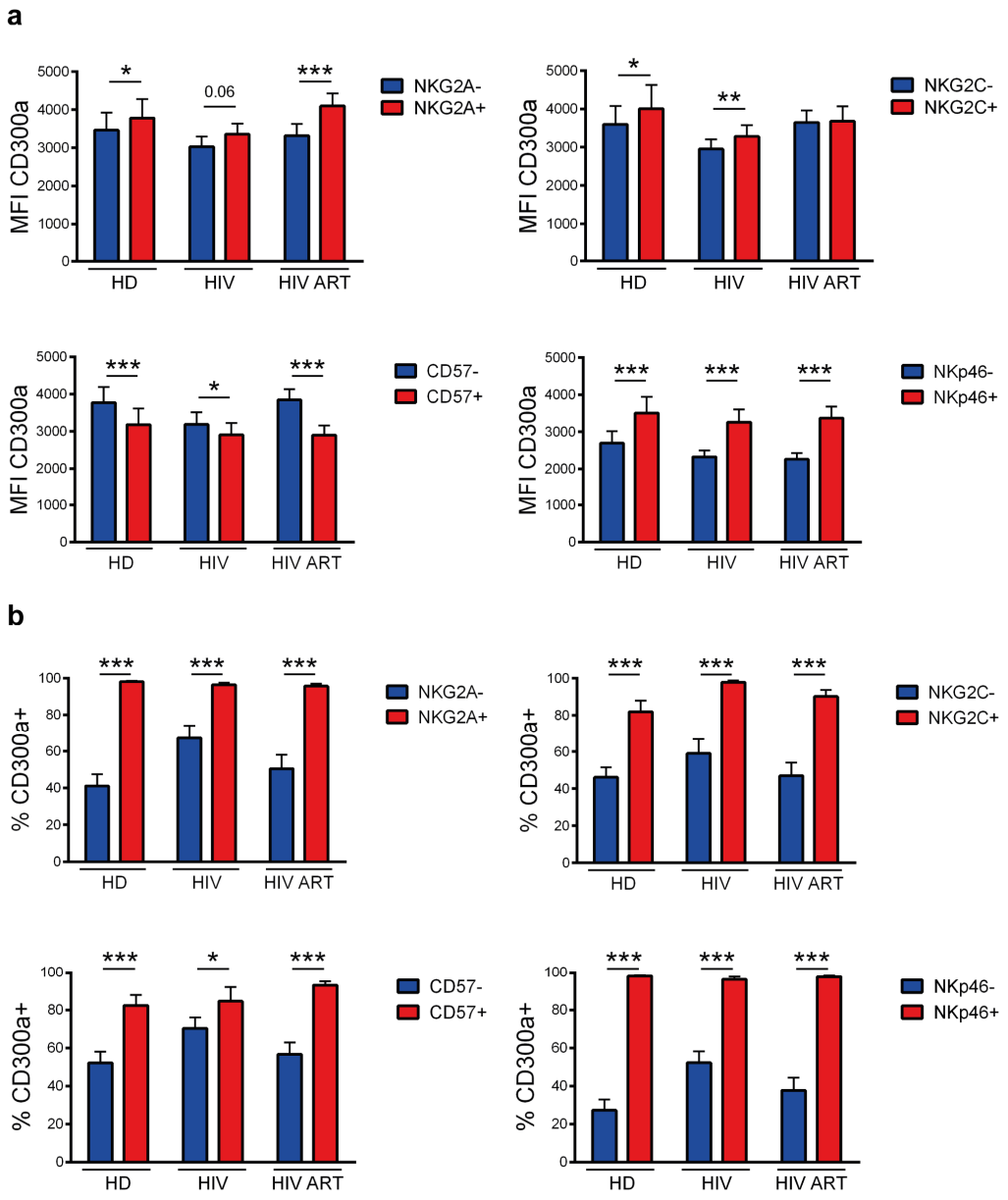

Fig. S4. CD300a expression on NK cell subsets according to the expression of NKG2A, NKG2C, CD57 and NKp46 receptors from healthy donors and HIV-1 infected patients. (**a**) Bar graphs showing the MFI of CD300a on $CD56^{dim}$ NK cells from healthy donors (HD), cART naïve HIV-1 infected subjects (HIV) and patients on cART (HIV ART). (**b**) Bar graphs representing the percentage of CD300a+ cells on $CD56^{neg}$ NK cells from HD, HIV and HIV ART patients. The mean with the SEM is represented. *p<0.05, ** p<0.01, *** p<0.001.

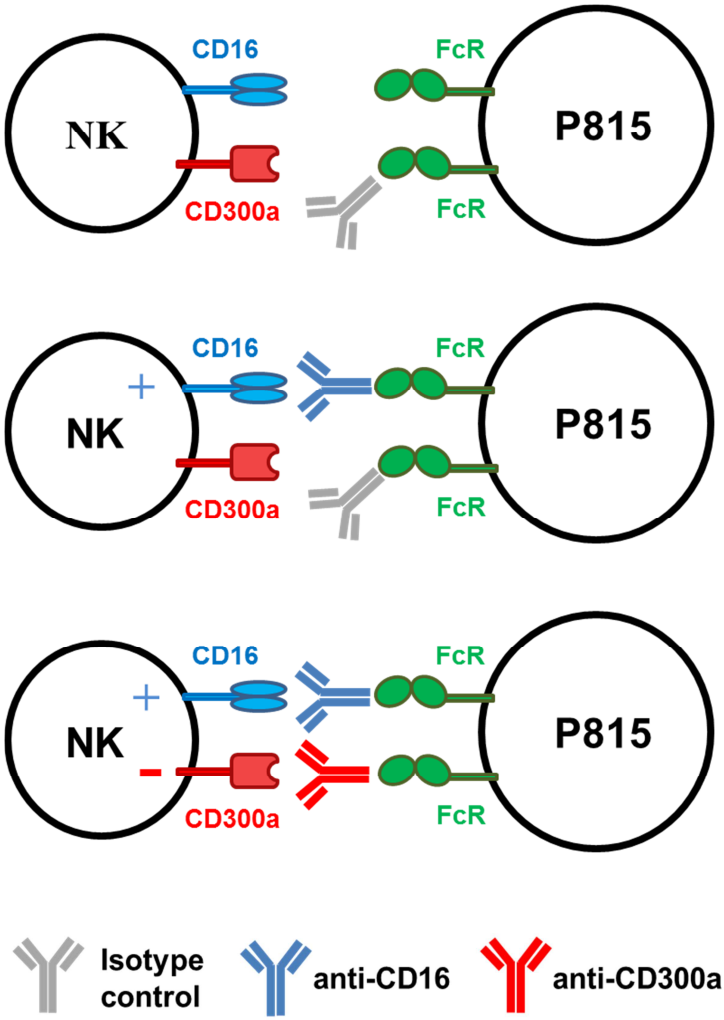

Fig. S5. Schematic representation of redirected lysis assay. Redirected lysis assay was carried out by co-culturing NK cells with the FcR-bearing cell line P815. This assay included three experimental conditions, in which different mAbs were added: isotype control (top), anti-CD16 plus isotype control (middle) and anti-CD16 plus anti-CD300a (below). The isotype control only binds FcRs from P815 cells, and therefore it does not transmit any signal on NK cells. NK cell activation is induced by triggering the CD16 receptor with a specific mAb, whose Fc portion binds FcR on P815 cells. The crosslinking of CD300a with a specific mAb, which is also able to bind FcR on P815, induces inhibitory signals on NK cells.

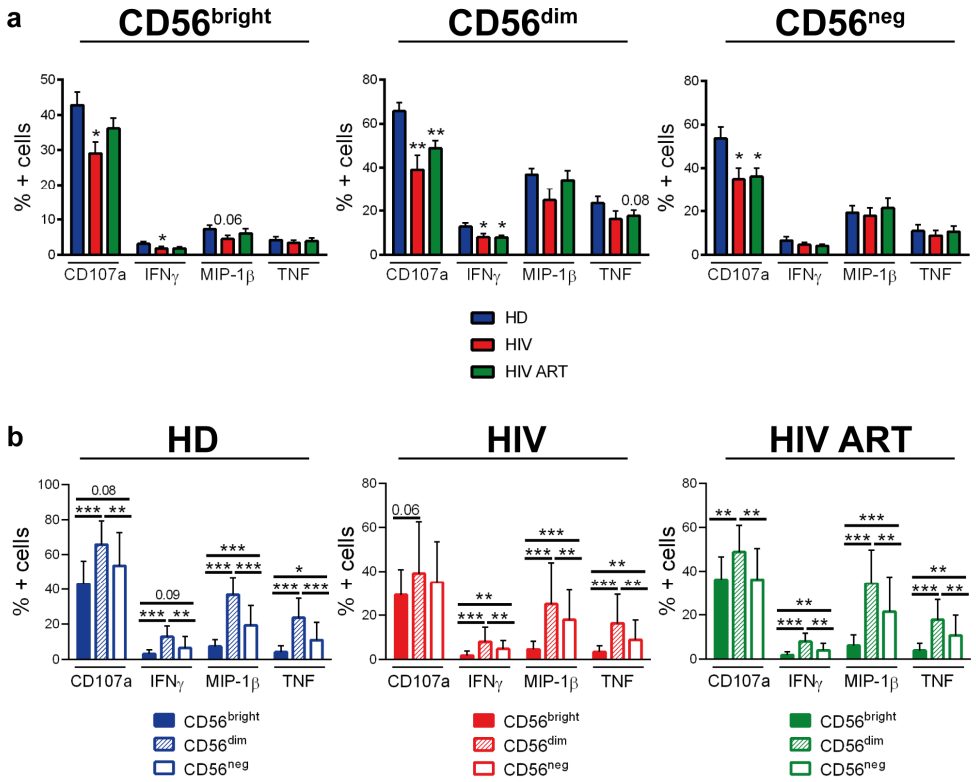

Fig. S6. CD16-mediated NK cell degranulation and cytokine production. (a) Bar graphs showing the percentage of CD56^{bright} (left panel), CD56^{dim} (middle panel) and CD56^{neg} (right panel) NK cells positive for CD107a, IFNγ, MIP-1β and TNF after the stimulation through the CD16 receptor, comparing healthy donors (HD), untreated HIV-1 infected subjects (HIV) and patients under cART (HIV ART). Statistical analyses are made only between HD with HIV and HIV ART. The mean with the SEM is represented. (b) Bar graphs showing the percentage of cells positive for CD107a, IFNγ, MIP-1β and TNF, within CD56^{bright} vs CD56^{dim} vs CD56^{neg} NK cells after the stimulation through the CD16 receptor, in HD (left panel), HIV (middle panel) and patients HIV ART (right panel). The mean with the SD is represented. *p<0.05, ** p<0.01, *** p<0.001.

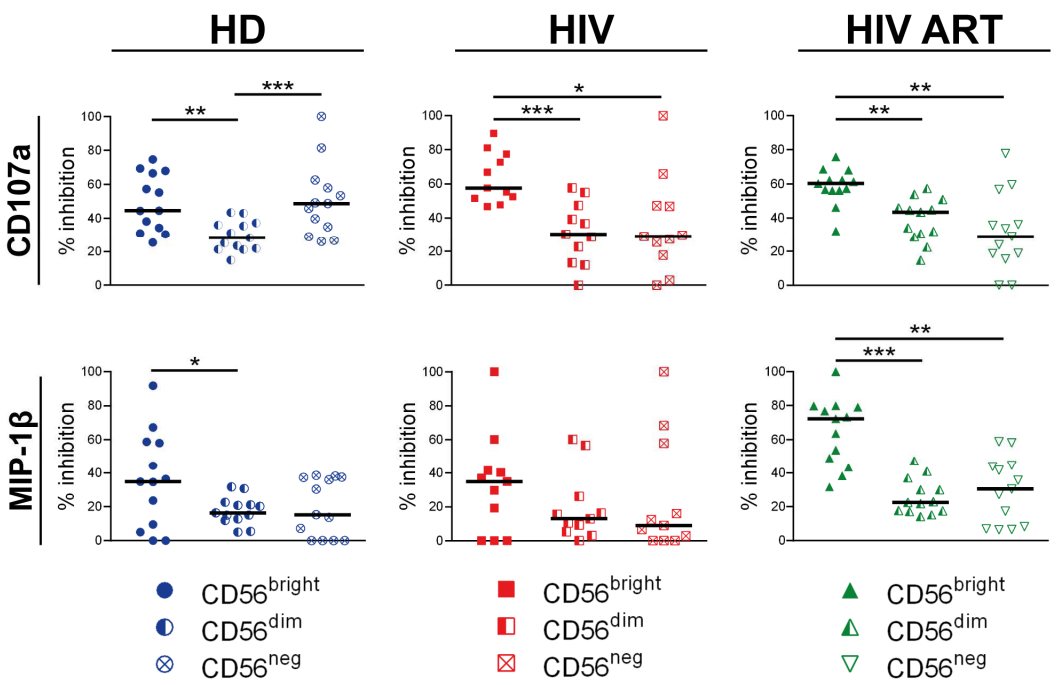

Fig. S7. Percentage of CD300a-mediated inhibition of the degranulation and MIP-1β production by NK cells. Dot plots showing the percentage of CD300a-mediated inhibition of degranulation (CD107a) and MIP-1β production by $CD56^{bright}$ vs $CD56^{dim}$ vs $CD56^{neg}$ NK cells, from healthy donors (HD), untreated HIV-1 infected subjects (HIV) and patients under cART (HIV ART). Each dot represents a subject and the median is shown. *p<0.05, ** p<0.01, *** p<0.001.

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Altered Expression of CD300a Inhibitory Receptor on CD4+ T Cells From Human Immunodeficiency Virus-1-Infected Patients: Association With Disease Progression Markers

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The ability of the CD300a inhibitory receptor to modulate immune cell functions and its involvement in the pathogenesis of many diseases has aroused a great interest in this molecule. Within human CD4+ T lymphocytes from healthy donors, the inhibitory receptor CD300a is differentially expressed among different T helper subsets. However, there are no data about the expression and regulation of CD300a receptor on CD4+ T cells from human immunodeficiency virus (HIV)-1-infected patients. The objective of this study was to investigate the expression of CD300a on CD4+ T cells from HIV-infected patients on suppressive combined antiretroviral therapy (cART) and cART naïve patients. Our results have demonstrated that the expression levels of this inhibitory receptor were higher on CD4+ T cells from HIV-1 infected subjects compared with healthy donors, and that cART did not reverse the altered expression of CD300a receptor in these patients. We have observed an increase of CD300a expression on both PD1+CD4+ and CD38+CD4+ T cells from HIV-1 infected people. Interestingly, a triple positive (CD300a+PD1+CD38+) subset was expanded in naïve HIV-1 infected patients, while it was very rare in healthy donors and patients on cART. Finally, we found a negative correlation of CD300a expression on CD4+ T lymphocytes and some markers associated with HIV-1 disease progression. Thus, our results show that HIV-1 infection has an impact in the regulation of CD300a inhibitory receptor expression levels, and further studies will shed light into the role of this cell surface receptor in the pathogenesis of HIV infection.

Keywords: CD300, CD300a, human immunodeficiency virus-1, CD4 T cells, PD1

INTRODUCTION

In order to mount an adequate and effective response against offenses, the immune system is orchestrated in part by a balance between stimulatory and inhibitory signals transmitted by a variety of receptors found on the surface of immune cells. Human CD300a is a member of the CD300

receptor family whose genes are located on chromosome 17 (1, 2). CD300a is a type I transmembrane protein formed by an immunoglobulin (Ig)V-like extracellular domain, a transmembrane region, and a long cytoplasmic tail containing three classical and one non-classical immunoreceptor tyrosine-based inhibitory motifs, which provides the receptor with an inhibitory capacity (1–5). Regarding to the ligands, CD300a is known to recognize phosphatidylserine (PS) and phosphatidylethanolamine (PE), phospholipids that are found in the inner leaflet of plasma membrane in resting cells and are exposed to the outer leaflet when cells get activated, transformed, infected, or dead (6–8).

Human CD300a receptor is found on the surface of both myeloid and lymphoid cells. Within human CD4+ T lymphocytes, it is differentially expressed among different subsets, and newborns CD4+ T cells significantly express lower levels of this receptor than adults (9, 10). Human Th1 cells expressing CD300a tend to be polyfunctional and after stimulation express the transcription factor eomesodermin (Eomes) (9). CD300a inhibitory receptor has the capacity to modulate CD4+ T cell responses through a signaling pathway that involves the src homology 2 domain containing protein tyrosine phosphatase (SHP)-1 (3, 11). Specifically, the co-ligation of the T cell receptor (TCR) and CD300a inhibited the calcium mobilization evoked by TCR alone and modulated the interferon (IFN)γ production on Th1 polarized cells (11). Importantly, over the past few years, several publications have emphasized the significant role that CD300a has in complex biological processes such as cytokine production and phagocytosis, and in a diversity of diseases including autoimmune disorders, allergic and inflammatory diseases, hematological malignancies, viral infections, etc. (2, 12–15).

Several studies have described the role of CD300a and its ligands PS and PE in the mechanisms that viruses use to enter host cells and also to evade the attack of the immune system. It is well known that one of the strategies used by viruses is apoptotic mimicry. Through this mechanism, which consists of enclosing viruses in a lipid bilayer obtained from the plasma membrane of host cells, they impersonate apoptotic cells and debris by concentrating PS and PE within their membranes, as it is the case of enveloped viruses; or by covering themselves in cell-derived PS and PE-containing vesicles, as it happens for many non-enveloped viruses (16–18). In this context, it has been shown that the interaction of CD300a with PS and PE enhances the infection of Dengue virus and other mosquito-borne viruses such as Yellow fever, West Nile, and Chikungunya viruses (14). In addition, Grauwet et al. have described that the US3 protein kinase of pseudorabies virus induces the exposure of PS and PE on the infected cells, a process that depends on the kinase activity of US3 and on group I p21-activated kinases. In consequence, the binding of CD300a on NK cells to the infected cells is increased, leading to an inhibition of NK cell-mediated killing (15). As a matter of fact, NK cells express high levels of CD300a (4, 5, 8, 10).

There are few reports describing the potential implication of CD300a during human immunodeficiency virus (HIV) infection. In a first study, a positive correlation between the mRNA levels of CD300a and the levels of basic leucine zipper transcription factor ATF-like (BATF) was described on HIV-specific CD8+ T cells. PD1, a marker of exhausted T cells, inhibits their function by upregulating BAFT, which has been suggested to increase other negative feedback pathways, including the expression of the CD300a inhibitory receptor, indicating that this molecule could also have a role in T cell exhaustion during HIV infection (19). Other report described a downregulation of CD300a expression on B cells from HIV-infected individuals, which could explain in part the B cell hyper-activation and dysfunction found in these patients (20). The downregulation in CD300a expression on B cell subsets was not corrected by effective antiretroviral therapy. On the other hand, a correlation between CD4+ T cell count and CD300a expression on memory B cells was observed in patients whose viremia was controlled by combined antiretroviral therapy (cART) (20). Finally, we have recently described the expression of CD300 receptors on monocytes from chronically HIV-1 infected patients under cART and its association with monocyte cytokine production. However, regarding to CD300a expression on monocytes, no differences were found between healthy donors and cART-treated HIV-1-infected patients (21).

In terms of CD4+ T cells, the expression of the CD300a receptor in HIV-infected patients and its regulation is still unknown. In this study, we have found that the cell surface levels of this inhibitory receptor are increased on CD4+ T cells after HIV-1 infection and it is not reverted by cART. Interestingly, a triple positive (CD300a+PD1+CD38+) subset was expanded in cART naïve HIV-1 infected patients, while it was very rare in healthy donors and cART-treated patients. We have also found an association between CD300a expression on CD4+ T lymphocytes and HIV disease progression markers.

MATERIALS AND METHODS

Subjects and Samples

In this work, frozen peripheral blood mononuclear cells (PBMCs) from healthy adult donors and chronically HIV-1 infected patients were analyzed. Blood samples from healthy donors ($n = 19$) were obtained from the Basque Biobank for Research (http://www.biobancovasco.org). All subjects provided written and signed informed consent in accordance with the Declaration of Helsinki. The study was approved by the Basque Ethics Committee for Clinical Research (PI2014017 and PI2013108). Plasma and PBMC cryopreserved samples from asymptomatic HIV-1 infected patients on suppressive cART (viral load < 20 copies/mL) for at least 6 months ($n = 16$) and from cART naïve HIV-1 infected subjects (viral load > 9,300 copies/mL) ($n = 20$), were collected from the Virgen del Rocío University Hospital in Seville (Spain). Clinical data of cART-treated and cART naïve HIV-1 infected patients are described in Tables S1 and S2 in Supplementary Material, respectively. All patients provided written and signed informed consent in accordance with the Declaration of Helsinki. The study was approved by the Virgen del Rocío University Hospital Ethics Committee for Research (15/2009).

Laboratory Methods

CD4 T-cell counts were determined in fresh whole blood using an Epic XL-MCL flow cytometer (Beckman-Coulter, Brea, CA,

USA) according to the manufacturer's instructions. Plasma HIV-1 RNA concentration was measured using quantitative polymerase chain reaction (COBAS Ampliprep/COBAS Taqman HIV-1 test, Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's protocol. The detection limit for this assay was 20 HIV RNA copies per milliliter.

High-sensitive C-reactive protein (hsCRP) and β2-microglobulin were determined with an immunoturbidimetric assay using COBAS 701 (Roche Diagnostics, GmbH, Mannheim, Germany). D-dimer levels were determined using an automated latex enhanced immunoassay (HemosIL, D-Dimer HS 500, Instrumentation Laboratory) in plasma samples stored at −20°C.

Flow Cytometry Analysis

To perform flow cytometry-based procedures, the following fluorochrome-conjugated monoclonal mouse anti-human antibodies were used: PerCP-Cy5.5 anti-PD1 (clone EH12.1), PE-Cy7 anti-CD3 (clone SK7), APC anti-CD38 (clone HIT2) and BV421 anti-CD4 (clone RPA-T4) from BD Biosciences; PE anti-CD300a (clone E59.126) from Beckman Coulter; APC-eFluor780 anti-CD27 (clone O323) from eBioscience and BV510 anti-CD45RA (clone HI100) from BioLegend. Frozen PBMCs were thawed at 37°C and washed with PBS two times. Afterward, cells were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies) to test for viability, followed by a second step of incubation with different fluorochrome-conjugated antibodies to assess the expression of surface markers. In both steps, PBMCs were incubated for 30 min on ice protected from the light and were washed twice with 2.5% of bovine serum albumin in PBS. Finally, cells were fixed with 200 µl of 4% of paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C and then other 200 µl of PBS were added. Samples were acquired with a flow cytometer FACS Canto II (BD Biosciences), using the FACS Diva software (BD Biosciences). Data were analyzed with FlowJo 10.0.7 software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assays were performed to measure soluble markers in the plasma samples from HIV-1-infected patients: sCD163 (Macro163™, IQ Products) and sCD14 (Human CD14 ELISA Kit, Diaclone Immunology Products) levels were determined. ELISA experiments were performed following the manufacturers' protocol.

Data Representation and Statistical Analysis

GraphPad Prism software (version 6.01) was employed for the graphical representation and statistical analysis. Data were represented in scatter dot plots with the median or bar graphs showing the mean with SEM. Prior to statistical analyses, data were tested for normal distribution with D'Agostino and Pearson normality test. Then, parametric tests were applied when the normality test was passed, while non-parametric tests were utilized when it was not. Differences between healthy donors and naïve and cART-treated HIV-1 infected patients were evaluated with the non-parametric unpaired Mann–Whitney test, and the comparisons within cell subsets from each subject were made with the non-parametric Wilcoxon matched-pairs test. The same software was used to carry out correlation analysis. In this case, parametric Pearson correlation test or non-parametric Spearman test was employed depending on data distribution. Boolean gate analysis were done utilizing the FlowJo 10.0.7 software and data were represented in pie chart graphs using SPICE v5.3 software and bar graphs showing the mean with SEM utilizing GraphPad Prism software.

RESULTS

CD300a Expression on CD4+ T Lymphocytes From HIV-Infected Patients

The human CD300a inhibitory receptor is known to be differentially expressed on CD4+ T cell subpopulations (9–11). Here, we have analyzed the CD300a expression levels on CD4+ T cell subsets from HIV-1 infected patients in comparison with healthy donors. CD27 and CD45RA cell surface receptors were used as markers to identify different CD4+ T subpopulations: naïve (CD27+CD45RA+), memory (CD27+CD45RA−), effector/memory (CD27−CD45RA−), and terminal differentiated effector/memory (TEM) (CD27−CD45RA+) cells (Figure S1A in Supplementary Material). In agreement with previous publications, the frequency of each subset varied between the different groups of patients (Figure S1B in Supplementary Material) (22–24). First, we examined the expression of CD300a on CD4+ T cell subsets from healthy donors, cART naïve, and patients on cART. Similar to other published findings (10), we have found that in healthy donors, naïve CD4+ T cells exhibited the lowest CD300a expression levels while TEM cells displayed the highest. Naïve cells were negative or expressed low levels of CD300a and almost all TEM cells were positive and expressed high levels of CD300a (**Figure 1A**). On the other hand, memory and effector/memory CD4+ T cells are divided into two well defined CD300a+ and CD300a− subpopulations (**Figure 1A**). Regarding CD4+ T cells from HIV-1 infected patients, we observed a similar CD300a expression pattern among the four subpopulations (**Figure 1A**).

Next, we compared the percentage of CD300a+ cells on CD4+ T cells from healthy donors with the cells from cART naïve and cART-treated HIV-1 infected patients. In general, the results did not show significant differences between groups (Figure S2 in Supplementary Material). In addition, we also checked the median fluorescence intensity (MFI) of CD300a within the CD300a+ cells in the same CD4+ T cell subpopulations, as a measure of the amount of CD300a molecules on the surface of CD300a+ cells. We observed a significant upregulation of CD300a expression on CD4+ T cells from HIV-1-infected patients when compared with cells from healthy donors. This was observed in every CD4+ T cell subset, with the exception of TEM cells, which only exhibited significant differences between healthy donors and cART-treated patients (**Figure 1B**). Therefore, our results demonstrated that HIV-1 infection increases CD300a expression

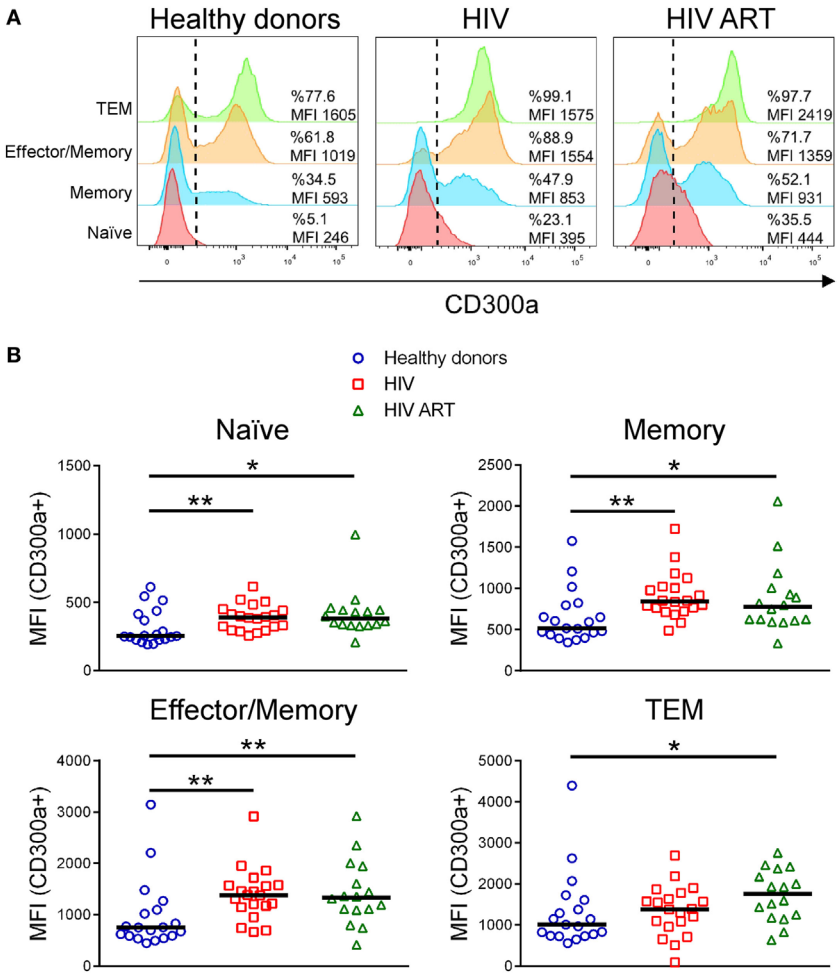

FIGURE 1 | CD300a inhibitory receptor expression on CD4+ T cell subsets from healthy donors and human immunodeficiency virus (HIV)-1 infected patients. **(A)** Representative histograms showing the percentage of CD300a+ cells and the median fluorescence intensity (MFI) of CD300a within positive cells on CD4+ T cell subpopulations. Data from a representative healthy donor, a cART naïve (HIV) and a patient on cART (HIV ART) are shown. **(B)** Dot plots showing the MFI of CD300a within positive cells on CD4+ T cell subsets from healthy donors and cART naïve (HIV) and cART-treated (HIV ART) HIV-1-infected patients. Each dot represents a subject and the median is shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

levels on CD4+CD300a+ cells and that cART does not reverse the upregulation of CD300a expression found in these patients.

CD300a Expression on PD1+CD4+ T Cells

During chronic HIV-1 infection, the persistent antigen exposure gives rise to T cell exhaustion, which is characterized by a reversible loss of effector functions and proliferative capacity. Exhausted T cells are characterized by a higher expression of inhibitory receptors such as PD1, TIM-3, and LAG-3 (25, 26). The PD1 inhibitory receptor, inductively expressed on T cells upon activation, has been identified as a major regulator of T cell exhaustion during chronic HIV infection (19, 27, 28). We decided to measure the expression of CD300a receptor on PD1+CD4+ T lymphocytes from HIV-1 infected patients. First, we were able to reproduce the results obtained in previous works (22, 24), since we observed an increase in the percentage of PD1+ cells on memory, effector/memory and TEM CD4+ T cells from HIV-viremic patients that are not receiving cART (Figure S3 in Supplementary Material). The frequency of PD1+ cells was significantly diminished with cART on the same CD4+ T cell subsets from HIV-1-infected patients (Figure S3A in Supplementary Material).

Afterward, we analyzed the percentage of CD300a+ cells within PD1+ and PD1− CD4+ T lymphocytes from healthy donors, cART naïve, and cART-treated HIV-1 infected patients (Figure S3B in Supplementary Material). Specifically, we focused on memory, effector/memory and TEM CD4+ T cells. Naïve CD4+ T cells were not analyzed since they express negligible levels of PD1, as we (Figure S3A in Supplementary Material) and others have shown (24, 29). Regarding to effector/memory and TEM CD4+ T cell subsets we saw that PD1+ cells exhibited a higher percentage of CD300a+ cells than PD1− cells, in both healthy donors and HIV-1 infected patients with the exception of TEM cells from cART-treated patients, which did not display

any difference between PD1+ and PD1− cells (**Figure 2A**). On the other hand, the frequency of CD300a+ cells in the PD1+ and PD1− memory CD4+ T lymphocytes were somehow different between the groups of patients (**Figure 2A**). Importantly, when we measured the MFI of the CD300a on PD1+CD300a+ and PD1−CD300a+ cells, we observed that PD1+ cells in all CD4+ T cell subsets always exhibited higher levels of expression of CD300a than PD1− cells (**Figure 2B**).

Finally, we compared the MFI of CD300a on PD1+CD300a+ cells from healthy donors with the cells from HIV-1 infected patients. We observed that PD1+ cells from both cART naïve and cART-treated HIV-1-infected patients displayed a higher MFI of CD300a than cells from healthy donors (**Figure 2C**). Altogether, these results indicate that HIV-1 infection induces an increase on the expression of CD300a inhibitory receptor on CD4+ T cells that otherwise already express the checkpoint PD-1.

CD300a Expression on CD38+CD4+ T Cells

Human immunodeficiency virus-1 infection induces T cell (CD4+ and CD8+) dysfunction and activation, which consequently upregulates the expression of the surface receptor CD38 on these cells. In fact, high expression of CD38 on T lymphocytes

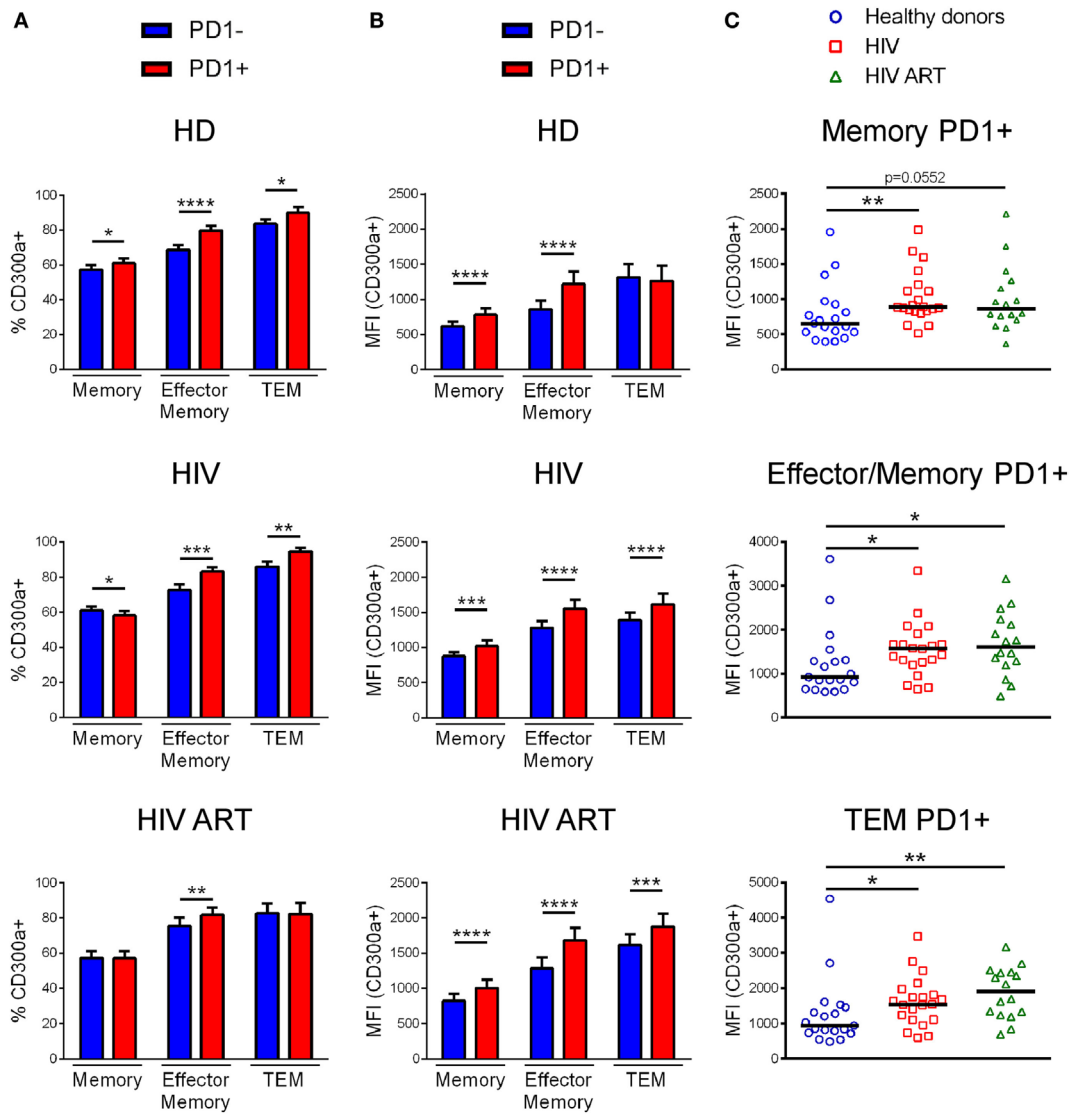

FIGURE 2 | CD300a expression on PD1+CD4+ T lymphocytes. Bar graphs showing the percentage of CD300a+ cells **(A)** and the median fluorescence intensity (MFI) of CD300a within positive cells **(B)** in PD1+CD4+ and PD1-CD4+ T lymphocytes from healthy donors, cART naïve [human immunodeficiency virus (HIV)] and patients on cART (HIV ART). Error bars represent the SEM. **(C)** Dot plots representing the MFI of CD300a within CD4+ T cells expressing both CD300a and PD1 from healthy donors, cART naïve (HIV) and cART-treated (HIV ART) HIV-1 infected patients. Each dot represents a subject and the median is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

is known to predict HIV infection progression (30–32). Therefore, we decided to utilize the marker CD38 in order to investigate the expression of CD300a receptor on activated CD4+ T cells from HIV-1 infected people. In agreement with previous publications (30, 33), we observed that, except for the naïve subset, all CD4+ T cells from cART naïve HIV-1 infected patients express higher percentages of CD38+ cells than the cells from healthy donors or cART-treated patients (Figure S4A in Supplementary Material). There were no significant differences in the frequency of CD38+ cells on naïve CD4+ T cells from healthy subjects and infected patients (Figure S4A in Supplementary Material). In fact, resting naïve CD4+ T cells express constitutively low levels (MFI) of CD38, making this receptor a proper activation marker only for T cells with a non-naïve phenotype (30). Hence, naïve CD4+ T cells were not considered in the following analysis.

We determined the percentage of CD300a+ cells on CD38+CD4+ T lymphocytes, focusing on memory, effector/memory, and TEM CD4+ T cells, from both healthy donors and infected patients (Figure S4B in Supplementary Material). In all subjects, memory and effector/memory CD4+ T cells expressing CD38 exhibited a lower percentage of CD300a+ cells than CD38− cells (**Figure 3A**). The same result was obtained from TEM cells

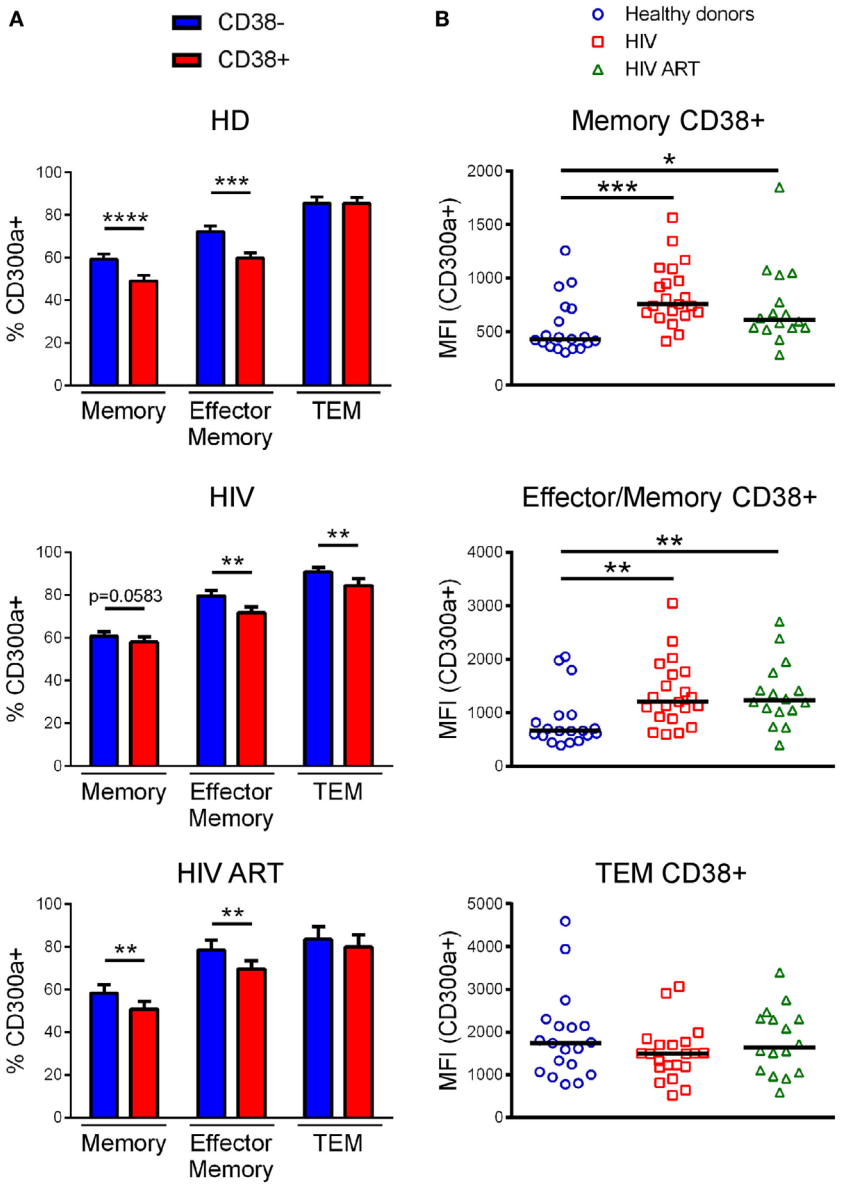

FIGURE 3 | CD300a expression on CD38+CD4+ T lymphocytes. **(A)** Bar graphs representing the percentage of CD300a+ cells within CD38+CD4+ and CD38−CD4+ T lymphocytes from healthy donors, cART naïve [human immunodeficiency virus (HIV)] and patients on cART (HIV ART). Error bars represent the SEM. **(B)** Dot plots representing the median fluorescence intensity of CD300a within CD4+ T cells expressing both CD300a and CD38 from healthy donors, cART naïve (HIV), and patients on cART (HIV ART). Each dot represents a subject and the median is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

but only in cART naïve patients (**Figure 3A**). Importantly, when we compared the MFI of CD300a on CD38+CD300a+ cells from healthy donors with the cells from cART naïve and cART-treated patients, we saw a higher expression of the CD300a receptor on memory and effector/memory CD4+ T cells from HIV-1-infected patients (**Figure 3B**).

We finally carried out a Boolean gate analysis, in order to compare the co-expression of CD300a, PD1, and CD38 receptors on non-naïve CD4+ T cells from healthy donors and cART naïve and cART-treated HIV-1 infected patients. Regarding memory and effector/memory CD4+ T lymphocytes, we saw a higher percentage of triple positive (CD300a+PD1+CD38+) and double positive (CD300a+PD1+CD38−, CD300a+PD1−CD38+ and CD300a−PD1+CD38+) cells in cART naïve patients than in healthy donors or cART-treated patients. The percentage of triple negative cells (CD300a−PD1−CD38−) was lower in cART naïve patients. TEM CD4+ T cells displayed similar results, mainly in the frequency of triple-positive cells (**Figure 4**). Altogether, we observed that in terms of CD300a, PD1, and CD38 expression, CD4+ T cells from healthy donors and cART-treated patients were very similar, while the CD4+ cells from cART naïve HIV-1 infected patients exhibited a clearly different phenotype with a very interesting appearance of a significant triple positive (CD300a+PD1+CD38+) cell subset (**Figure 4**).

Association of CD300a Expression With Markers of HIV-1 Infection Progression

Once we have described that CD300a expression is upregulated on CD4+ T lymphocytes from HIV-1 infected patients, we investigated if these findings could have any clinical relevance to determine HIV-1 disease progression. To do that, correlation analysis were carried out between CD300a expression and markers that are currently used for clinical monitoring of HIV-1 infected patients, including viral load and CD4+ T cell count, and also several soluble markers from plasma such as β2-microglobulin, D-dimer, hsCRP, soluble CD163 (sCD163),

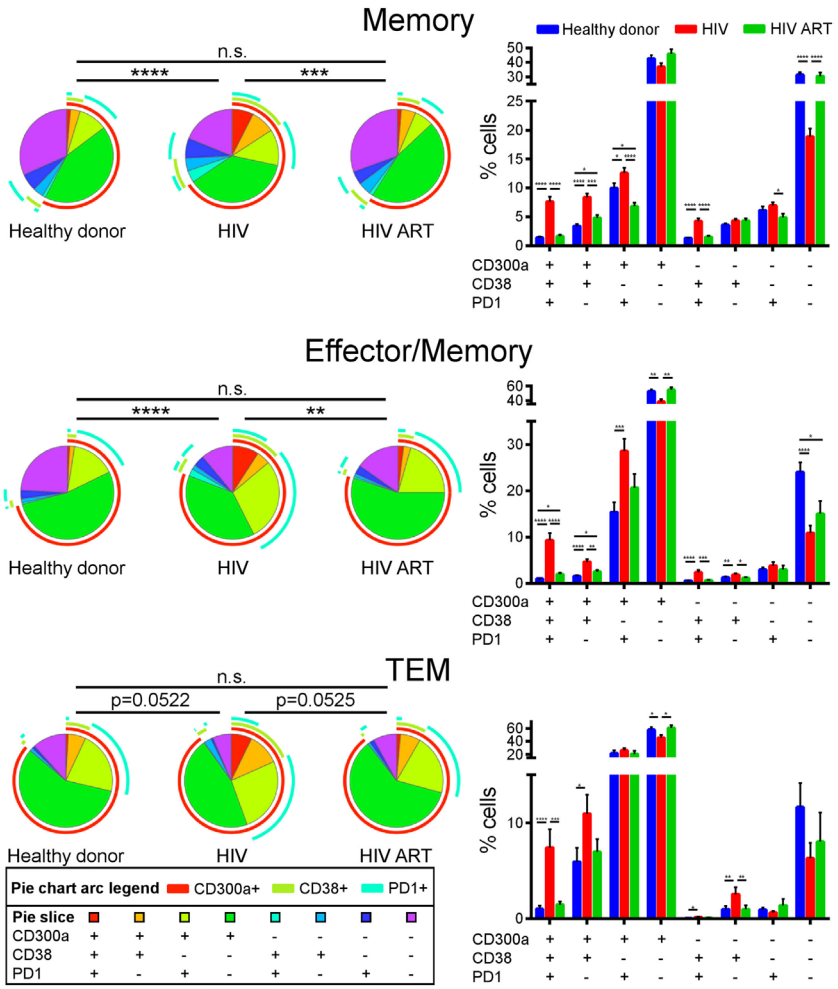

FIGURE 4 | Co-expression of CD300a, PD1, and CD38 on CD4+ T cells. Boolean gate analysis showing the frequency of memory, effector/memory, and TEM CD4+ T cells expressing CD300a, PD1, and CD38, in healthy donors, cART naïve [human immunodeficiency virus (HIV)], and patients on cART (HIV ART). Data are represented in pie chart graphs and bar graphs showing the mean with SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and soluble CD14 (sCD14). Figure S5 in Supplementary Material shows the values of each soluble marker and CD4+ T cell counts in both cohorts of patients. As expected, HIV-1-infected patients under cART exhibited a higher CD4+ T cell count than non-treated patients. In addition, cART-treated patients had lower serum levels of β2-microglobulin and sCD163 than cART naïve patients (Figure S5 in Supplementary Material). No correlation was found between the percentage of CD300a+ cells and viral load or D-dimer and sCD163 levels (data not shown). On the other hand, we did observe a significant correlation between CD4+ T cell counts, β2-microglobulin, hsCRP, and sCD14 levels and the frequency of CD300a+ cells in untreated HIV-1 infected patients. The percentage of CD300a+ cells within memory CD4+ T lymphocytes was positively associated with CD4+ T cell count, while it was negatively correlated with β2-microglobulin and hsCRP levels (**Figure 5**). Moreover, naïve CD4+ T cells from cART naïve patients also exhibited a negative association between the percentage of CD300a+ cells and sCD14 plasma levels, and the same tendency was observed on memory CD4+ T cells (**Figure 5**). In contrast, related to the effector/memory CD4+ T cells, we observed a somehow contradictory correlation pattern of the frequency of CD300a+ cells with the progression markers,

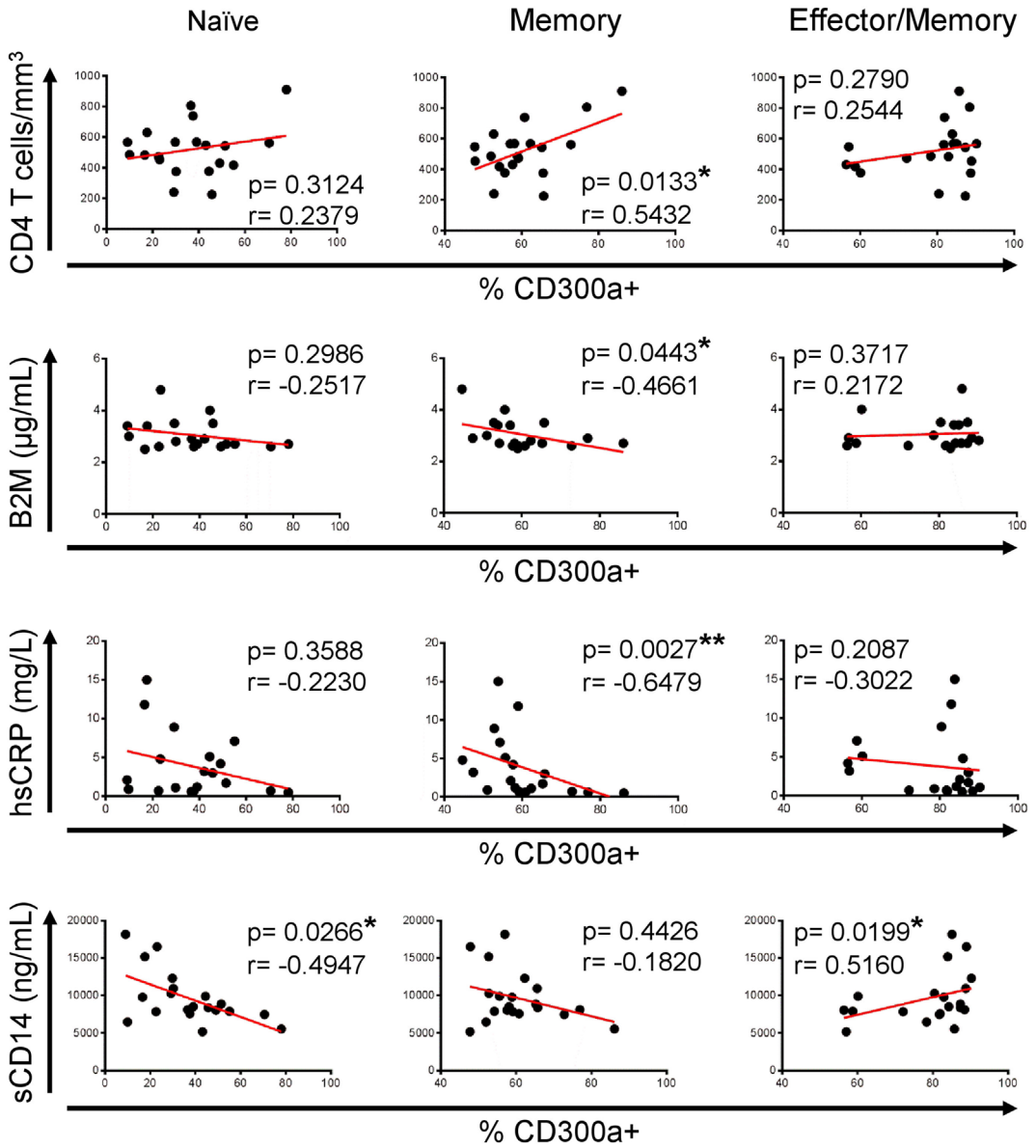

FIGURE 5 | Association of CD300a expression on CD4+ T lymphocytes with markers of human immunodeficiency virus (HIV)-1 infection progression in cART naïve HIV-1 infected patients. Correlation analysis of the percentage of CD300a+ cells on naïve (left column), memory (middle column), and effector/memory (right column) CD4+ T lymphocytes, with CD4+ T cell counts and β2-microglobulin (B2M), high sensitive C-reactive protein (hsCRP), and soluble CD14 (sCD14) levels in cART naïve HIV-1 infected patients. *$p < 0.05$, **$p < 0.01$.

as for example, the positive correlation between the percentage of CD300a+ cells and sCD14 levels (**Figure 5**), making it difficult to obtain a clear interpretation of the results in this CD4+ T cell subset from untreated patients.

Interestingly, and in opposition to the analyses performed with samples from untreated patients, regarding naïve or memory CD4+ T cells from cART-treated patients, we did not see any correlation between the studied parameters, but an opposite tendency was noticed (**Figure 6**). Furthermore, effector/memory cells from HIV ART displayed a significant negative correlation between the percentage of CD300a+ cells and CD4+ T cell numbers (**Figure 6**). TEM CD4+ T cells from both untreated and cART-treated HIV-1 infected patients did not show any significant correlation (data not shown) with the CD4+ T cell count and serum levels of soluble markers. Altogether, these results suggest that a higher frequency of CD300a+ cells on CD4+ T cells could be associated to a better progression in cART naïve HIV-1-infected people, while this association disappears after the introduction of cART.

DISCUSSION

The ability of the CD300a inhibitory receptor to modulate immune cell effector functions and its involvement in the pathogenesis

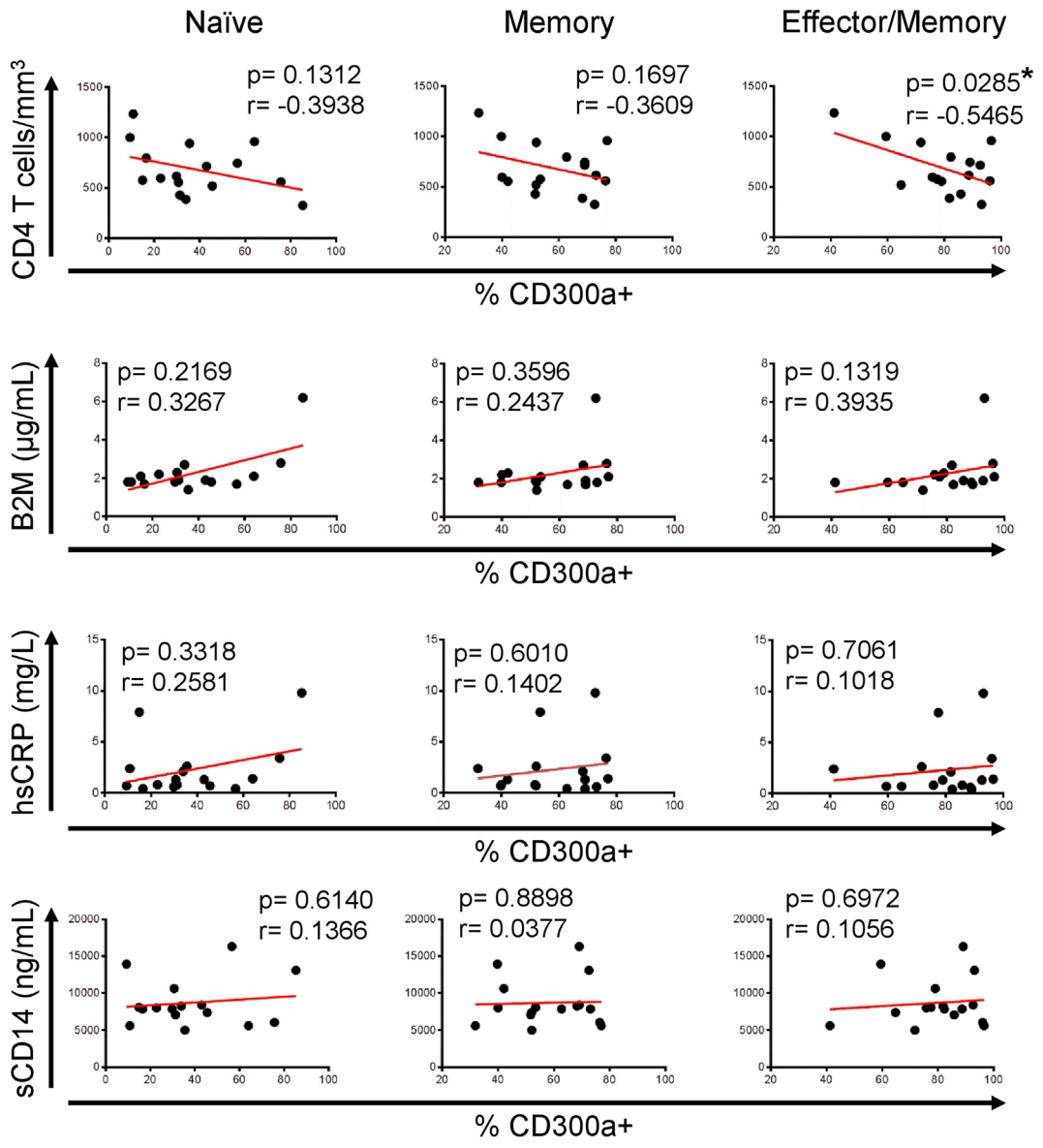

FIGURE 6 | Association of CD300a expression on CD4+ T lymphocytes with markers of human immunodeficiency virus (HIV)-1 infection progression in HIV-1 infected patients on cART. Correlation analysis of the percentage of CD300a+ cells on naïve (left column), memory (middle column), and effector/memory (right column) CD4+ T lymphocytes, with CD4+ T cell counts and β2-microglobulin (B2M), high sensitive C-reactive protein (hsCRP), and soluble CD14 (sCD14) levels in HIV-1-infected patients on cART. *$p < 0.05$.

of several diseases has recently triggered a great interest in this molecule (1, 2, 12, 13). The CD300a receptor is known to have an important role in various viral infections, enhancing viral entry and promoting viral escape from the immune system (14, 15, 34). However, there are only a few publications about CD300a on HIV-1 infection. These publications indicate a possible role for CD300a in the exhaustion of HIV-specific CD8+ T cells (19) and in the B cell hyper-activation and dysfunction found in HIV-1 infected patients (20). In this work, we report that the expression levels of the CD300a inhibitory receptor are increased on CD4+ T cells from HIV-1-infected patients. Moreover, we found a CD4+ T cell population co-expressing PD1, CD38, and CD300a that is expanded in cART naïve HIV-1 infected patients, while in healthy donors and cART-treated patients is rare. We also discovered an association of CD300a expression with some markers of disease progression in non-treated HIV-1-infected patients.

The differential expression of CD300a inhibitory receptor among CD4+ T cell subpopulations was previously described in healthy donors (9–11). Here, we first observed that the expression pattern in the CD4+ T cell subsets in HIV-1 infected patients was similar to the one exhibited by healthy donors: naïve CD4+ T cells displayed the lowest levels of CD300a while TEM cells the highest and memory and effector/memory CD4+ T cells expressed intermediate levels. But importantly, we have discovered that the expression levels of the CD300a receptor in HIV-1 infected patients were significantly altered when we compared them with the expression on CD4+ T cells from healthy donors. Hassouneh et al. have described a higher percentage of CD300a+ cells on total CD4+ T cells in healthy donors, mainly in middle-age and old people, which are seropositive for cytomegalovirus (CMV) (35). Published data have shown that there is a high incidence of CMV positive blood donors in Spain (36) and also of CMV positive organ donors (see report from the Spanish National Transplant Organization at www.ont.es/infesp/Memorias/Memoria%20Donación%202017.pdf). On the other hand, it is also well known that a high percentage of HIV-1 infected people are co-infected with CMV (50–80%) (37, 38). Our results showed no significant differences in the frequency of CD300a+ cells on total CD4+ T cells and in the different cell subsets (Figure S2 in Supplementary Material), which somehow could be explained by the high incidence of CMV in both healthy donors and HIV-1-infected patients. On the other hand, we observed a significant upregulation of CD300a MFI on CD4+CD300a+ T cells from HIV-1-infected patients (**Figure 1**). Future studies are required to determine the role of CMV infection on the altered expression of CD300a observed in HIV-1-infected patients.

The expression of CD300a molecule is regulated by different stimuli. For instance, CD300a expression is upregulated after TLR9 stimulation on memory B cells (20) and its expression also increases on monocytes stimulated with IFNγ and cultured under hypoxia conditions (39, 40). The reason of why the CD300a receptor is upregulated on HIV-1-infected CD4+ T cells is still unknown. Though, as other immunomodulatory receptors such as PD1, the CD300a inhibitory receptor may be inductively expressed after T cell activation as a regulatory mechanism during HIV-1 infection, and probably the cytokine environment has an important role determining the expression levels of CD300a. It has been previously published that Th1 polarization in the presence of IL-12 induces the generation of mostly CD300a+ cells in healthy donors (9). Moreover, patients with HIV infection display an increased IL-12 production *in vivo* and *ex vivo* (41). Thus, it could be possible that the higher IL-12 production, among others, during acute/early HIV infection, may induce the upregulation of CD300a and this overexpression might be maintained during chronic HIV infection. Clearly, more studies are required to investigate the factors leading to an increase in the expression levels of CD300a during HIV infection. On other hand, our results did not show significant differences in CD300a expression levels on CD4+ T cells between cART naïve and cART-treated HIV-1 infected people, meaning that cART does not reverse the upregulation of CD300a found in infected patients. This is in line with previous results published by us where the altered levels of CD300a expression on B cells are not reversed by cART (20). The maintenance of the higher expression levels of CD300a inhibitory receptor in cART-treated HIV-1-infected subjects could be a reflection of the continuous immune activation in these patients, even after cART. It is well known that although cART decreases viral load to undetectable levels, as HIV is not completely eradicated, the activation of the immune system still occurs (32, 42–45).

Consistent with the results described by Quigley et al., who showed a positive correlation between CD300a mRNA levels and BATF, a transcription factor downstream of PD1 that increases inhibitory pathways on HIV-specific exhausted CD8+ T cells (19), here, we have discovered a higher frequency of CD300a+ cells on PD1+ cells in comparison with PD1− cells within most of CD4+ T cell subsets from both healthy donors and HIV-1 infected patients. It is well known that PD1 is an inhibitory receptor that is upregulated after T cell activation as a negative feedback mechanism (27–29). Several publications have proposed that PD1, apart from inducing immune exhaustion, identify a particular T cell differentiation stage and effector function (46–48). For instance, memory PD1+CD4+ T lymphocytes from healthy donors and HIV-1 infected children preferentially secreted IFNγ and IL-17A (49). Previously, it has been described that in healthy donors, CD4+ T cells expressing CD300a were higher producers of IFNγ than CD300a− cells, and that they were more polyfunctional (9, 11). Therefore, CD300a receptor, as PD1, may represent a CD4+ T cell subset with specific effector functions, at least in healthy donors. But even more relevant for this study, the expression levels of the CD300a inhibitory receptor were significantly higher on PD1+CD4+ T lymphocytes from HIV-1-infected patients when compared with the same cells from healthy donors.

It is well known that HIV-1 induces T cell activation and consequently increases the expression of CD38 (30, 50). A higher CD38 expression on CD4+ T cells from viremic HIV-1-infected people is a biomarker of poor prognosis and is strongly associated with short survival in patients with advanced infection (30–32, 51). In this study, we saw a decrease in the percentage of CD300a+ cells within CD38+CD4+ T lymphocytes from both healthy people and HIV-1 infected patients, in comparison with CD38−CD4+ T cells. But importantly, CD38+ cells from HIV-1

infected patients exhibited higher expression levels of CD300a than the CD38+ cells from healthy donors, which is consistent with a general upregulation of CD300a expression levels on different CD4+ T cell populations after HIV-1 infection, regardless of the exhaustion or activation status of the cells. Finally, Boolean gate analysis showed that in terms of CD300a, PD1, and CD38 expression pattern, the phenotype of CD4+ T cells from healthy donors was very similar to the one of cART-treated HIV-1 infected people, while naïve patients for cART exhibited a different pattern. It is very possible that the differences found between cART-treated and non-treated HIV-1 infected subjects are mainly due to their disparities in PD1 and CD38 expression. Of note, these analyses revealed an expansion of a triple positive cell subset (CD300a+PD1+CD38+) in untreated patients that is very rare in healthy donors and HIV-1 patients on cART. The increased percentage of this triple positive population is very possibly the consequence of an increase in PD1+ and CD38+ cells in cART naïve patients rather than an increase in the frequency of CD300a+ cells. This subset may represent a highly activated population characterized by high expression of immune checkpoints. In the future, it will be very interesting to study in depth this subpopulation, both phenotypically (immune checkpoints) and functionally (proliferation, cytokine production, etc.).

Finally, we investigated if there was any association between CD300a expression and markers of HIV-1 disease progression. An increase in CD4+ T cell counts has been broadly interpreted as good prognosis in HIV-1-infected people (31, 52). Moreover, there are various soluble molecules that have been related to HIV-1 infection progression. Among others, high β2-microglobulin and hsCRP values in plasma reflect the activation of immune system (53, 54) and illustrate disease progression (54, 55). Furthermore, high plasma levels of sCD14, a marker of microbial translocation, have also been related to disease progression and poor response to cART in HIV-infected patients (56–58). Our results showed that in untreated HIV-1-infected patients, the percentage of cells expressing CD300a was positively correlated with CD4+ T cell numbers, while was negatively associated to β2-microglobulin, hsCRP, and sCD14 levels, suggesting that a higher frequency of CD300a+ cells could be indicative of good prognosis in active HIV-1 infection. In contrast, our results did not show the same tendency regarding to CD4+ T cells from HIV-infected patients receiving cART. In fact, an opposite pattern between naïve cART and treated patients was found. This could suggest that this receptor might play different roles depending on the levels of virus replication and T cell activation. Since T cell activation supports viral replication, in untreated HIV-1 patients, a higher expression of the CD300a inhibitory receptor might be beneficial. However in patients under effective cART, because viral levels decrease and consequently permit the antiviral effects to prevail, higher expression levels of CD300a inhibitory receptor could be counterproductive for T cell activation. Undoubtedly, further studies are required to test this hypothesis.

In summary, our study demonstrates that chronically HIV-1 infected patients exhibit an altered expression of CD300a inhibitory receptor on CD4+ T lymphocytes that is not reverted by effective cART. Moreover, we have discovered a negative correlation between CD300a levels and some markers of HIV-1 infection progression in cART naïve HIV-1 infected patients. These are promising findings that will lead to new research aimed at further understanding the role of the CD300a inhibitory receptor in CD4+ T lymphocytes during chronic HIV-1 infection.

ETHICS STATEMENT

Institutional and Ethical Review Board approvals were obtained and written informed consent was obtained from all healthy donors and patients. The study was approved by the Basque Ethics Committee for Clinical Research and Virgen del Rocío University Hospital Ethics Committee for Biomedical Research.

AUTHOR CONTRIBUTIONS

JV designed and performed experiments, analyzed and interpreted the data, designed the figures, and wrote the manuscript. IT analyzed the results and made the figures. LG-U analyzed the results. AO participated in the interpretation of the data. ML and MG clinically characterized the patients and participated in the interpretation of the data. LT-D and ER-M collected samples, performed experiments, and analyzed the data. SG-O analyzed the results and participated in the interpretation of the data. OZ participated in the design of the study and interpreted the data. FB conceived and designed the study, interpreted the data, and wrote the manuscript. All the authors critically reviewed, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01709/full#supplementary-material.

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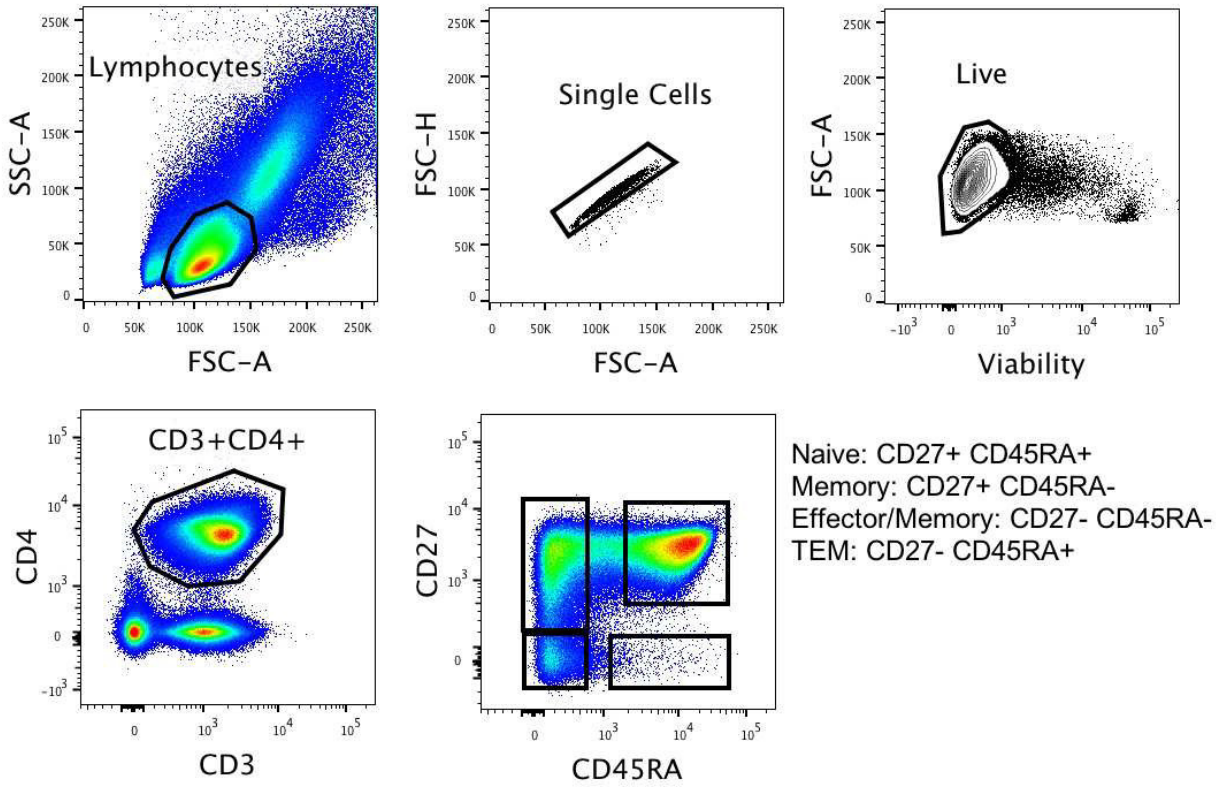
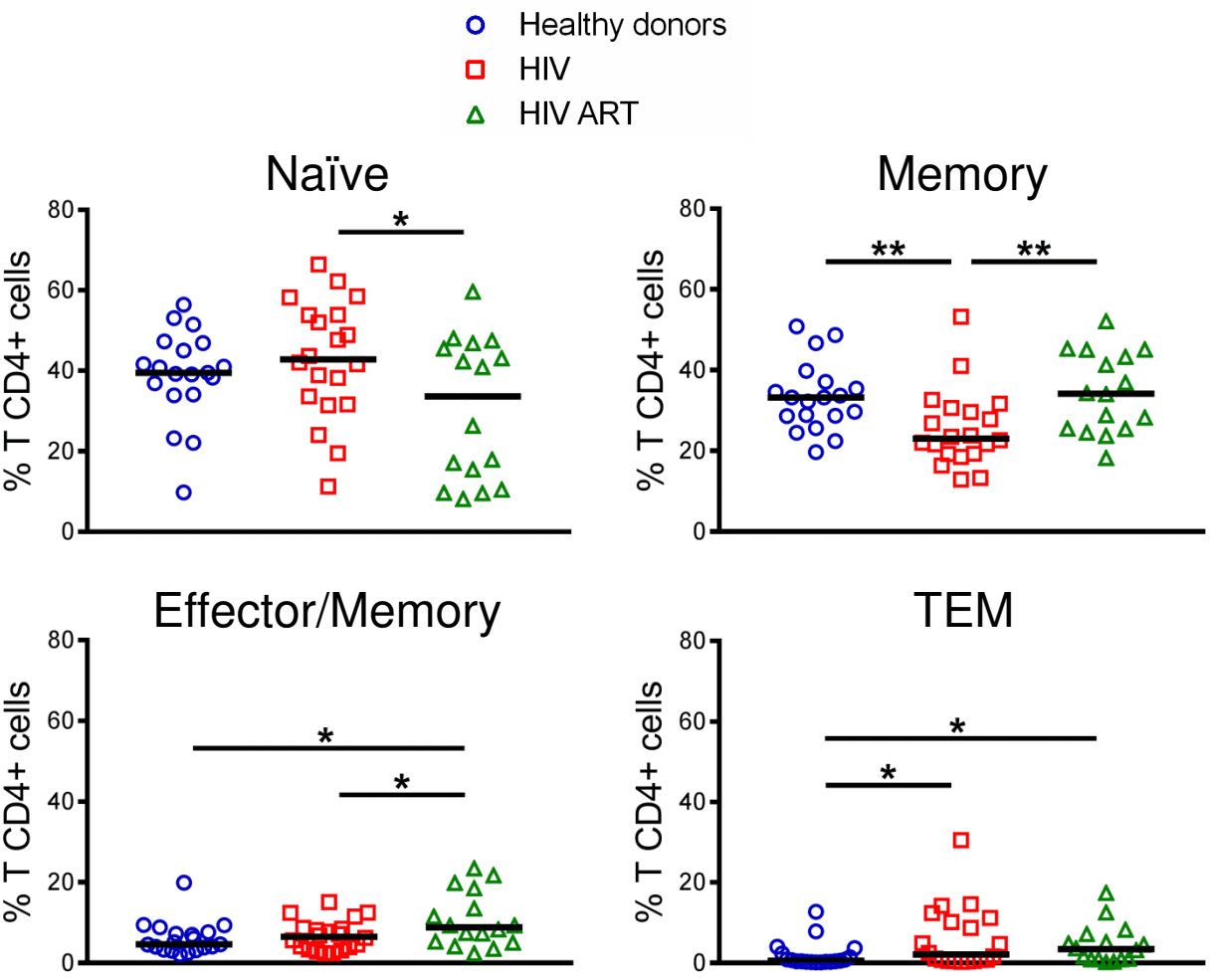
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

A

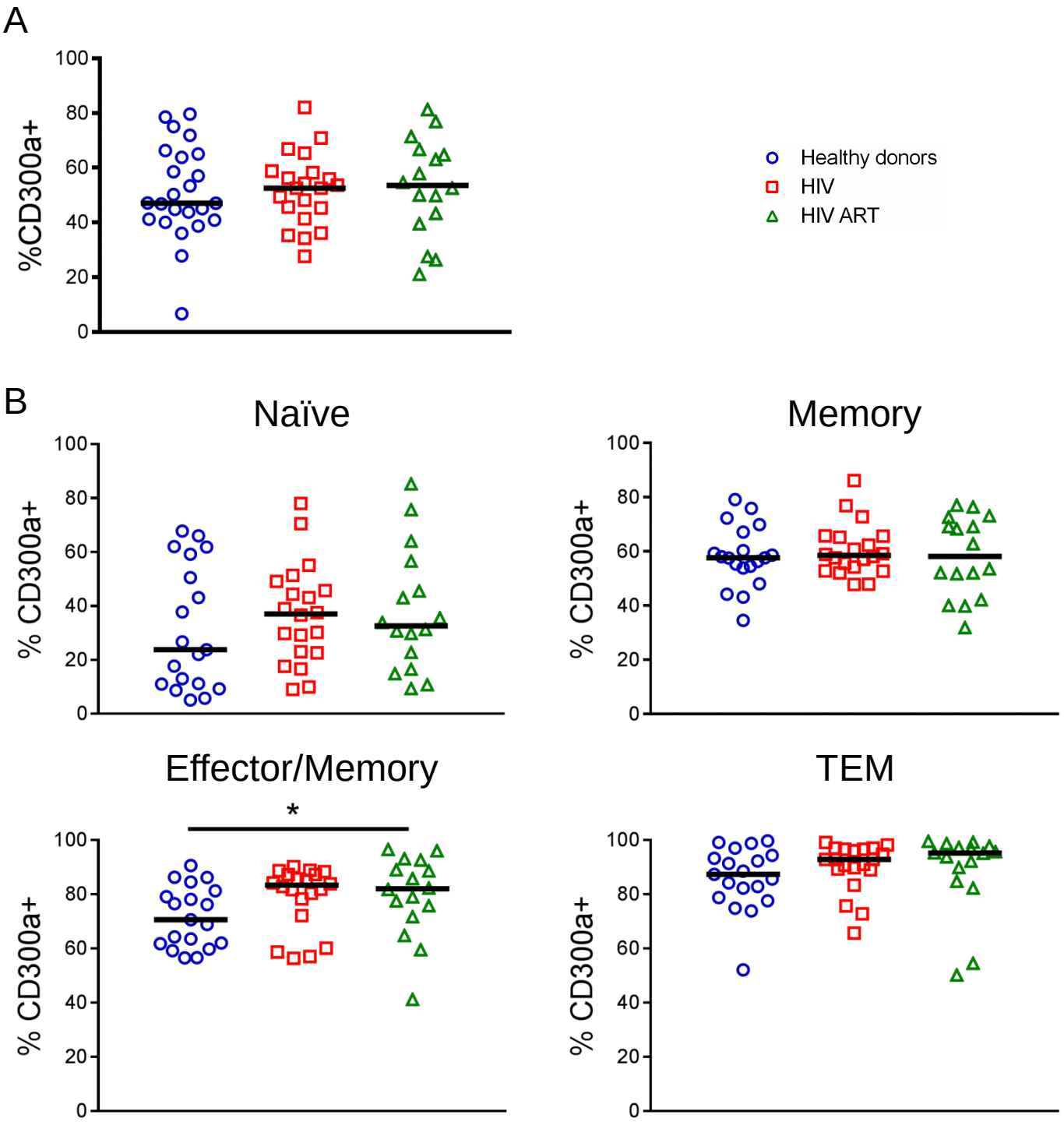


Naive: CD27+ CD45RA+
Memory: CD27+ CD45RA-
Effector/Memory: CD27- CD45RA-
TEM: CD27- CD45RA+

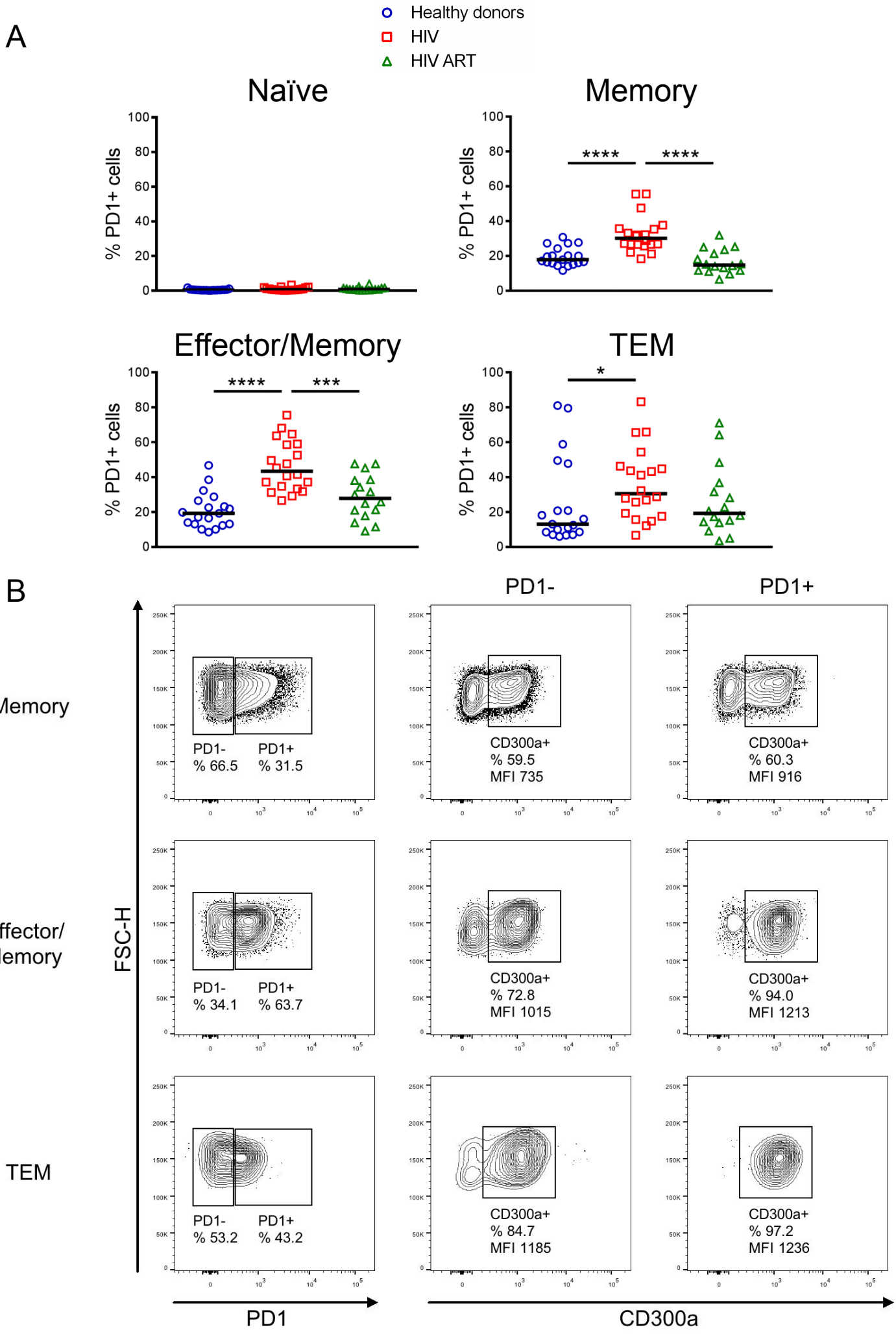
B



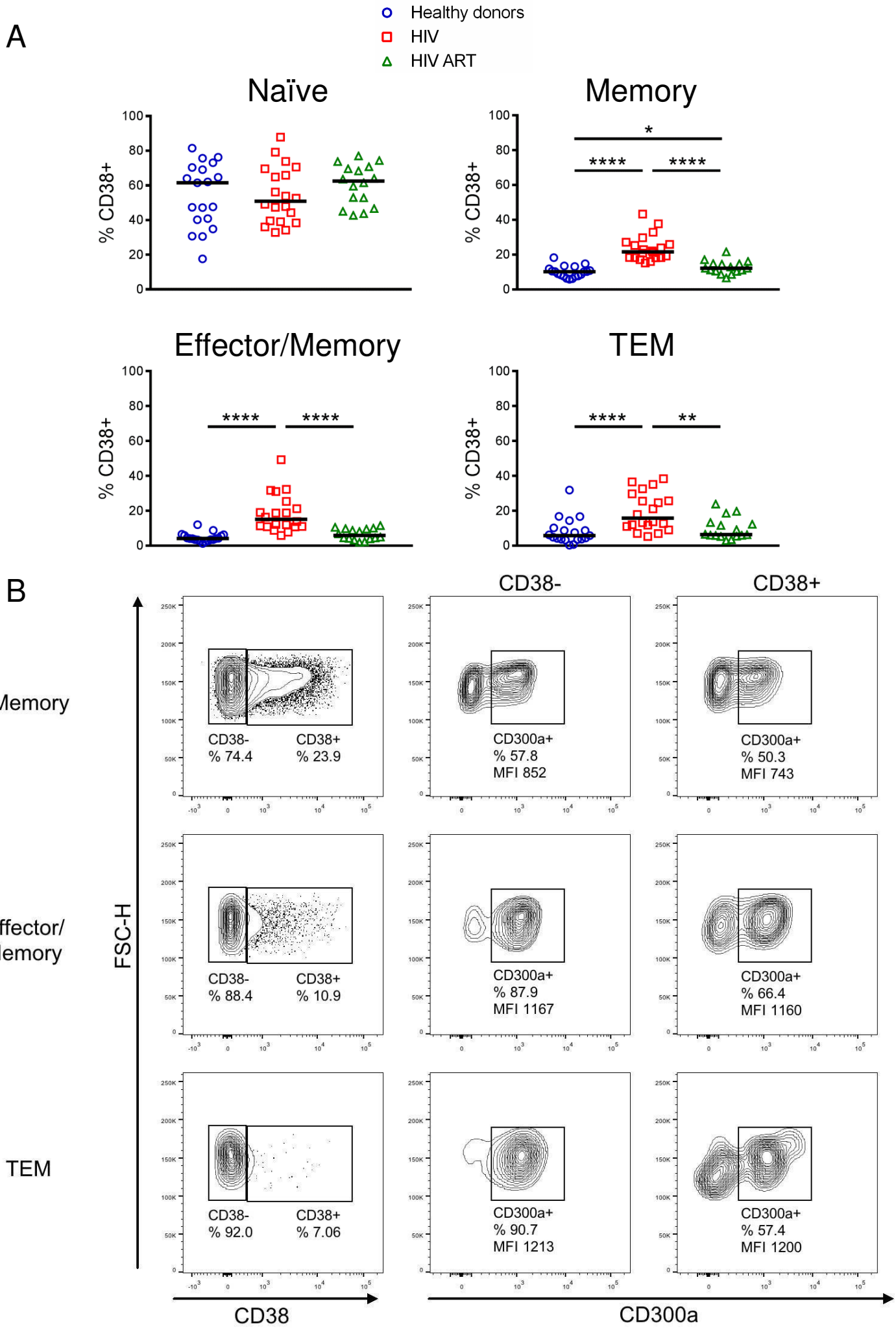
Supplementary Figure 1. CD4+ T cell subsets from healthy donors and HIV-1 infected patients. (A) Pseudocolor plots representing the gating strategy utilized during the study, data from a representative healthy donor is shown. Lymphocytes were electronically gated based on their forward and side scatter parameters, then live cells were selected and CD4+ T lymphocytes were detected by the expression of both CD3 and CD4. Four CD4+ T subsets were differentiated based on the expression of CD27 and CD45RA: naïve (CD27+CD45RA+), memory (CD27+CD45RA-), effector/memory (CD27-CD45RA-) and terminal differentiated effector/memory (TEM) (CD27-CD45RA+) cells. (B) Dot plot graphs showing the percentage of each CD4+ T cell subpopulation from healthy donors, cART naïve (HIV) and patients on cART (HIV ART). Each dot represents a subject and the median is shown. *$p<0.05$, ** $p<0.01$.



Supplementary Figure 2. Percentage of CD300a+ cells on CD4+ T cells from healthy donors and HIV-1 infected patients. Dot plots showing (A) the percentage of CD300a+ cells in total CD4+ T cells and (B) within CD4+ T cell subsets from healthy donors, naïve for cART (HIV) and patients on cART (HIV ART). Each dot represents a subject and the median is shown. *p<0.05.


A
Healthy donors
HIV
HIV ART
Naïve
Memory
Effector/Memory
TEM
% PD1+ cells
****
***
*
B
PD1-
PD1+
Memory
Effector/
Memory
TEM
FSC-H
PD1
CD300a
PD1-
% 66.5
PD1+
% 31.5
CD300a+
% 59.5
MFI 735
CD300a+
% 60.3
MFI 916
PD1-
% 34.1
PD1+
% 63.7
CD300a+
% 72.8
MFI 1015
CD300a+
% 94.0
MFI 1213
PD1-
% 53.2
PD1+
% 43.2
CD300a+
% 84.7
MFI 1185
CD300a+
% 97.2
MFI 1236

Supplementary Figure 3. Percentage of PD1+ and PD1- cells within CD4+ T lymphocytes. (A) Dot plots representing the percentage of PD1+ cells within CD4+ T cells from healthy donors, cART naïve (HIV) and patients on cART (HIV ART). Each dot represents a subject and the median is shown. (B) Contour plots showing the percentage of PD1+ and PD1- cells, and the percentage of CD300a+ cells within PD1+ and PD1- cells on CD4+ T lymphocytes. Data from a representative untreated HIV-1 infected patient is shown. *$p<0.05$, *** $p<0.001$, **** $p<0.0001$.


A
Healthy donors
HIV
HIV ART
Naïve
Memory
Effector/Memory
TEM
% CD38+
100
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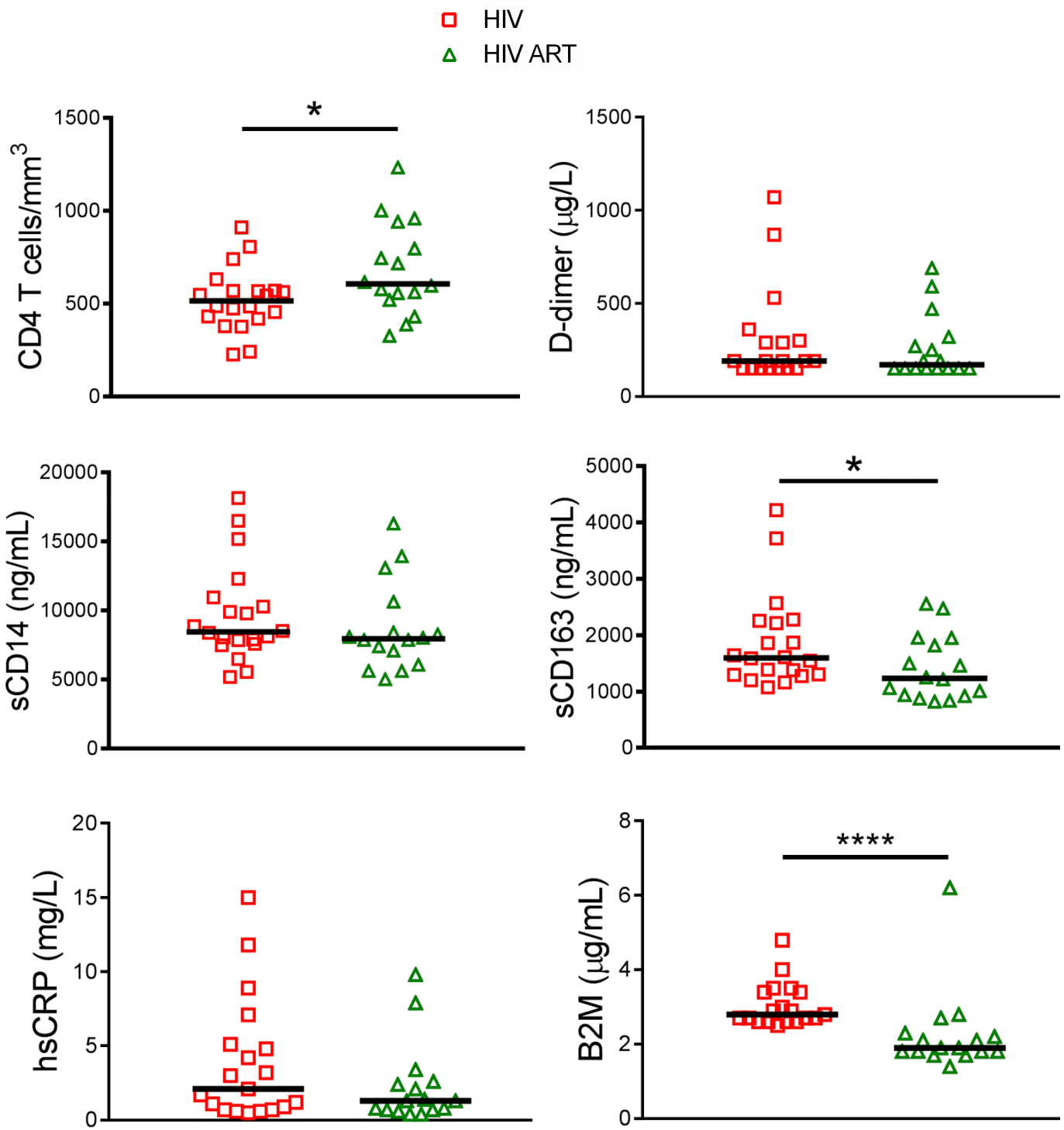
B
CD38-
CD38+
Memory
Effector/
Memory
TEM
FSC-H
CD38
CD300a
CD38-
% 74.4
CD38+
% 23.9
CD300a+
% 57.8
MFI 852
CD300a+
% 50.3
MFI 743
CD38-
% 88.4
CD38+
% 10.9
CD300a+
% 87.9
MFI 1167
CD300a+
% 66.4
MFI 1160
CD38-
% 92.0
CD38+
% 7.06
CD300a+
% 90.7
MFI 1213
CD300a+
% 57.4
MFI 1200

Supplementary Figure 4. Percentage of CD38+ and CD38- cells within CD4+ T lymphocytes. (A) Dot plots representing the percentage of CD38+ cells within CD4+ T cells from healthy donors, cART naïve (HIV) and patients on cART (HIV ART). Each dot represents a subject and the median is shown. (B) Contour plots showing the percentage of CD38+ and CD38- cells, and the percentage of CD300a+ cells within CD38+ and CD38- cells on CD4+ T lymphocytes. Data from a representative untreated HIV-1 infected patient is shown. *$p<0.05$, ** $p<0.01$, **** $p<0.0001$.



Supplementary Figure 5. Markers of HIV-1 disease progression in untreated and patients on cART (HIV ART). Dot plots representing CD4+ T cell count and plasma levels of D-dimer, soluble CD14 (sCD14), soluble CD163 (sCD163), high sensitive C-reactive protein (hsCRP) and beta-2-microglobulin (B2M) from cART naïve and patients on cART (HIV ART). Each dot represents a subject and the median is shown. *p<0.05, **** p<0.0001.

S1 Table. Clinical data of cART naïve HIV-1-infected patients.

PATIENTS	**SEX**	**AGE**	**CD4**	**VL**
28898	MAN	28	910	199000
29589	MAN	36	569	120000
25998	MAN	33	562	12900
29208	MAN	49	418	51900
27315	MAN	46	454	233000
28285	MAN	28	376	65400
28178	MAN	23	241	268000
26183	MAN	24	568	20600
27213	MAN	30	547	22800
28109	MAN	22	806	61400
28547	MAN	25	486	11400
29384	MAN	29	484	72600
30385	MAN	47	226	47500
27090	MAN	20	630	97600
29452	MAN	35	543	43400
30995	MAN	32	431	75700
27594	MAN	30	378	93200
26558	MAN	27	739	9350
25966	WOMAN	26	473	32400
25909	MAN	20	567	21700

S2 Table. Clinical data of cART treated HIV-1-infected patients

PATIENTS	SEX	AGE	cART years	CD4 T count	VL
30318	MAN	31	3	519	<20
30135	MAN	45	9	596	<20
29987	MAN	69	15	387	<20
29984	MAN	45	7	958	<20
29971	WOMAN	28	2	796	<20
29970	MAN	68	16	560	<20
29969	WOMAN	53	12	744	<20
29965	WOMAN	35	2	1001	<20
29935	MAN	62	7	555	<20
29925	MAN	39	12	577	<20
29919	MAN	60	24	325	<20
29909	MAN	52	3	616	<20
29895	MAN	41	5	941	<20
29879	MAN	58	12	1233	<20
29862	MAN	32	3	429	<20
29854	MAN	28	2	716	<20

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CD300a identifies a CD4+CD45RA- T lymphocyte subset with a higher susceptibility to HIV-1 infection

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Abstract:
Several CD4+ T cell receptors such as some chemokine receptors, PD1 or CTLA-4 have been previously associated to a higher susceptibility to HIV-1 infection. Human CD300a is found on some CD4+ T lymphocytes and HIV-1 infection increases its expression on this cell type. Moreover, the receptor is known to promote the infection by Dengue and other viruses. Thus, in this study we investigated if CD300a expressing CD4+ T cells might be more susceptible to HIV-1 infection than cells negative for the receptor. First, CD4+RA- T cells obtained from untreated HIV-1 infected patients were expanded and a higher percentage of CD300a expressing cells were observed within HIV-1 infected CD4+RA- T cells than within the uninfected cells. Furthermore, CD4+RA- T cells from healthy donors were in vitro activated and infected with HIV-1. Our results showed a significantly higher percentage of HIV-1 infected cells within the CD300a+ subset than in CD300a- subset. This CD300a expressing CD4+RA- T cell subset displayed higher expression of CCR5 and activation markers CD38, HLA-DR and PD1 after activation and HIV-1 infection. Therefore, CD300a might be a potential biomarker of susceptibility to HIV-1 infection on memory CD4+ T lymphocytes.

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Key points:

- Ex vivo expanded HIV-1 infected CD4+RA- T lymphocytes from untreated HIV-1 infected patients are predominantly CD300a+.
- CD4+RA- T lymphocytes expressing CD300a are more susceptible to HIV-1 infection in vitro than cells negative for this receptor.

ABSTRACT

Several CD4+ T cell receptors such as some chemokine receptors, PD1 or CTLA-4 have been previously associated to a higher susceptibility to HIV-1 infection. Human CD300a is found on some CD4+ T lymphocytes and HIV-1 infection increases its expression on this cell type. Moreover, the receptor is known to promote the infection by Dengue and other viruses. Thus, in this study we investigated if CD300a expressing CD4+ T cells might be more susceptible to HIV-1 infection than cells negative for the receptor. First, CD4+RA- T cells obtained from untreated HIV-1 infected patients were expanded and a higher percentage of CD300a expressing cells were observed within HIV-1 infected CD4+RA- T cells than within the uninfected cells. Furthermore, CD4+RA- T cells from healthy donors were in vitro activated and infected with HIV-1. Our results showed a significantly higher percentage of HIV-1 infected cells within the CD300a+ subset than in CD300a- subset. This CD300a expressing CD4+RA- T cell subset displayed higher expression of CCR5 and activation markers CD38, HLA-DR and PD1 after activation and HIV-1 infection. Therefore, CD300a might be a potential biomarker of susceptibility to HIV-1 infection on memory CD4+ T lymphocytes.

INTRODUCTION

CD4+ T lymphocytes are the main target of HIV-1, principally due to the expression on the cell surface of CD4 receptor and CCR5 and CXCR4 co-receptors[1,2]. Although CCR5 and CXCR4 are known to be the major co-receptors for HIV-1, other chemokine receptors as CXCR6, CCR2 and CX3CR1 have been associated to HIV-1 infection or disease progression[3,4]. In addition, the protease CD26 and the integrins α4β7 and lymphocyte function-associated antigen 1 (LFA-1) act as HIV cofactors and have been also related to an increased susceptibility of CD4+ T cells to HIV-1 infection[4]. Moreover, other surface receptors have been suggested to identify CD4+ T cell subsets that are more permissive to HIV infection. This is based on the observations that memory CD4+ T cells expressing high levels of PD1 and low levels of CTLA-4 were preferentially lost in advanced HIV-1 infection[5] and also that CD161 identified a Th17 cell subset that was especially depleted in HIV-1 infected patients[6,7].

Human CD300a is an inhibitory receptor expressed on the surface of several immune cell types[8–10]. Our group has previously studied the expression and function of CD300a on various cell types from HIV-1 infected patients, including B cells, monocytes, CD4+ T cells and NK cells[11–15]. Among others findings, we have described higher expression levels of CD300a on CD4+ T cells from HIV-1 infected patients than in healthy donors, and this up-regulation was not reverted with combined antiretroviral therapy (cART)[14]. CD300a has the capacity to interact with phosphatidylserine (PS) and phosphatidylethanolamine (PE)[16]. Carnec *et al* have described that CD300a receptor binds directly Dengue Virus (DENV) recognizing predominantly PE and to a lesser extent PS from the viral envelope, and enhances viral entry. Furthermore, CD300a also promotes cell infection by Yellow Fever, West Nile and Chikungunya viruses[17]. Thus, the main objective of this work was to investigate if the expression of CD300a could lead CD4+ T lymphocytes to be more susceptible to HIV-1 infection.

METHODS

Freshly isolated and enriched memory CD4+CD45RA- (CD4+RA-) T lymphocytes from naïve for cART HIV-1 infected patients were activated with phytohemagglutinin (PHA) for 24h and expanded by culturing them for 13 days with interleukin (IL)-2. On the other hand,

CD4+RA- T cells from healthy donors were also activated and then infected with HIV-1 (BaL) overnight and cultured with IL-2 for 7 days (Supplementary Figure 1). Samples were analyzed with flow cytometry-based procedures.

Detailed methodology is provided in supplemental material, available on Blood Web site.

RESULTS AND DISCUSSION

CD4+RA- T lymphocytes, that include effector memory and central memory CD4+ T cells, have been described as main targets of HIV and, in particular, central memory cells are known to highly contribute to HIV reservoir[18,19]. CD4+RA-T lymphocytes can be divided in CD300a- and CD300a+ populations in both healthy donors and HIV-1 infected patients[14,20] (Supplementary Figure 2). With the aim to investigate the relationship between CD300a expression and HIV-1 infection susceptibility, in this study we first ex vivo expanded CD4+RA- T lymphocytes from untreated HIV-1 infected patients, and determined the percentage of CD300a+ cells within the in vivo HIV-1 infected cells (p24+) (Supplementary Figure 1A and 2). Our results showed a significantly higher percentage of CD300a expressing cells within HIV-1 infected CD4+RA- T cells (p24+) than within the uninfected cells (p24-), at both day 7 and day 13 of expansion (Figure 1). Hence, these results strongly suggest that in untreated patients the HIV-1 infected cells are predominantly CD300a+. Nevertheless, as the CD4+RA- T cells were infected by HIV-1 in vivo, it is unclear if CD300a expressing cells are more vulnerable to HIV-1 infection or if the infected CD300a+ subset is more expanded during the culture.

Therefore, in order to study the susceptibility of CD300a+ cell subset to HIV-1 infection, CD4+RA- T cells from healthy donors were infected in vitro with HIV-1 (Supplementary Figure 1B) and the percentage of infection (p24+) on CD300a+ and CD300a- CD4+RA- T lymphocytes was determined. First, we confirmed that CD4+RA- T cells were efficiently infected by HIV-1 in vitro and that the infection did not induce any change in the percentage of CD300a+ cells at different time points, suggesting that during the culture period CD300a+ cells did no expand at a higher rate (Supplementary Figure 3). Importantly, we observed a higher percentage of HIV-1 infected cells (p24+) within CD300a+ CD4+RA- T cells than within CD300a- subset (Figure 2A). After that, we separately infected CD300a+ and CD300a- CD4+RA- T cells to discard the possibility that the higher susceptibility of

CD300a+ cells to be infected by HIV-1 was somehow related to the fact that both cell subsets were in contact during the pre-activation with PHA, infection and the subsequent culture in the presence of IL-2. Thus, we sorted CD300a- and CD300a+ CD4+RA- T cell subsets from three healthy donors before cells were activated and infected in vitro with HIV-1. This experiment, not only confirmed our previous results, but also showed a greater difference in the percentage of HIV-1 infection between CD300a expressing CD4+RA- T cells and cells negative for the receptor (Figure 2B).

The cause of why CD300a expressing CD4+RA- T cells are more susceptible to HIV-1 infection is unknown. CD300a could work as an attachment factor for HIV-1 through its interaction with PS and PE from the viral envelope, as it does occur with DENV[17]. In fact, HIV is known to contain both PS and PE in its envelope[21,22]. However, other factors might be contributing to the susceptibility to HIV-1 infection. Thus, in order to better characterize CD300a+ and CD300a- CD4+RA- T cell subsets, we determined the expression of the co-receptor CCR5 and activation markers CD38, HLA-DR and PD1. We found a higher expression of CCR5 within CD300a+ cells than in CD300a- CD4+RA- T cells at all time points (before and after the activation and infection) (Figure 2C). Moreover, we observed that CD300a+ population displayed a higher percentage of CD38+, HLA-DR+ and PD1+ cells after activation and infection with HIV-1 (Figure 2C). The fact that CD300a+ CD4+RA- T cells express more CCR5, a co-receptor with a critical role in HIV entry[1,23], and that they seem to be predisposed to be more activated, which also promotes viral infection and replication[24,25], might partially explain the higher susceptibility to HIV-1 infection of this CD4+ T cell subset. Nevertheless, it has to be mentioned that we did not observe a correlation between the percentage of CCR5+ cells and the frequency of infected (p24+) cells (Supplementary Figure 4), which it may suggest that the differential expression of CCR5 between the CD300a+ and CD300a- subsets is not a highly relevant aspect and/or that other factors may be also involved in the higher susceptibility of CD300a+ CD4+RA- T cells to HIV-1 infection.

In conclusion, in this study we have described that in vivo HIV-1 infected CD4+RA- T lymphocytes are predominantly CD300a+. Also, we have demonstrated that the presence of CD300a represents a memory CD4+ T cell subset, which is more susceptible to HIV-1 infection. These results highlight the potential of CD300a as a biomarker of susceptibility to HIV-1 infection in CD4+ T lymphocytes and its potential use as a future therapeutic target in this disease.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHORSHIP

J.V. designed and performed the experiments, analyzed and interpreted the data, designed the figures, and wrote the manuscript. L.T-D. and M.R.J-L. designed the experiments and interpreted the data. I.T. analyzed the results and made the figures. A.O. participated in the interpretation of the data. L.L-C. and C.R-O. characterized the patients and participated in the interpretation of the data. E.R-M. participated in sample collection, designed the experiments and interpreted the data. O.Z. participated in the design of the study and interpreted the data.

F.B. conceived and designed the study, interpreted the data, and wrote the manuscript. All the authors critically reviewed, edited, and approved the final manuscript.

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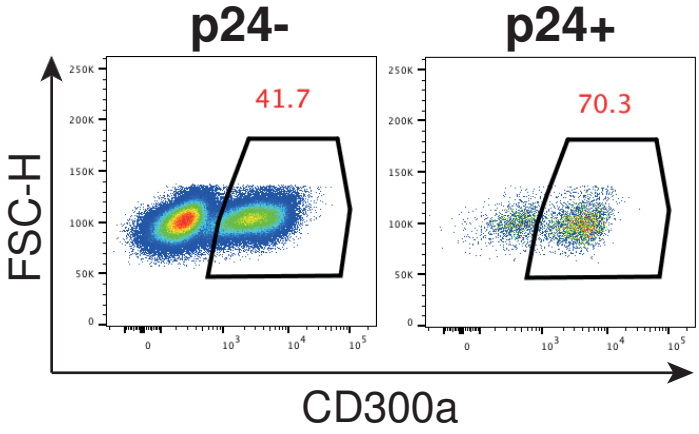
FIGURE LEGENDS

Figure 1. CD300a expression on in vivo HIV-1 infected CD4+RA- T lymphocytes. Pseudocolor plots showing the percentage of CD300a+ cells within non-infected (p24-) and HIV-1 infected (p24+) CD4+RA- T cells from a representative untreated HIV-1 infected patient at day 13 of culture (left). Before-after graphs showing the percentage of CD300a+ cells within non-infected (p24-) and HIV-1 infected (p24+) CD4+RA- T cells from untreated HIV-1 infected subjects at day 7 and day 13 of culture. Each dot represents a patient (right). *$p<0.05$.

Figure 2. In vitro HIV-1 infection and phenotypical characterization of CD4+RA- T lymphocytes. **(A)** Pseudocolor plots showing the percentage of infected (p24+) cells within CD300a- and CD300a+ CD4+RA- T cells from a representative healthy donor at day 7 of culture (left). Before-after graphs showing the percentage of infected (p24+) cells within CD300a- and CD300a+ CD4+RA- T cells from healthy donors at day 3 and day 7 of culture. Each dot represents a patient (right). **(B)** Pseudocolor plots representing the percentage of infected (p24+) cells within CD300a- and CD300a+ CD4+RA- T cells from three healthy donors that have been sorted before infection and cultured for 7 days **(C)** Bar graphs representing the percentage of CCR5+, CD38+, HLA-DR+ and PD1+ cells within CD300a- and CD300a+ CD4+RA- T cells from healthy donors before incubation (day -2) and at day 3 and day 7 of culture. Each dot represents a donor and the median is shown. *$p<0.05$, ** $p<0.01$.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p24+
41.7
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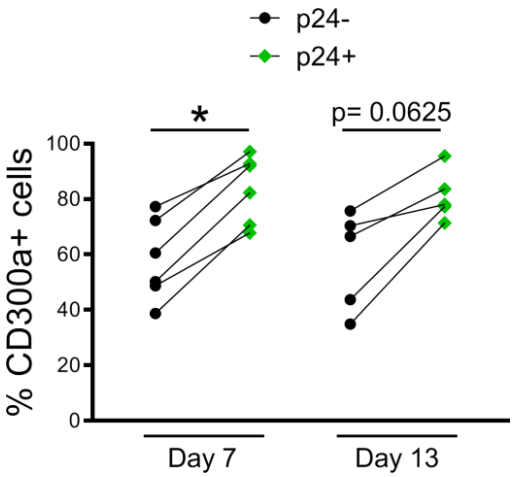
FSC-H
CD300a


p24-
p24+
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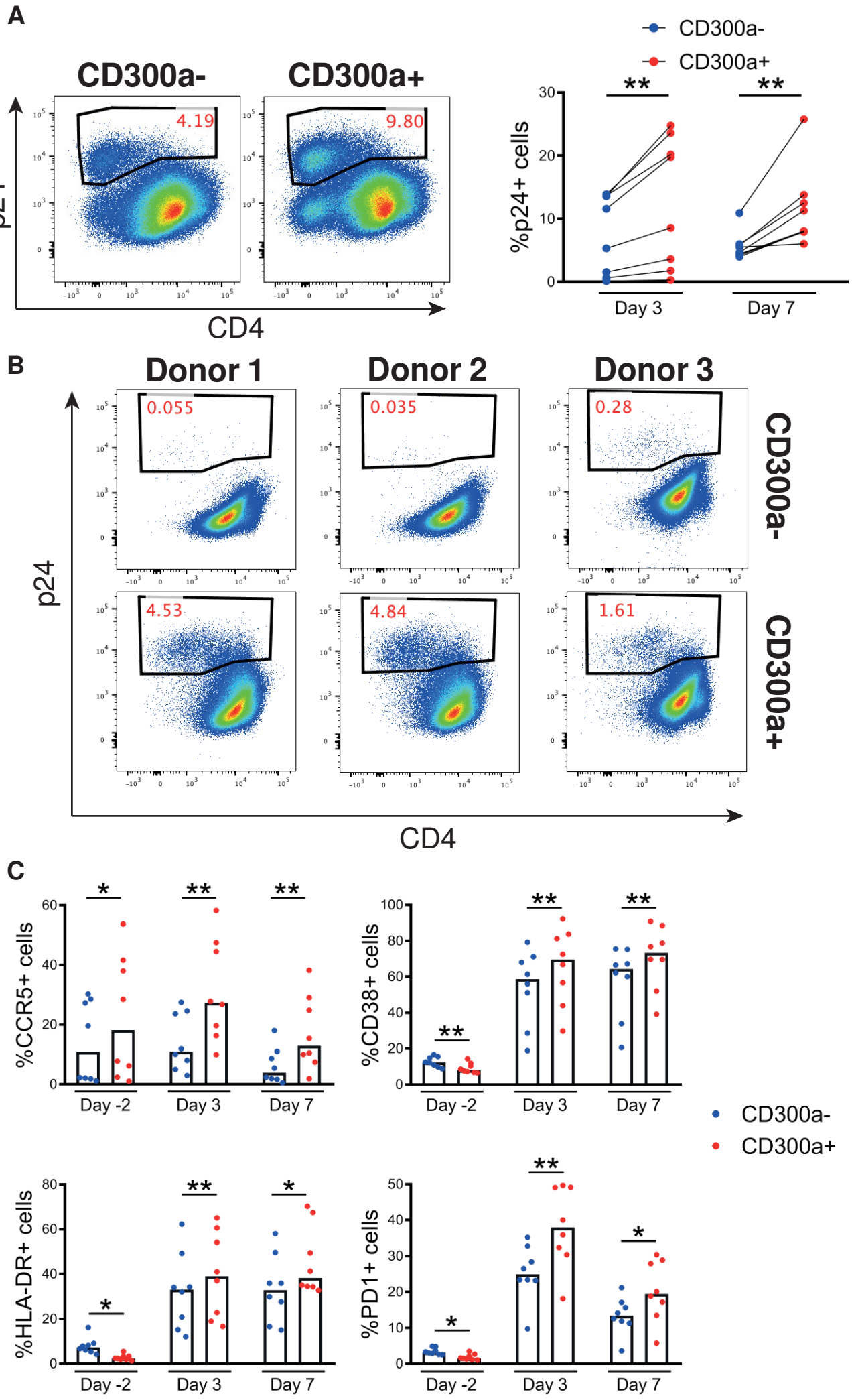
p= 0.0625
% CD300a+ cells
Day 7
Day 13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CD300a-
CD300a+
4.19
9.80
p24
CD4
%p24+ cells
Day 3
Day 7
B
Donor 1
Donor 2
Donor 3
0.055
0.035
0.28
4.53
4.84
1.61
CD300a-
CD300a+
C
%CCR5+ cells
%CD38+ cells
%HLA-DR+ cells
%PD1+ cells
Day -2
Day 3
Day 7

METHODS

Subjects and samples

In this work, freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors (n = 8) and naïve for combined antiretroviral therapy (cART) HIV-1 infected patients (n = 6) were studied. Whole blood samples from HIV-1 infected patients were collected from patients attending to the Infectious Diseases Unit at Virgen del Rocío University Hospital in Seville (Spain) and buffy coats from healthy donors were obtained from the Regional Center of Blood Transfusion of Seville. The study was approved by the Basque Ethics Committee for Clinical Research (PI2014017 and PI2013108) and the Virgen del Rocío University Hospital Ethics Committee for Research (15/2009). All subjects provided written and signed informed consent in accordance with the Declaration of Helsinki. Clinical data of HIV-1 infected subjects are described in Supplementary Table 1.

Laboratory measurements

Plasma HIV-1 RNA levels were measured using quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1 test; Roche Molecular Systems, Basel, Switzerland) with a detection limit of 20 HIV-RNA copies/ml. Absolute numbers of CD4+ T cells were determined with an Epics XL-MCL flow cytometer (Beckman-Coulter).

Cell isolation and cell culture

PBMCs from all subjects were isolated by a density gradient. Cell preparation tubes (CPT) (BD, Vacutainer®) were used to obtain cells from untreated HIV-1 infected patients and Ficoll-Paque (Sigma-Aldrich) was utilized for healthy donors. Then, PBMCs were counted and CD4+CD45RA- (CD4+RA-) T lymphocytes were enriched with the Memory CD4+ T cell Isolation Kit (Miltenyi Biotec) following the manufacturer's protocol. Enriched CD4+RA- T cells from both healthy donors and infected patients were cultured at a concentration of 10^6 cells/ml in culture flasks of 25 cm^2 in R10 medium (RPMI 1640 containing 10% of Fetal Bovine Serum, 1% GlutaMax and 1% of penicillin/streptomycin). Afterwards, cells were activated with 5 µg/ml of

phytoheamagglutinin (PHA) (Sigma-Aldrich) at 37℃ and 5% CO_2 for 24 hours (Supplementary Figure 1). In three healthy donors, the sorting of CD300a+ and CD300a- subsets from CD4+RA- T lymphocytes was carried out before the activation with PHA, utilizing the FACSAria Flow Cytometer using FACS Diva software (BD Biosciences). In this set of experiments, cells were cultured in 24 well plates in the same conditions. As a negative control an uninfected condition was included for each experiment. As CD4+RA- T cells from patients were already infected in vivo with HIV-1, in order to achieve a suitable number of infected cells for our analysis, after the activation with PHA cells were washed with R10, rested overnight and expanded with 10 ng/ml of interleukin (IL)-2 (R&D Systems) for 13 days. Regarding CD4+RA- T cells from healthy donors, after the activation with PHA, cells were washed and infected overnight with HIV-1 (BaL) (NIH AIDS Reagent Program [https://www.aidsreagent.org/index.cfm]) at a MOI of 200:1. Lastly, cells were incubated with 10 ng/ml of IL-2 in the presence of the virus for 7 days (Supplementary Figure 1). In all cases, the R10 medium with IL-2 was renewed each 3 days.

Flow cytometry

Flow cytometry-based procedures were performed in order to study the cell populations of interest at different time points. Specifically, regarding CD4+RA- T cells from HIV-1 infected patients the percentage of CD300a+ cells within HIV-1 infected cells was analyzed at day 7 and day 13 of incubation (Supplementary Figure 1). The percentage of HIV-1 infected cells at day 3 and day 7 and the expression of CCR5, HLA-DR, CD38 and PD1 receptors at day -2, day 3 and day 7 were analyzed within CD300a+ and CD300a- CD4+RA- T cells from healthy donors (Supplementary Figure 1). For that, cells were first stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermofisher) to detect dead cells following the manufacturer's protocol, washed with PBS with 2% Bovine Serum Albumin (BSA) and then stained for 30 min at 4℃ in the dark with fluorochrome-conjugated monoclonal antibodies (mAbs) to determine the expression of surface receptors. The mouse anti-human mAbs utilized for the extracellular staining were PerCP-Cy5.5 anti-PD1 (clone EH12.1), APC anti-CD38 (clone HIT2), BV421 anti-CCR5 (clone 2D7) and BV510 anti-HLA-DR (clone G46-6) from BD Biosciences; PE-Cy7 anti-CD4 (clone RPA-T4) from Thermofisher and PE

anti-CD300a (Clone E59.126) from Beckman Coulter. After the staining of surface receptors, cells were washed with PBS with 2% BSA and were fixed and permeabilized using Cytofix/Cytoperm Plus Kit (BD Biosciences) following manufacturer's instructions. Then, they were incubated for 30 min at 4℃ in the dark with a FITC-conjugated anti-p24 mAb (clone KC57, Beckman Coulter) for the detection of HIV infected cells. Lastly, stained cells were washed again, resuspended in 250 µl of PBS and acquired with the FACS Canto II flow cytometer using FACS Diva software (BD Bioscience).

Statistical analysis and data representation

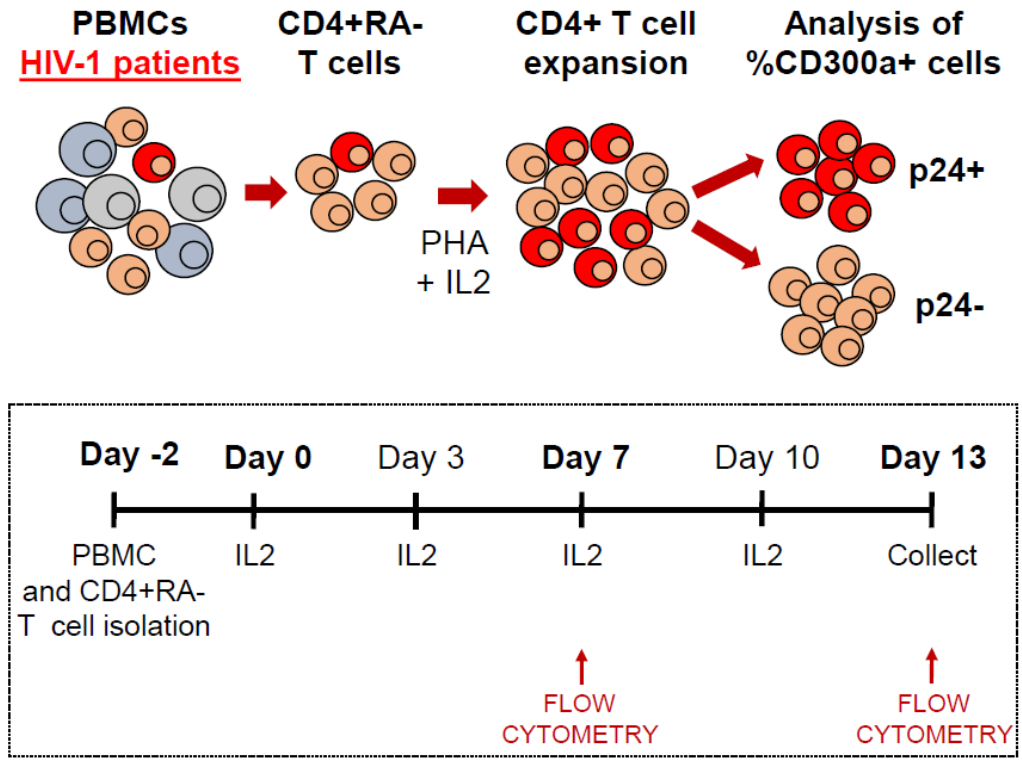
Data obtained from the flow cytometer were analyzed utilizing FlowJo software (version 10.0.7) (Treestar, Ashland, OR) and graphical representation and statistical analysis were carried out with GraphPad Prism software (version 6.01). Due to the sample size, the non-parametric Wilcoxon matched-pairs test was used for all the analysis. Correlations were assessed using the Spearman's rho correlation coefficient.

SUPPLEMENTARY MATERIAL

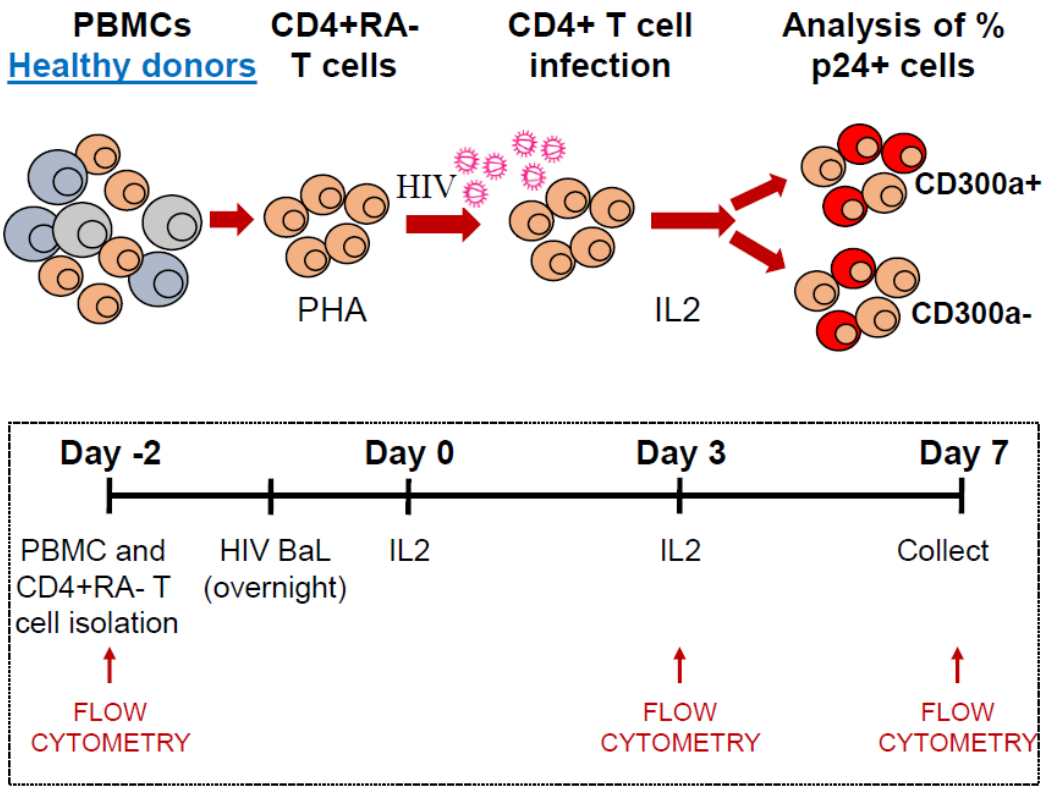
Supplementary Table 1. Clinical data HIV-1 infected patients

Patient	**Sex**	**Age (years)**	**CD4+ T cells/mm^3**	**Viral load (RNA copies/ml)**
VIH-1	Male	32	612	4,350
VIH-2	Male	57	524	2,390
VIH-3	Male	32	495	33,600
VIH-4	Female	53	588	9,310
VIH-5	Male	49	252	19,100
VIH-6	Male	37	393	26,800

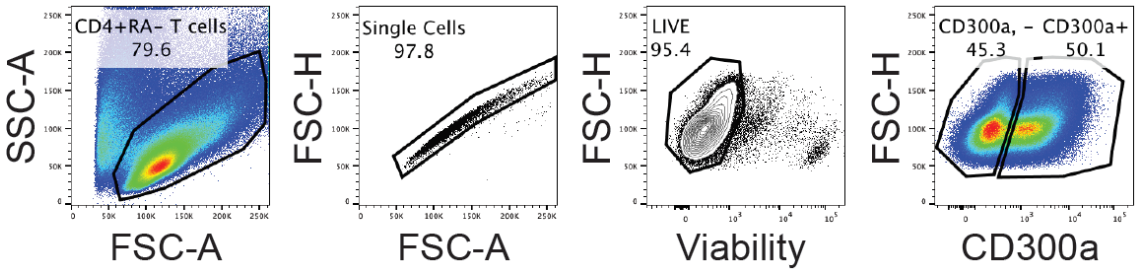
A


PBMCs
HIV-1 patients
CD4+RA- T cells
CD4+ T cell expansion
Analysis of %CD300a+ cells
PHA + IL2
p24+
p24-
Day -2
Day 0
Day 3
Day 7
Day 10
Day 13
PBMC and CD4+RA- T cell isolation
IL2
IL2
IL2
IL2
Collect
FLOW CYTOMETRY
FLOW CYTOMETRY

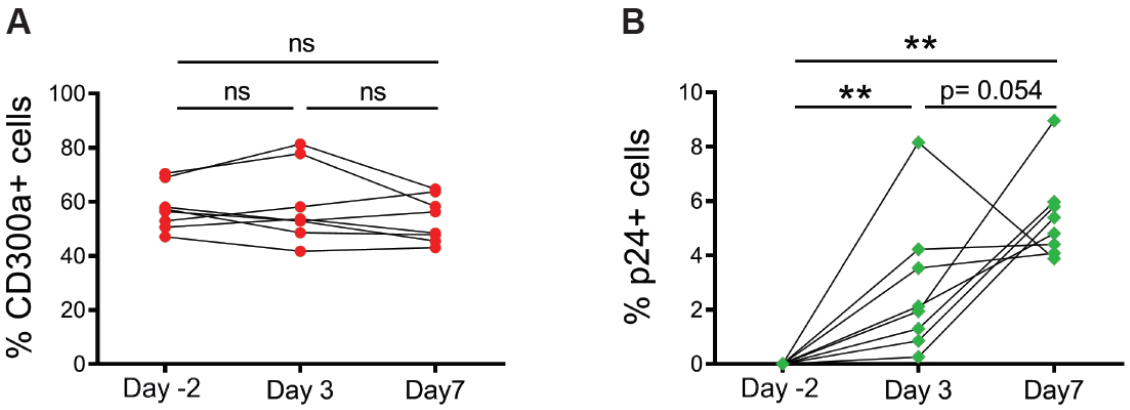
B


PBMCs
Healthy donors
CD4+RA- T cells
CD4+ T cell infection
Analysis of % p24+ cells
HIV
PHA
IL2
CD300a+
CD300a-
Day -2
Day 0
Day 3
Day 7
PBMC and CD4+RA- T cell isolation
HIV BaL (overnight)
IL2
IL2
Collect
FLOW CYTOMETRY
FLOW CYTOMETRY
FLOW CYTOMETRY

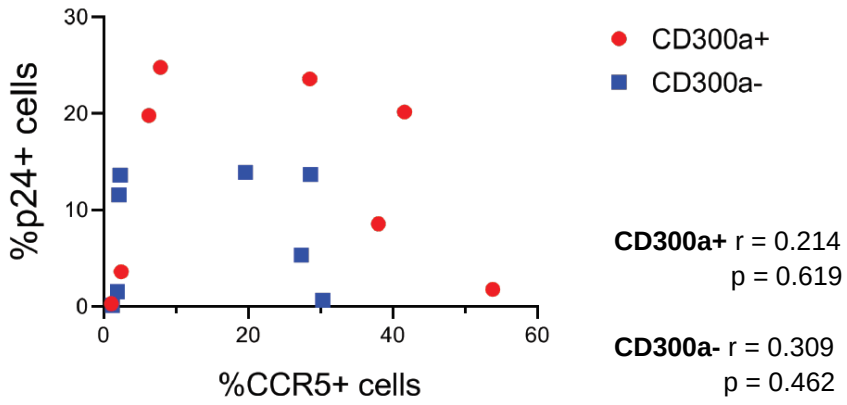
Supplementary Figure 1. Schematic representation of cell culture protocol for CD4+RA- T cell activation, HIV-1 infection and expansion. **(A)** PBMCs from naïve for cART HIV-1 infected patients were freshly isolated by density gradient and CD4+RA- T cells were enriched by negative selection (day -2). Then, cells were stimulated with PHA for 24 hours, rested in R10 overnight and then they were cultured during 13 days with IL-2 in order to expand HIV-1 infected cells. The medium with IL-2 was renewed each 3 days and samples were analyzed by flow cytometry at day 7 and day 13. **(B)** PBMCs from healthy donors were freshly isolated by density gradient and CD4+RA- T cells were enriched by negative selection (day -2). Afterwards, cells were stimulated with PHA for 24 hours, and then they were infected with HIV-1 (BaL) overnight and cultured during 7 days with IL-2. An uninfected condition was included for each experiment as a negative control. The medium with IL-2 was renewed each 3 days and samples were analyzed by flow cytometry at day -2, day 3 and day 7.



Supplementary Figure 2. Gating strategy used for the identification of CD300a- and CD300a+ subsets. First, previously enriched CD4+RA- T cells were electronically gated according to forward and side scatter parameters and single cells were selected based on their area and height. Then, dead cells were discarded gating the negative cells for the viability marker. Finally, CD300a- and CD300a+ subsets were selected.



Supplementary Figure 3. Percentage of CD300a+ and HIV-1 infected CD4+RA- T cells at different time points. Before-after graphs representing the percentage of CD300a+ **(A)** and p24+ **(B)** CD4+RA- T cells from healthy donors before activation and infection (day -2) and at day 3 and day7. Each dot represents a subject. **p<0.01.



Supplementary Figure 4. Correlation between the percentages of HIV-1 infected CD4+RA- T cells and CCR5 expressing cells. Correlation between the percentage of p24+ cells at day 3 after infection and the percentage of CCR5+ cells before infection (day -2) within CD300a- and CD300a+ CD4+RA- T cells from healthy donors.

4. DISCUSSION

Human CD300 receptors are known to be expressed on the surface of several immune cell types from both lymphoid and myeloid lineages[1,2]. Nevertheless, ours is the first comprehensive study examining the expression of CD300a, CD300c, CD300e and CD300f receptors on different subpopulations of T and B lymphocytes, NK cells, monocytes and DCs in peripheral blood from healthy subjects. Our results showed a differential CD300 expression pattern, not only among immune cell types and subsets, but also between cells from adult peripheral blood and from cord blood. In general, in resting conditions, lymphocytes only express the inhibitory CD300a receptor, with the exception of a small percentage of CD300c+ cells within the rare tissue-like memory B lymphocytes and $CD56^{bright}$ NK cells. Myeloid cells, however, express the four studied CD300 receptors.

The differential expression of CD300 molecules on adult versus neonatal immune cells could be explained due to the fact that they are exposed to different signals and different cytokine environment that might be altering their expression. It is well known that multiple factors have been described to modify the expression of CD300 molecules in healthy adults. For example, CD300a expression is known to be modulated by TGF-β and by the stimulation with anti-CD3 and anti-CD28 mAbs in CD4+ T cells, by TLR-mediated stimulation in B cells, monocytes and pDCs, and by the stimulation with several cytokines such as IL-4, IFNγ or IFNα in the same cell types[39,41,42,57–60]. CD300c expression is also regulated by cytokines and stimulation via TLR. Specifically, IL-2, IL-15 and IL-4 regulate the expression of the receptor on NK cells, while LPS and CpG modulate its expression on monocytes and pDCs respectively[20,60,74]. In addition, we have described that LPS is able to modulate the expression of CD300a, CD300c, CD300e and CD300f on monocytes from adult individuals, but significantly less on neonatal monocytes. For a better understanding of the involvement of CD300 molecules in adult and neonatal immune system, we also studied the ability of these receptors to modulate monocyte function. It is broadly known that monocytes have a key role in the first response against microbes by accumulating in sites of inflammation where they get activated and secrete cytokines[142,143]. It was previously described that CD300c and CD300e are able to activate adult monocytes, inducing the expression of activation markers and cytokine production[22,74,76]. In this doctoral thesis, we discovered that

CD300c- and CD300e-mediated monocyte activation is lower in neonates than in adults. The differences in the basal expression and in the LPS-induced modulation of CD300c and CD300e cell surface expression could explain the lower cytokine production by neonatal monocytes. Therefore, we conclude that healthy donors display a differential CD300 expression pattern depending on the cell lineage and cell population or subpopulation, and that CD300 expression, regulation and function differ between neonates and adults.

The expression of CD300 receptors is also known to be altered in multiple pathological conditions such as allergies, leukemia, psoriasis, etc.[1,2]. Furthermore, it has been previously described that viral infections, such as those by CMV, rabies virus or HIV, modulate CD300 receptors expression on different immune cell types[42,106–108]. HIV-1 infection is known to induce the alteration of the expression of multiple surface receptors that modulate the effector functions of different cell types[144,145]. For example, the impairment of NK cell activity during HIV-1 infection is partially due to alterations in the expression of a variety of receptors such as natural cytotoxicity receptors (NCRs), killer cell immunoglobulin-like receptors (KIRs) and C-type lectin-like receptors, which transmit activating and inhibitory signals and modulate NK cell effector functions[146–148]. There was only one publication describing the modulation of CD300 expression by HIV-1 infection, which reported a down-regulation of CD300a on B cells from HIV-1 infected patients, including untreated subjects and those under cART[42]. In this doctoral thesis, we have analyzed the cell surface expression of CD300 receptors on monocytes, NK cells and CD4+ T lymphocytes from HIV-1 infected patients. Because it is well known that the suppressive anti-viral treatment is able to revert the altered expression of many cell surface receptors[145,148] we have studied untreated and cART treated patients. Regarding monocytes, we did not observe differences between healthy donors and HIV-1 infected patients under cART. However, we have results showing that monocytes from untreated patients exhibit alterations in CD300 receptors, as for example a significant increase in the CD300a cell surface levels (unpublished data). On the other hand, we did find an increase of CD300a expression on CD4+ T lymphocytes from HIV-1 infected patients, both treated and untreated, and on some NK cell subsets only in untreated HIV-1 infected subjects, when compared with healthy donors. It is intriguing the observation that the altered expression of CD300 receptors expression during HIV-1 infection is reverted by cART only in cells that belong to the innate arm

of the immune system (monocytes and NK cells), while in B cells[42] and CD4+ T cells the anti-retroviral treatment was not able to restore the CD300a expression to normal levels. Undoubtedly, further research is required in order to investigate how is regulated the expression of CD300a by HIV-1 infection in different cell types.

Monocytes have an important role in chronic inflammation and immune system activation, characteristic of chronically HIV-1 infected patients on cART[149,150]. Thus, we studied the association of CD300 receptors expression on monocytes from cART treated patients with disease progression markers and monocyte cytokine production. As it is discussed above, we did not observe differences in CD300 expression between healthy and infected people, probably because cART reverts the alteration of the expression of these receptors, at least regarding CD300a which is overexpressed on monocytes from untreated HIV-1 infected patients (unpublished data). We observed a positive correlation of CD300e and CD300f expression on monocytes with CD4+ T cell counts and a higher TNF production in cART treated HIV-1 infected patients. Importantly, it is well known that in spite of successful cART the virus is not completely eradicated, leading to viral rebound after treatment interruption. Thus, several therapeutic vaccines as MVA-B have been tested with the aim to solve this problem[151,152]. Here, we observed that MVA-B vaccination induce a higher cytokine production in response to LPS by monocytes, although no association was found with CD300 receptors expression. In conclusion, CD300 receptors expression is associated to CD4+ T cell counts and higher TNF production by monocytes in cART treated HIV-1 infected patients, but not in patients vaccinated with MVA-B.

In addition to monocytes, the innate immune response against HIV infection is also provided by NK cells. Antibody dependent cellular cytotoxicity (ADCC) plays a critical role in NK cell mediated killing of HIV infected cells and has been related to long-term control of the infection, which leads to a better disease prognosis[146,153,154]. Nevertheless, HIV-specific effector antibody responses by NK cells are decreased in HIV-1 infected patients, even if they are under cART[145,146] and the alteration of NK cell surface receptors are known to contribute to this dysfunction[146,148]. Here, we have discovered that the CD300a+ subset is expanded on $CD56^{neg}$ NK cells from untreated HIV-1 infected patients and that CD300a is expressed at higher levels in immature $CD56^{dim}$ NK cells, which are characterized by the expression of CD94/NKG2A and the absence of CD57, a subpopulation that is significantly decreased in HIV-1 infected

patients[155,156]. Considering that CD56^{neg} NK cells are dysfunctional cells expanded during HIV-1 infection[146,148,153] we suggest that this could have a role in the impairment of NK cell function during HIV-1 infection. As a matter of fact, we have demonstrated that CD300a is able to inhibit CD16-mediated NK cell degranulation and cytokine production in HIV-1 infected patients at higher levels than in healthy subjects. A previous study has described the capacity of CD300a to inhibit NK cell killing of infected cells by other virus[110]. Hence, CD300a might diminish antibody-dependent NK cell killing of HIV-1 infected cells by inhibiting degranulation and cytokine and chemokine production by NK cells.

HIV infection, as its name indicates, induces an immune deficiency caused by the progressive loss and dysregulation of CD4+ T lymphocytes[132,157] and this cell type is known to highly contribute to HIV reservoir[158,159]. Hence, we studied the expression and potential implication of CD300a in this cell type during HIV-1 infection. We first discovered an overexpression of CD300a on CD4+ T lymphocytes from untreated and cART treated HIV-1 infected patients, when compared with healthy donors. This could partially be explained by the presence of a different cytokine environment in HIV-1 infected patients, which might be responsible for the altered CD300a expression levels. It is remarkable that we have also observed a positive association of the percentage of CD4+CD300a+ cells with CD4+ T cell counts and a negative correlation with plasma levels of β2-microglobulin, C-reactive protein and soluble CD14, suggesting that CD300a expression on CD4+ T cells could be indicative of good prognosis in this disease. In addition to the expression of the receptor, we were interested in investigating if CD300a could be involved in the infection of CD4+ T lymphocytes by HIV-1. CD4+CD45RA- (CD4+RA-) T lymphocytes display two well differentiated populations based on the presence or absence of CD300a in their cell surface in both healthy and HIV-1 infected patients. Thus, we studied the susceptibility of CD300a- and CD300a+ CD4+ T lymphocytes to HIV-1 infection. Our results showed that in vivo HIV-1 infected CD4+RA- T cells are predominantly CD300a+ and, more importantly, that CD300a expressing cells from healthy donors were more susceptible to HIV-1 infection in vitro than cells that are negative for the receptor. One explanation of the higher susceptibility to infection of the CD300a+ subset on memory CD4+ T lymphocytes could be that this population expressed more CCR5 and was more activated, two factors that are known to promote HIV-1 infection[157,160,161]. However, it could also be

postulated that CD300a might have a role in the attachment or entry of the virus. CD300a may promote the binding and infection of HIV particles through the interaction with its ligands PS and PE, which are present in the viral envelope, in a similar manner as CD300a binds DENV and enhances infection[122]. In conclusion, HIV-1 infection altered CD300a expression on CD4+ T lymphocytes and importantly, CD300a identifies a memory CD4+ T cell subset more susceptible to HIV-1 infection.

In summary, CD300 receptors have an important role in the regulation of the immune system in normal conditions and during HIV-1 infection. The results of this doctoral thesis will contribute to a better understanding of how is modulated the phenotype and function of several immune cells in healthy adults versus newborns and during HIV-1 infection. The observed results on CD4+ T lymphocytes suggest that CD300a might be a potential biomarker of disease progression and/or susceptibility to HIV-1 infection. Importantly, the fact that CD300a inhibits NK cell function in HIV-1 infected patients and that CD300a expressing CD4+ T cells are preferentially infected by HIV-1 suggest that CD300a could also be utilized in a future as a therapeutic target in HIV-1 infection.

5. CONCLUSSIONS

1. The expression pattern of CD300 receptors is different among immune cell populations and subpopulations as well as between adults and newborns.

2. Monocytes responsiveness to CD300c and CD300e-mediated activation is lower in newborns than in adults.

3. The expression of CD300e and CD300f molecules on monocytes from HIV-1 infected patients under cART is associated to CD4+ T cell counts and higher TNF production in response to LPS.

4. The vaccination with a modified vaccinia Ankara-based HIV-1 vaccine induces a higher cytokine production by monocytes in response to LPS in cART treated HIV-1 infected patients. However, no association was found with CD300 receptors expression.

5. CD300a expression is altered on the $CD56^{neg}$ NK cell subset only in untreated HIV-1 infected patients.

6. CD300a inhibits CD16-mediated NK cell degranulation and cytokine production in HIV-1 infected patients.

7. CD300a expression is altered on CD4+ T cells from HIV-1 infected patients and is associated with the disease progression markers CD4+ T cell counts and plasma levels of β2-microglobulin, C-reactive protein and soluble CD14.

8. CD300a identifies a CD4+CD45RA- T cell subset with a higher susceptibility to HIV-1 infection.

CONCLUSIONES

1. El patrón de expresión de los receptores CD300 es distinto entre poblaciones y subpoblaciones de células inmunitarias, así como entre adultos y recién nacidos.

2. La activación de los monocitos mediada por CD300c y CD300e es menor en recién nacidos que en adultos.

3. La expresión de las moléculas CD300e y CD300f en monocitos de pacientes infectados por VIH-1 bajo cART se asocia con el número de linfocitos T CD4+ y una mayor producción de TNF en respuesta al LPS.

4. La vacunación con la vacuna "modified vaccinia Ankara-based HIV-1 type B" (MVA-B) induce una mayor producción de citoquinas en respuesta al LPS en monocitos de pacientes infectados por VIH-1 bajo cART. Sin embargo, no se asocia con la expresión de los receptores CD300.

5. La expresión del receptor CD300a está alterada en las células NK $CD56^{neg}$ en pacientes infectados por VIH-1 no tratados.

6. CD300a inhibe la degranulación y la producción de citoquinas mediada a través del receptor CD16 en células NK de pacientes infectados por VIH-1.

7. La expresión del receptor CD300a está alterada en linfocitos T CD4+ de pacientes infectados por VIH-1 y se asocia con el número de linfocitos T CD4+ y niveles en plasma de β2-microglobulina, proteína C reactiva y CD14 soluble, marcadores de progresión de la infección por VIH-1.

8. CD300a identifica una población de linfocitos T CD4+CD45RA- con una mayor susceptibilidad a la infección por VIH-1.

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7. SUMMARY

Introducción

Los receptores CD300 son proteínas transmembrana de tipo I compuestos por un dominio extracelular tipo inmunoglobulina (Ig)V. Los receptores activadores, tienen una cola citoplasmática corta con residuos aminoacídicos cargados en la región transmembrana que facilitan la asociación con proteínas adaptadoras que contienen motivos activadores basados en tirosina (ITAMs). Los receptores inhibidores, sin embargo, tienen un tallo citoplasmático largo con motivos inhibidores basados en tirosina (ITIMs). En el humano, la familia de moléculas CD300 consta de 8 miembros: los receptores activadores CD300b, CD300c, CD300d, CD300e y CD300h, los receptores inhibidores CD300a y CD300f, y el CD300g, que además del dominio tipo IgV, contiene también un dominio extracelular tipo mucina y carece de motivos inhibidores o activadores en su cola citoplasmática. Aunque aún no se conocen los ligandos de todos los miembros de la familia, se sabe que las moléculas CD300 son capaces de unirse a distintas moléculas. Por ejemplo, los receptores CD300a y CD300c humanos reconocen fosfatidilserina (PS) y fosfatidiletanolamina (PE), dos fosfolípidos que se encuentran en la cara interna de la membrana plasmática en condiciones de reposo pero que se exponen a la cara externa cuando la célula está activada, entra en apoptosis o se infecta. También se conoce que algunos miembros de la familia de receptores se unen a moléculas como lipopolisacárido (LPS), ceramida, esfingomielina etc.

Los receptores CD300 se encuentran en la superficie de diferentes tipos celulares del sistema inmunitario, tanto del linaje linfoide como del mieloide. Existen numerosos factores que modulan la expresión de estos receptores. Por ejemplo, la expresión de los receptores CD300a y CD300c se modula tras la estimulación a través de receptores tipo toll (TLR), la presencia de distintas citoquinas u otros factores como la hipoxia, el factor de crecimiento transformante (TGF)-β, etc. Las moléculas CD300 tienen la capacidad de modular numerosas funciones de distintas células inmunes. En general, el receptor CD300a tiene un efecto inhibidor sobre la funcionalidad de distintos tipos celulares, mientras que los receptores CD300c y CD300e tienen un efecto activador. CD300f sin embargo, es capaz de transmitir señales tanto activadoras como inhibidoras. Entre las funciones que son capaces de regular, se encuentran la modulación de las

señales mediadas por el receptor de células T (TCR) y de células B (BCR), distintos TLRs, y receptores Fc como FcgRIIa o FcεRI. Se ha demostrado, que los receptores CD300 tienen un papel importante en procesos como proliferación celular, activación, producción de citoquinas y quimiocinas, etc.

Numerosos estudios han resaltado la importancia de la familia de las moléculas CD300 en distintas patologías. De hecho, se ha descrito que estos receptores tienen un papel relevante en la regulación del sistema inmunitario en el desarrollo de las enfermedades alérgicas (asma, rinitis alérgica, alergias alimentarias, etc.), enfermedades autoinmunes y autoinflamatorias (psoriasis, colitis, diabetes, esclerosis múltiple, etc), cáncer (leucemia linfoblástica aguda, leucemia mieloide aguda, linfomas, cáncer de pulmón) y también en distintas infecciones causadas por bacterias y virus. Con respecto a infecciones virales, trabajos previos han demostrado que la expresión de los receptores CD300 se ve afectada por diferentes virus como el virus de la rabia, el citomegalovirus (CMV) o el virus de la inmunodeficiencia humana (VIH). Lo que es más importante, se sabe que algunas de las moléculas CD300 tienen un papel importante en mecanismos de escape viral, como es el caso del virus de la pseudorrabia porcina (PRV) o en mecanismos de unión o de entrada en las células huésped, siendo el caso del virus del dengue (DENV) y el norovirus murino (MNV).

Aunque no hay demasiada información publicada, varios estudios sugieren que los receptores CD300 podrían tener un papel importante durante la infección por el VIH. Esta enfermedad que provoca una inmunodeficiencia causada por la pérdida progresiva de los linfocitos T CD4+, es considerada un problema global de salud pública. La introducción de la terapia antirretroviral combinada (cART) y el desarrollo de numerosas combinaciones de drogas antirretrovirales ha mejorado de forma muy considerable la calidad de vida de los pacientes. Sin embargo, la presencia de linfocitos T CD4+ latentemente infectados por el VIH hace que la completa erradicación del virus sea una tarea muy complicada. Por ello, en los últimos años se han realizado numerosos estudios con el fin de desarrollar nuevas estrategias para eliminar el reservorio viral y/o aumentar la respuesta celular y humoral VIH-específica, como por ejemplo, diversas vacunas terapéuticas frente al VIH. Por lo tanto, la necesidad de nuevas estrategias para conseguir la eliminación completa del VIH resalta la importancia de la búsqueda de nuevos biomarcadores y/o dianas terapéuticas.

Hipótesis y Objetivos

Los miembros de la familia de receptores CD300 se expresan en células tanto del linaje linfoide como mieloide. Postulamos que el patrón de expresión de estos receptores es diferente dependiendo de la edad. Además, predecimos que ciertos estímulos y condiciones patológicas, incluyendo la infección por VIH-1, modulan la expresión de las moléculas CD300, lo que conlleva una posible alteración de la respuesta inmune. Finalmente, teniendo en cuenta el papel del receptor CD300a en la patogénesis de distintas infecciones virales, postulamos que la expresión de esta molécula podría tener un efecto en la susceptibilidad de las células T CD4+ a la infección por el VIH-1.

Por lo tanto, el objetivo principal de este proyecto ha sido investigar la expresión, regulación de la expresión y función de los receptores CD300 en distintas células inmunitarias de individuos sanos, y estudiar cómo esto se altera durante la infección por el VIH-1. Para ello, establecimos los siguientes objetivos:

1- Estudiar la expresión, regulación y función de las moléculas CD300 en distintas células inmunitarias de adultos y neonatos sanos.
2- Investigar la expresión de los receptores CD300 y su asociación con la producción de citoquinas en monocitos de pacientes infectados por el VIH-1 bajo cART antes y después de la vacunación con la vacuna "modified vaccinia Ankara-based HIV-1 vaccine type B" (MVA-B).
3- Estudiar la expresión del receptor CD300a en distintos subtipos de células NK e investigar su capacidad para inhibir las funciones efectoras mediadas a través del receptor CD16 en células NK de pacientes infectados por VIH-1.
4- Analizar la expresión de CD300a en distintos subtipos de células T CD4+ de pacientes infectados por VIH-1 e investigar su asociación con marcadores de progresión de la enfermedad.
5- Estudiar la susceptibilidad a la infección por VIH-1 de células T CD4+ que expresan CD300a.

Material, Métodos y Resultados

La expresión y función de los receptores CD300 humanos en células mononucleares circulantes es distinta en neonatos y adultos

Se conoce que los recién nacidos son más susceptibles a las infecciones que los adultos. Esta susceptibilidad se puede apreciar en las diferencias cualitativas y cuantitativas de la respuesta inmune innata y adaptativa de los neonatos. De hecho, la distinta expresión de receptores de superficie puede provocar alteraciones en el umbral de activación de las células inmunes de los neonatos. En este trabajo, determinamos si la expresión y la función de los receptores CD300a, CD300c, CD300e y CD300f son diferentes en las células inmunes de los neonatos en comparación con las de los adultos. Para ello, se utilizó la citometría de flujo multiparamétrica para analizar la expresión de las moléculas CD300 en células mononucleares de sangre periférica de adulto y de cordón umbilical. Observamos que la expresión del receptor inhibidor CD300a está reducida de forma significativa en células del sistema inmunitario adaptativo de los recién nacidos y que las células presentadoras de antígeno, como las células dendríticas o los monocitos, de los neonatos muestran diferencias en el patrón de expresión de las moléculas CD300 en comparación con los adultos. También observamos que la regulación de la expresión de los receptores CD300 en respuesta al LPS en monocitos de recién nacidos es diferente a la observada en monocitos de adultos, y que la activación de los monocitos mediada por CD300c y CD300e es también cuantitativamente distinta. Los resultados de este trabajo, que muestran diferencias en la expresión y función de los receptores CD300, podrían ayudar a explicar las distinciones entre el sistema inmunitario de los recién nacidos y el de los adultos.

Fenotipo y producción de citoquinas de monocitos en pacientes infectados por VIH-1 vacunados con la vacuna MVA-B: relación con la expresión de las moléculas CD300

Los monocitos son una parte importante del reservorio viral y numerosos estudios han sugerido que son unos de los responsables principales de la inflamación crónica que se observa en los pacientes infectados por VIH bajo cART. La familia de receptores CD300 tiene un papel importante en distintas patologías, incluyendo infecciones virales. Los monocitos expresan CD300a, CD300c, CD300e y CD300f en su superficie y se conoce que el LPS, y otros estímulos, regulan la expresión de estos receptores. En este

trabajo, se investigó mediante técnicas de citometría de flujo la expresión de las moléculas CD300 y la producción de citoquinas en respuesta a LPS en monocitos de pacientes infectados por VIH-1 antes y después de la vacunación con "modified vaccinia Ankara-based HIV-1 vaccine type B" (MVA-B), una vacuna terapéutica frente al VIH-1. Nuestros resultados mostraron que la expresión de algunos miembros de la familia CD300 se correlacionaba con marcadores de progresión de la enfermedad como el número de linfocitos T CD4+ y marcadores de inflamación como la expresión de CD163. Además, observamos que los monocitos exhibían una mayor producción de citoquinas en respuesta al LPS después de la vacunación con MVA-B, prácticamente en los mismos niveles que los de los donantes sanos. Finalmente, también describimos una correlación entre la expresión de los receptores CD300e y CD300f y la producción de la citoquina factor de necrosis tumoral (TNF) en respuesta al LPS, únicamente en monocitos de pacientes infectados por VIH-1 antes de la vacunación. En resumen, nuestros resultados describen el impacto de la infección por VIH-1 y de la vacunación con MVA-B en el fenotipo y la producción de citoquinas de los monocitos.

CD300a inhibe las funciones efectoras mediadas a través del CD16 en células NK de pacientes infectados por VIH-1

Las células "natural killer" o NK juegan un papel importante en la inmunidad antiviral mediante diversos mecanismos. Específicamente, la citotoxicidad celular dependiente de anticuerpo (ADCC), mediada por el receptor CD16, tiene un papel crítico en las respuestas anti-VIH. Las funciones efectoras de las células NK se regulan mediante distintos receptores de superficie activadores e inhibidores, que pueden estar alterados durante la infección por el VIH. CD300a es un receptor inhibidor altamente expresado en células NK, previamente relacionado con ciertas infecciones virales y que es capaz de disminuir la capacidad citotóxica de las células NK frente a células infectadas por el PRV. Anteriormente se ha descrito que, en distintos tipos celulares del sistema inmunitario, la expresión de CD300a está alterada por la infección por el VIH-1. En este trabajo, mediante citometría de flujo, estudiamos la expresión y función del receptor CD300a en distintos subtipos de células NK provenientes de donantes sanos y pacientes infectados por VIH-1 sin tratamiento y bajo cART. En primer lugar, observamos una expansión de células NK $CD56^{neg}$ que expresaban CD300a únicamente en pacientes infectados por VIH-1 sin tratamiento. Además, vimos que CD300a se expresaba

predominantemente en células NK $CD56^{dim}$ inmaduras. Se ha descrito previamente que estos subtipos de células NK se ven especialmente afectados por la infección por el VIH. A continuación, se realizó un ensayo de lisis redirigida utilizando anticuerpos específicos frente a CD16 y a CD300a con el fin de estudiar la actividad NK mediada por anticuerpo e investigar la capacidad inhibidora de CD300a. Los resultados mostraron que CD300a inhibía la degranulación y producción de citoquinas de las células NK, tanto en donantes sanos como en sujetos infectados por VIH-1, y lo que es más importante, el efecto inhibidor fue mayor en los pacientes. Por lo tanto, proponemos que el receptor inhibidor CD300a podría utilizarse en un futuro como una nueva diana terapéutica con el fin de aumentar las funciones efectoras de las células NK en pacientes infectados por VIH-1.

Expresión alterada del receptor inhibidor CD300a en células T CD4+ de pacientes infectados por VIH-1: asociación con marcadores de progresión de la enfermedad

La capacidad del receptor CD300a para modular funciones efectoras de células inmunitarias y su papel en la patogénesis de numerosas enfermedades ha generado un gran interés en esta molécula. Se sabe que en donantes sanos, el CD300a se expresa de forma diferente en las distintas subpoblaciones de los linfocitos T CD4+. Sin embargo, se desconocía la expresión y la regulación del receptor CD300a en linfocitos T CD4+ de pacientes infectados por VIH-1. Por lo tanto, el objetivo de este trabajo fue investigar la expresión del receptor CD300a en células T CD4+ de pacientes infectados por VIH-1 sin tratamiento y bajo cART mediante citometría de flujo multiparamétrica. Demostramos que los niveles de expresión de CD300a son mayores en células T CD4+ pertenecientes a pacientes infectados por VIH-1 que en las de los donantes sanos, y que el cART no revierte los niveles de expresión del receptor. Observamos también un incremento en la expresión del CD300a en células T PD1+CD4+ y CD38+CD4+ de sujetos infectados. Además, observamos una población de células T CD4+ que co-expresa CD300a, PD1 y CD38, que está expandida en pacientes infectados por VIH-1 sin tratamiento. Finalmente, encontramos una correlación negativa entre la expresión de CD300a en células T CD4+ y marcadores de progresión, concretamente el número de linfocitos T CD4+ y niveles en plasma de β2-microglobulina, proteína C reactiva y CD14 soluble. En conclusión, nuestros resultados muestran que la infección por VIH-1 tiene un impacto en la regulación de la expresión del receptor CD300a en células T

CD4+ y que este receptor podría utilizarse en un futuro como biomarcador de progresión de la infección por VIH-1.

CD300a identifica una población de linfocitos T CD4+CD45RA- con una mayor susceptibilidad a la infección por el VIH-1

Trabajos previos han asociado distintos receptores, como pueden ser PD1, CTLA-4 o algunos receptores de quimiocinas, con una mayor susceptibilidad a la infección por VIH-1. Se conoce que el receptor CD300a se expresa en algunas células T CD4+ y que la expresión del receptor aumenta con la infección por VIH-1 en este tipo celular. Además, está descrito que CD300a promueve la infección por el virus del Dengue y otros virus. Así, en este estudio investigamos si las células T CD4+ que expresan CD300a podrían ser más susceptibles a la infección por VIH-1 que las que no lo expresan. En primer lugar, expandimos linfocitos T CD4+CD45RA- de pacientes infectados por VIH-1 sin tratamiento y observamos, mediante citometría de flujo, un mayor porcentaje de células CD300a+ en células que estaban infectadas por el VIH-1, en comparación con células no infectadas. Después, activamos e infectamos células T CD4+CD45RA- de donantes sanos in vitro con el VIH-1. Nuestros resultados mostraron un porcentaje significativamente mayor de células infectadas por VIH-1 en la población CD300a+ que en la población CD300a-. Estas células T CD4+CD45RA- que expresaban CD300a, mostraban una mayor expresión del co-receptor CCR5 y de los marcadores de activación CD38, HLA-DR y PD1 después de la activación e infección por VIH-1. Por lo tanto, estos hallazgos sugieren que CD300a podría utilizarse, en un futuro, como biomarcador de susceptibilidad a la infección por VIH-1 en linfocitos T CD4+ memoria.

Discusión

Los receptores CD300 humanos se expresan en células tanto del linaje linfoide como del linaje mieloide. Nosotros hemos estudiado el patrón de expresión de los receptores CD300a, CD300c, CD300e y CD300f en distintas subpoblaciones de linfocitos T y B, células NK, monocitos y células dendríticas en sangre periférica de donantes sanos. Nuestros resultados mostraron que la expresión de las moléculas CD300 era distinta en

las diferentes poblaciones y subpoblaciones, pero además, observamos también diferencias entre células del sistema inmunitario adulto y el neonatal. La diferencia en la expresión de los receptores CD300 en células inmunitarias de adultos y de neonatos podría explicarse por la exposición a diferentes señales y citoquinas que puedan estar modulando su expresión. Por ejemplo, hemos descubierto que el LPS es capaz de modular la expresión de estos receptores en monocitos de adulto, sin embargo en neonatos, el efecto es significativamente menor. Además, observamos que CD300c y CD300e son capaces de inducir la producción de citoquinas en monocitos de adultos y neonatos sanos, aunque en menor medida en estos últimos.

Se conoce que la expresión de los receptores CD300 está alterada en distintas enfermedades incluyendo infecciones virales como la del citomegalovirus, el virus de la rabia y la infección por VIH-1. La infección por VIH-1 induce la modulación de la expresión de numerosos receptores de superficie en distintos tipos celulares. Previamente nuestro grupo descubrió que la expresión de CD300a se encontraba disminuida en linfocitos B de pacientes infectados por VIH-1, tanto en aquellos que estaban bajo cART como en los que no. En este trabajo, hemos estudiado la expresión de los receptores CD300 en distintos tipos celulares. Nuestros resultados sugieren, que la infección por VIH-1 modula la expresión de CD300a y que esta alteración se revierte por el cART en células del sistema inmunitario innato como monocitos y NKs, pero no en células del sistema inmunitario adaptativo como células B o células T.

En este trabajo, en el contexto de la infección por VIH-1, estudiamos en primer lugar los monocitos, los cuales tienen un papel importante en la inflamación y activación crónica del sistema inmunitario, condición típica de pacientes infectados por VIH-1 bajo cART. Nuestros resultados mostraron que la expresión de los receptores CD300 en monocitos de pacientes infectados por VIH-1 bajo cART se asocia con el número de linfocitos T CD4+ y la producción de la citoquina TNF. Esta asociación no se observó en pacientes vacunados con la vacuna frente al VIH MVA-B.

En segundo lugar estudiamos las células NK, las cuales tienen un papel clave en la respuesta antiviral frente al VIH. De hecho, la citotoxicidad celular mediada por anticuerpo (ADCC) de las células NK se ha relacionado con un mejor control de la infección y, por lo tanto, un mejor pronóstico de los pacientes. Aquí, descubrimos que la población de células que expresan CD300a estaba aumentada en células NK $CD56^{neg}$ de pacientes infectados por VIH-1 sin tratamiento, una subpoblación de células NK que

se conoce que está expandida en sujetos infectados por VIH-1 y que presenta una funcionalidad alterada. Es de remarcar, que demostramos que el receptor CD300a tiene la capacidad de inhibir las funciones efectoras inducidas por CD16 de las células NK de pacientes infectados por VIH-1. Anteriormente se ha descrito que CD300a disminuye la capacidad de las células NK de matar células infectadas por PRV. Por lo tanto, proponemos que el receptor CD300a podría estar inhibiendo la capacidad de las células NK de matar células infectadas por VIH-1.

Es bien sabido, que la infección por VIH provoca una inmunodeficiencia causada por la pérdida progresiva de los linfocitos T CD4+ y que este tipo celular contribuye de forma considerable al reservorio viral. Por lo tanto, en este proyecto estudiamos la expresión de CD300a en distintos subtipos de células T CD4+ y observamos que la expresión del receptor estaba aumentada en pacientes infectados por VIH-1 en comparación con los sujetos sanos. Además, encontramos una correlación de la expresión de CD300a en células T CD4+ con el número de células T CD4+ y niveles en plasma de β2-microglobulina, proteína C reactiva y CD14 soluble, sugiriendo que este receptor podría utilizarse en un futuro como biomarcador de progresión de la enfermedad. Por último, se conoce que en linfocitos T CD4+ memoria (CD45RA-) se diferencian dos poblaciones en base a la presencia del receptor CD300a. En este trabajo, descubrimos que los linfocitos T CD4+ memoria que expresan CD300a son más susceptibles a la infección por VIH-1 que los que no lo expresan. La mayor susceptibilidad de esta población a la infección por VIH-1 podría explicarse, en parte, por una mayor expresión del co-receptor CCR5 y de marcadores de activación, los cuales son factores que promueven la infección. Sin embargo, otra explicación podría ser que CD300a promueva la infección por VIH-1 mediante la interacción con sus ligandos PS y PE presentes en la envoltura viral, como ocurre en el caso del virus del Dengue. En conclusión, CD300a podría utilizarse en un futuro también como biomarcador de susceptibilidad a la infección por VIH-1 en linfocitos T CD4+ memoria.

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HIGHLIGHTS

REVIEW

CD300 receptor family in viral infections

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The CD300 molecules constitute an evolutionarily significant family of receptors that are expressed on myeloid and lymphoid cells, but also on other cell types, such as tuft cells. Many of the CD300 receptors have been shown to recognize lipids, e.g. phosphatidylserine and phosphatidylethanolamine. Over the past couple of years, accumulating evidence has shown that this family of receptors is involved in the pathogenesis of many diseases. Specifically, CD300 molecules participate in the mechanisms that viruses employ to develop immune evasion strategies and to infect host cells. The participation of CD300 molecules in viral infection includes both lipid dependent and independent mechanisms, as for example in infections with dengue virus (DENV) and murine norovirus (MNV), respectively. CD300 receptors are also involved in viral escape mechanisms, for instance inhibiting NK cell-mediated cytotoxicity against infected cells. Moreover, it is becoming increasingly recognized that the expression of CD300 receptors is altered during viral diseases. Here, we review the involvement of human and murine CD300 molecules in viral binding and entry and in cellular responses to viruses, which highlights the potential of CD300 molecules in the search of new biomarkers for various stages of infection and therapeutic targets for the treatment of viral infections.

Keywords: CD300a · dengue virus · immune evasion · norovirus · virus entry

Introduction

An appropriate regulation of the immune system is dependent, among other factors, on a delicate balance between activating and inhibitory signals transmitted by a variety of receptors found on the surface of immune cells [1–6]. The CD300 receptors are type I transmembrane proteins expressed in both myeloid and lymphoid lineages and modulate immune responses by their stimulatory and inhibitory capabilities. These molecules have an IgV-like extracellular domain, a transmembrane domain and a cytoplasmic tail that could be short or long depending on their functionality. In humans, the CD300 family consists of eight members divided into two groups (Figure 1). Most of them (i.e., CD300b, CD300c, CD300d, CD300e, and CD300h) have a short cytoplasmic tail with a basic transmembrane residue or a leucine zipper-like sequence. These transmembrane domains facilitate the association with adaptor proteins containing immunoreceptor tyrosine-based activating motifs (ITAMs), such as DAP12 and FcRγ chain, or phosphatidylinositol 3-kinases (PI3K) binding motif (YxxM) such as DAP10, which enable their activating function. By contrast, other members of this family (i.e., CD300a and CD300f) exhibit an inhibitory capacity due to their long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs). In addition, CD300f is also able to deliver activating signals through motifs reported to bind the p85α regulatory subunit of PI3K and growth factor receptor-bound protein 2 (Grb2). The exception is the CD300g receptor, which has an additional extracellular mucin-like domain and lacks structural motifs indicative of

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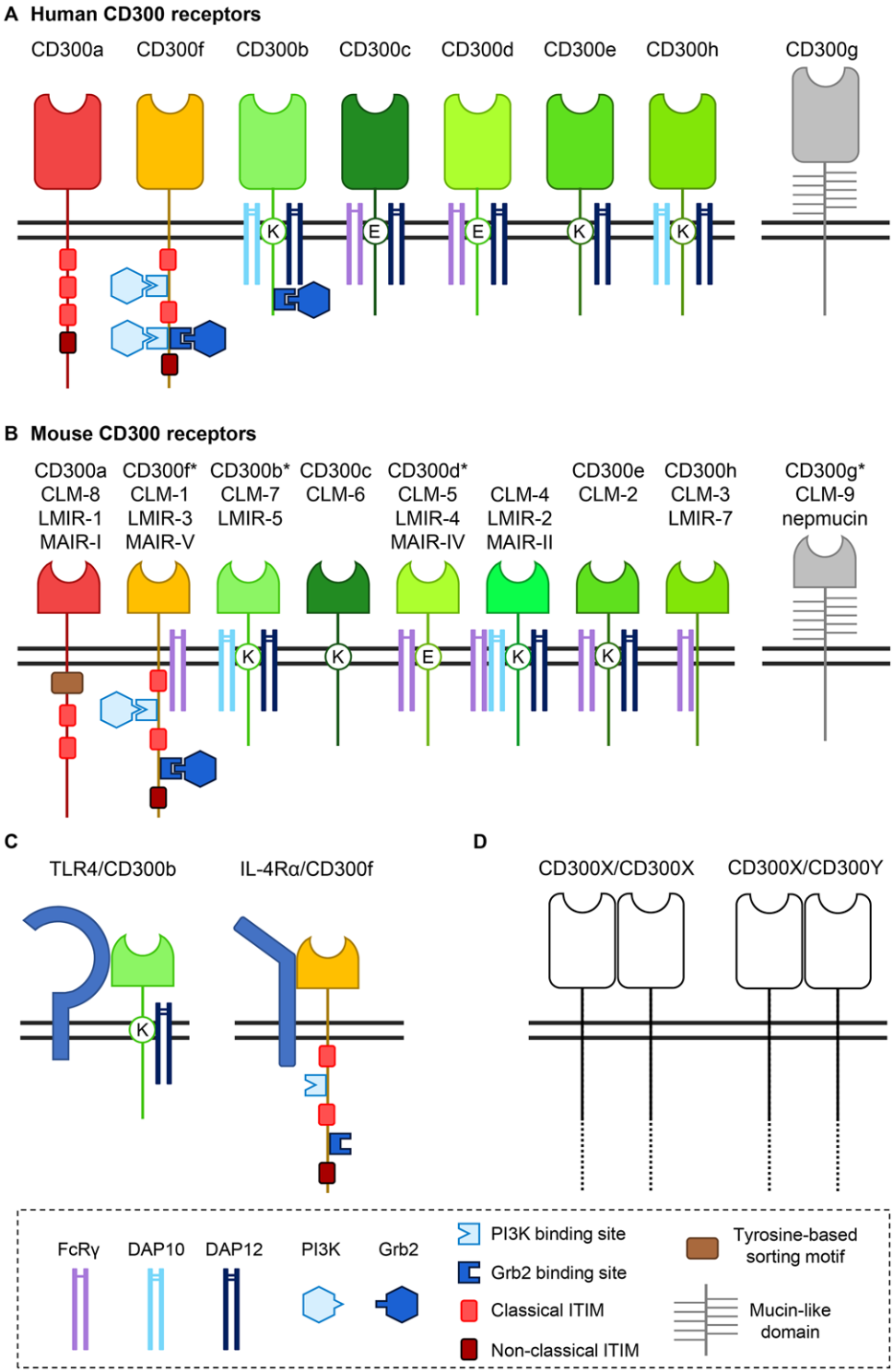

Figure 1. Human and mouse CD300 receptor family members and complexes. The CD300 receptors in humans (A) and mice (B) are differently represented in terms of the shape of the extracellular domain. The receptors with an activating function are shown in green, while the ones with an inhibitory capacity are indicated in red. CD300f, displaying dual function, is colored in orange and CD300g in gray. The motifs and binding sites from the intracellular tail, and the interaction with adaptor proteins (ITAM-bearing FcRγ and DAP12, DAP10 containing PI3K-binding motif and Grb2) and kinases (PI3K) are also indicated. Mouse CD300 molecules marked with an asterisk (*) are also named as CD300lX (e.g. CD300lf or CD300lb). This figure also shows the capacity of CD300 receptors to form complexes with other molecules, such as TLR4 and IL-4Rα (C), and their ability to interact with each other forming homo- and hetero-dimers (D).

Table 1. Ligands of human and mouse CD300 receptors

Species	CD300	Ligands	References
Human	CD300a	PS, PE	[24–26]
		Adenovirus E3/49K protein	[80, 81]
	CD300b	LPS	[21]
	CD300c	PS, PE	[24, 26]
		Adenovirus E3/49K protein	[80, 81]
	CD300e	SPH	[35]
	CD300f	Ceramide, SPH	[32]
Mouse	CD300a	PS, PE	[28, 29]
	CD300b	LPS, PS, PE, PC, TIM1, TIM4, PT, CT-B, C24:1	[21, 31, 34, 36–38]
	CD300c	PT	[37]
	CD300d	Norovirus	[82, 83]
	CD300e	SPH	[35]
	CD300f	PS, PC, Ceramide, SPC, HDL, LDL	[30, 33]
		Norovirus	[82, 83, 89, 90, 92, 93]

CT-B, cholera toxin B subunit; **C24:1,** 3-O-sulfo-β-D-galactosylceramide C24:1; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein; **LPS,** lipopolysaccharide; **PC,** phosphatidylcholine; **PE**, phosphatidylethanolamine; **PS,** phosphatidylserine; **PT,** pertussis toxin; **SPC,** sphingosylphosphocholine; **SPH,** sphingomyelin; **TIM,** T-cell immunoglobulin mucin.

stimulatory or inhibitory potential in the intracellular tail. Furthermore, CD300g is mostly expressed on the vascular endothelial cells of high endothelial venules of lymph nodes [7–13].

In mice, the CD300 family of receptors includes nine members (Figure 1), and they are also known as CMRF-35-like molecules (CLM), leukocyte mono-Ig-like receptors (LMIR), and myeloid associated Ig-like receptors (MAIR) or CD300-like (e.g. CD300lf instead of CD300f). While human and mouse CD300a and CD300f receptors are functional orthologs, the rest of the members of this family are not [7, 8, 12]. As in humans, mouse CD300f possesses ITIM motifs, and also Grb2 and PI3K-binding domains in its cytoplasmic tail [14–17]. Furthermore, mouse CD300f has also been demonstrated to bind the ITAM-containing adaptor FcRγ chain (Figure 1) [16]. An additional layer of complexity related to both the expression and signaling pathways of this receptor family is that their members can interact with each other to form homo and heterodimers, which is dependent on their immunoglobulin domains [18–20]. Furthermore, it has been described that CD300b also has the ability to form a complex with toll-like receptor 4 (TLR4), and therefore regulates LPS-induced responses on myeloid cells [21], while mouse CD300f associates with IL-4 receptor α and amplifies IL-4-induced immune cell responses [22] (Figure 1).

Although the specific ligands of every CD300 member are still unknown, it has been shown that they are able to bind lipids [7, 23]. For example, human CD300a and CD300c receptors recognize phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Table 1) [24–26]. In both resting and live cells, PS and PE are localized in the inner leaflet of the plasma membrane, while both lipids are translocated to the outer leaflet when cells undergo apoptosis, get activated, are transformed or infected [27]. Human CD300a binds PE with higher affinity than PS, while human CD300c binds both aminophospholipids with similar affinity [24–26]. On the other hand, it has been shown that mouse CD300a binds mainly to PS and modestly to PE [28, 29]. Other CD300 molecules that have been described to bind PS include mouse CD300b and CD300f [30, 31]. Other authors have demonstrated that CD300f binds ceramide and sphingomyelin [32, 33], human and mouse CD300b binds LPS [21] and mouse CD300b binds 3-O-sulfo-β-D-galactosylceramide C24:1 [34]. Recently, sphingomyelin has also been demonstrated to bind human and mouse CD300e [35]. Furthermore, other nonlipids ligands have been described to bind CD300b, including cholera toxin B subunit [36], pertussis toxin [37] and T-cell immunoglobulin mucin 1 (TIM-1) and TIM-4 receptors, although the binding appears to depend on PS [31, 38]. Pertussis toxin has also been found to interact with CD300c [37] (Table 1). Lastly, it has been demonstrated that CD300 molecules also work as receptors for certain viruses (see below and Table 1). Undoubtedly, more functional studies are required to determine the exact ligands of the CD300 molecules and complete structural studies are necessary to define their mode of binding.

Over the past few years, several publications have highlighted the important role the CD300 family of receptors has in complex biological processes such as phagocytosis, proliferation, and cytokine production [7, 12, 13, 17, 24, 25, 30, 31, 39, 40], and in a variety of diseases, including autoimmune disorders, allergic and inflammatory diseases, hematological malignancies, sepsis, etc. [7, 12, 17, 19, 21, 33, 41–58]. Here, we review several aspects involving the CD300 molecules and viral infections, including how viruses use these receptors to interact with and enter cells or to escape from the attack of the immune system. We also discuss how during the course of viral infections the expression of this set of molecules is altered, probably contributing to the pathogenesis of the disease.

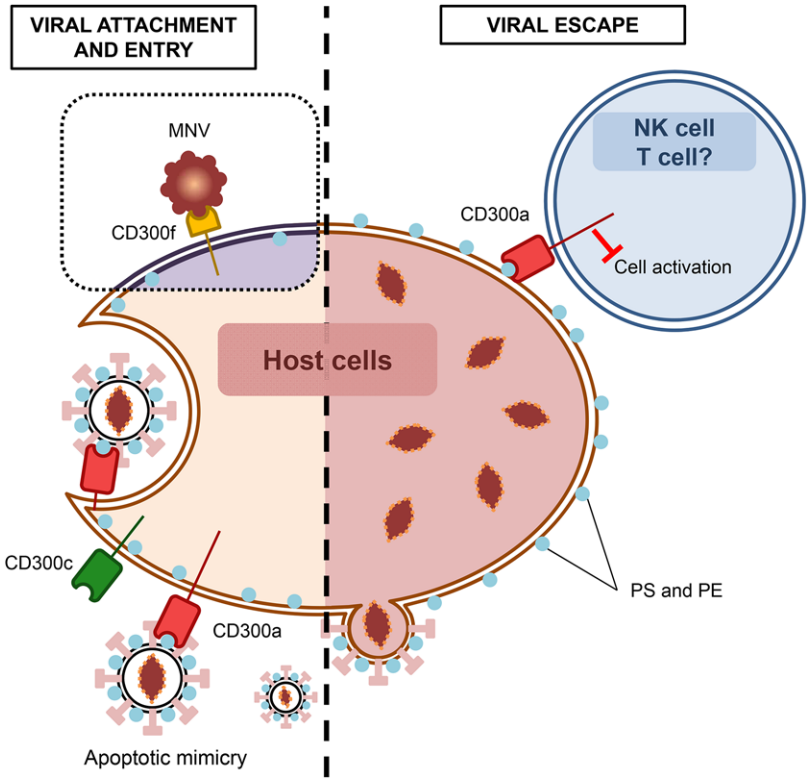

Figure 2. CD300 molecules in viral attachment, entry, and escape. When a host cell is infected by certain viruses, the asymmetrical distribution of phospholipids in the plasma membrane is lost, causing the externalization of phosphatidylserine (PS) and phosphatidylethanolamine (PE). The CD300a inhibitory receptor expressed on NK cells is able to recognize the PS and PE from the surface of pseudorabies virus-infected cells, decreasing NK cell-mediated cytotoxicity and, in consequence, avoiding viral clearance. A similar process may occur regarding $CD8^+$ T lymphocytes, since they also express CD300a and its expression have been associated to $CD8^+$ T-cell exhaustion during HIV-1 infection **(Viral Escape)**. On the other hand, through apoptotic mimicry, viruses enclose their capsids in a lipid bilayer obtained from the plasma membrane of host cells, leading to the incorporation of PS and PE to the viral envelope. Thus, surface receptors, such as CD300a, expressed on host cells bind PS and PE-containing viral particles and could also promote viral uptake. Other PS- and PE-binding CD300 molecules, such as CD300c, have the potential to bind viruses expressing these phospholipids in their envelopes. Lastly, mouse CD300f receptor binds murine norovirus particles and promotes the infection, in a PS- and PE-independent manner **(Viral Attachment and Entry)**.

CD300 receptors in viral binding and entry

Apoptotic mimicry

As obligate and opportunistic pathogens, viruses have developed many strategies in order to infect cells, replicate, and evade the immune system. They exploit cell surface receptors for host cell binding, entry, and downregulation of anti-viral responses [59–62]. It has been shown that one of the strategies used by viruses is the so-called viral apoptotic mimicry that aims to hijack cell's apoptotic recognition and clearance mechanisms for their own means [63]. During apoptosis, the asymmetrical distribution of phospholipids in the plasma membrane is lost, which results in the exposure of PS and PE in the outer leaflet. It is well established that externalized PS is essential for apoptotic cell clearance by professional (e.g. macrophages) and nonprofessional phagocytes [27, 64, 65]. Enveloped viruses deploy apoptotic mimicry by enclosing themselves in a lipid bilayer obtained from the plasma membrane of host cells and concentrating PS and PE within their membranes, thereby mimicking apoptotic cells and debris. Nonenveloped viruses, in turn, cover themselves in cell-derived PS and PE-containing vesicles [63, 66, 67] (Figure 2).

Just as PS expressed on apoptotic cells and debris, PS-bearing viruses interact with cell surface receptors through two mechanisms. One involves soluble molecules, such as growth-arrest-specific 6 (Gas6) that bridges the viral envelop PS to tyrosine kinase receptor Axl, Tyro3, and Mer [68–70], or milk fat globule-EGF factor 8 protein (MFG-E8) that bridges integrins to PS [71]. The other mechanism is mediated by surface receptors that directly recognize PS, such as TIM-1, -3, and -4 or brain angiogenesis inhibitor 1 (BAI1) [68, 69, 71, 72], which are expressed in professional phagocytes and other cell types [71]. TIM-1, for instance, promotes the infection of retroviruses and pseudotyped virus-like particles from filovirus, New World arenavirus, flavivirus, and alphavirus families [72, 73]. It has been demonstrated that TIM-1 also enhances the infection of replication competent viruses from the same families [73]. Importantly, PS receptors can also recognize PE in viral membranes and promote phagocytosis and viral entry [74]. Specifically, it has been shown that TIM-1 binds PE and promotes the infection by Ebola, Dengue (DENV), and West Nile viruses [74]. Recently, it has been demonstrated that ubiquitination of TIM-1 is required for DENV infection [75].

CD300a as a mediator of viral attachment and entry

The CD300a inhibitory receptor has been proposed as another PS-binding protein involved in viral entry. Human CD300a receptor ectopically expressed on 293T cells was able to bind pseudotyped lentiviral vectors with envelopes from Sindbis virus, Ross River virus, baculovirus (gp64), and vesicular stomatitis-G virus, but did not enhance their transduction, unlike, for example, TIM-1 and TIM-4 [69, 71]. Therefore, in this specific situation, it is unclear if CD300a serves as a binding receptor or, on the contrary, may help to avert viral entry. It is well known that CD300a binds apoptotic cells through PS and PE and transmits inhibitory signals that preclude the engulfment of dead cells by monocyte-derived macrophages (MDMs) [25]. By contrast, Carnec et al.

have described that human and mouse CD300a not only bind the four DENV serotypes, but also enhance the infection [28]. They discovered that this process depended on clathrin-mediated entry and was independent of the cytoplasmic tail of CD300a. Furthermore, blocking the receptor with antibodies (Abs) in MDMs naturally expressing CD300a lead to a decrease in the number of infected cells. However, other CD300a positive cells, such as monocytes and mast cells, were insensitive to the infection, suggesting a cell type-specific role of CD300a during DENV infection. Importantly, human CD300a interacted mainly with PE associated with the viral particles and to a lesser extent with PS [28], which somehow was expected since this receptor recognizes PE with higher affinity than PS [24, 25]. On the other hand, mouse CD300a interaction with viral particles was essentially dependent on virion-associated PS [28]. Moreover, this work demonstrated that, apart from DENV, CD300a was also able to increase the infectivity of other mosquito-borne viruses like Yellow fever, West Nile, and Chikungunya viruses [28].

Interestingly, several PS receptors, including CD300a, TIM-1, and Axl, have been identified as entry factors for DENV infection [28, 68, 75]. Nevertheless, it is quite possible that they accomplish complementary and nonredundant functions during DENV infection [75]. In this manner, CD300a may act as a DENV-binding molecule without promoting viral uptake [28]. Therefore, the CD300a receptor acts as an attachment factor for the studied PS and PE associated viral particles, although it may promote the viral internalization only in specific cases. These disparities may be explained, among others, by a difference in PE levels on the viral envelope between different viruses and by the cell type where this receptor is expressed, but this is still unknown. Also, the demonstration that the cytoplasmic tail of CD300a is not necessary for DENV infection raises the possibility that the ITIMs have no role in this process. However, it is very important to point out that the majority of these experiments were performed with CD300a transfected cells that otherwise are negative for this receptor [28]. Even not all CD300a positive cells were sensitive to the infection [28].

Although both src homology 2 domain containing protein tyrosine phosphatase (SHP)-1 and SHP-2 are recruited to the phosphorylated ITIMs of CD300a, it seems that this receptor inhibits immune receptor signaling only via SHP-1, at least in lymphocytes [76]. SHP-1 has been associated with negative signaling while SHP-2 has been associated with positive signals [77]. Furthermore, the expression of SHP-1 is mostly restricted to hematopoietic cells, while SHP-2 exhibits a broader pattern of expression [77–79]. Therefore, it could be speculated that, in each cell type, the relative availability of the different phosphatases that bind to phosphorylated ITIMs in the intracellular tail may determine, at least in part, the quality of the signaling pathway generated from CD300a after interacting with its ligands, including PS and PE associated viral particles. Further research is required to clarify the implication of CD300a in the attachment and internalization of different viruses in host cells and also the potential of inducing signals regulating the postentry step of virus infection. Nevertheless, it could be affirmed that, at least, this receptor has an important role in the binding of viruses that utilize apoptotic mimicry as a mechanism to enter host cells. It should not be forgotten that other members of the CD300 family are able to bind PS, for example, mouse CD300f [17, 30], mouse CD300b [31] and human CD300c, the latter also binding PE [24, 26]. Therefore, these proteins have a potential to serve, at least, as binding receptors for the PS and PE associated viral particles.

Lipid-independent enhancement of viral entry by CD300 receptors

Apart from binding to PS and PE associated viral particles, it has also been demonstrated that CD300 molecules are able to bind and facilitate the entry of viruses in a PS and PE independent manner. In a large-scale protein interaction screen, several human adenovirus E3 protein host cell interactions were identified and validated [80, 81]. Among them, it was shown that human adenovirus-D47 E3/49K protein binds to the CD300a and CD300c paired receptors [80, 81], which have extensive homology in their extracellular domains, but have distinct intracellular domains that determine their inhibitory and activating functions, respectively [7].

By using a screening method based in CRISPR/Cas9 technology, two groups identified mouse CD300f (also known as CD300lf) as crucial for murine norovirus (MNV) infection (Figure 2) [82, 83]. Further in vitro experiments showed that depletion of mouse CD300f or its blockade with polyclonal Abs or soluble CD300f (sCD300f) caused resistance to MNV infection, whereas the blockade of PS with annexin V had no effect [82, 83]. Both groups had apparently discordant results when they addressed the role of glycans, although previous works have reported that glycans are involved in MNV binding [84–88]. Haga et al. showed that the polyclonal Ab targeting mouse CD300f inhibited MNV-induced cytopathic effects and MNV progeny, but the binding was not reduced, suggesting the existence of other molecules, for example, sialic acids, involved in MNV binding. In fact, the use of neuraminidase to remove the terminal sialic acid caused a significant reduction in the yield of infectious particles from the MNV-S7 strain [82]. On the contrary, Orchard et al. showed that treating cells with the mannosidase I inhibitor kifunensine did not affect the binding of MNV to cells besides the significant reduction of cell surface carbohydrates [83]. However, they also demonstrated that a serum cofactor that is present in delipidated serum and is resistant to proteinase K and heat denaturation, is required, together with CD300f, for MNV binding [83]. This serum cofactor has been recently identified as bile acids [89].Very importantly, the in vivo significance of CD300f in MNV infection was tested in two mouse models. First, survival was superior when mice were challenged with MNV preincubated with sCD300f in comparison with control protein and second, CD300f$^{-/-}$ mice were resistant to MNV infection compared to littermate controls [83].

Norovirus infections exhibit strict host tropism. In this context, it was also demonstrated that mouse CD300f receptor determines MNV host tropism, as human cells and cells from other species were only infected by MNV when they ectopically expressed the

murine, but not the human CD300f [82, 83]. A rare chemosensory epithelial cell type known as tuft or brush cells has been shown to express CD300f and to represent the physiological target cell for MNV in the mouse intestine [90]. Since tuft cells have been described as orchestrators of anti-parasite type 2 responses in the gut [91], Wilen et al. addressed the relationship between type 2 immunity and MNV infection [90]. The authors found that type 2 cytokines IL-4 and IL-25, which induce tuft cell proliferation, promoted MNV infection in vivo. Furthermore, this group also found that IL-4 and IL-25 administration can replace the effect of commensal microbiota in promoting virus infection [90]. Structural and mutational studies have identified what parts of the IgV-like extracellular domain of mouse CD300f were relevant for the viral binding and entry and those that conferred species specificity [82, 83]. Interestingly, the serine palmitoyltransferase complex, that is required for sphingolipid biosynthesis, has been shown to have a very important role in the ability of MNV to bind and enter cells. Serine palmitoyltransferase activity is required for CD300f to adopt an optimal conformation that is tolerant for viral binding. Remarkably, addition of ceramide to serine palmitoyltransferase deficient cells restores the conformational changes of CD300f and the susceptibility to MNV infection [92]. Also, in a similar manner to the requirements of CD300a for DENV infection [28], the cytoplasmic tail of mouse CD300f was not required to infect HeLa and HEK293T cells [82, 83].

Kilic et al. solved the X-ray crystal structure of the MNV protruding domain and sCD300f receptor complex that consisted of one protruding domain dimer and a single sCD300f molecule [93]. The authors found that sCD300f binds to the topside of the MNV protruding domain from the CR10 strain capsid and involved hydrophilic and hydrophobic interactions. Very recently, Nelson et al. have also reported that sCD300f binds to the protruding domain of MNV strain CW3 with a 2:2 stoichiometry [89]. Of note, they have demonstrated that bile acids act as cofactors enhancing viral binding and infection. Addition of glycochenodeoxycholic acid enhanced MNV binding and infection of BV2 cells. There are two bile acid-binding sites at the protruding domain dimer interface that are distant from the sCD300f-binding sites. The authors also found that the affinity of monomeric sCD300f for the protruding domain is low and is divalent cation-dependent. Finally, by docking CD300f-bile acid-protruding domain cocomplex structures onto a cryo-EM-derived model of MNV, Nelson et al. have described that a virion is able to make multiple interactions with CD300f, suggesting that infection may be driven by the avidity of clustered CD300f molecules on the cell surface [89].

There are nine mouse CD300 receptors with variable degree of homology in their N-terminal domains between them. It has been tested if other members of this family were capable of working as MNV receptors and it was demonstrated that mouse CD300d (or CD300ld) also functions as an MNV receptor as shown by its ability to promote viral replication when ectopically expressed on CD300d negative cells [82, 83, 93]. Nevertheless, it seems that it does not have an essential role in vivo, as shown by the ability of CD300f$^{-/-}$ mice to resist MNV infection [83]. Interestingly, surface plasmon resonance analyses revealed that only mouse sCD300f exhibited a strong signal for binding to the protruding domain, whereas little binding was observed for mouse CD300d and CD300h and human CD300f [89]. Finally, although CD300h (or CD300lh) was identified in a CRISPR/Cas9 screening as a possible MNV receptor [83], its expression on target cells did not have any effect on MNV infection, very possibly indicating that CD300h is not a bona fide MNV receptor.

CD300 molecules and cellular responses to viral infections

One of the several strategies used by viruses to evade the immune response is to promote the binding of inhibitory receptors expressed on different immune cell types with their ligands on infected cells or directly with viral particles, inducing the downregulation of antiviral responses [59]. Importantly, to efficiently replicate, many viruses, such as HCV and HIV, increase the levels of intracellular calcium. This causes externalization of PS and PE [12, 59, 94–96], enabling the viruses to bind receptors that interact with these two aminophospholipids. Some of these receptors have inhibitory properties (i.e. CD300a and TIM-3) and are expressed on cytolytic immune cells such as NK cells and CD8$^+$ T cells. Therefore, after engaging PS and PE on infected cells, they may block activation signals on immune cells, thereby hindering viral clearance [97–100].

During chronic HIV-1 infection, persistent antigen exposure gives rise to T-cell exhaustion, which is characterized by a reversible loss of effector functions and diminished proliferative potential. Exhausted T cells are characterized by a higher expression of inhibitory receptors [101]. Among them, the PD1 inhibitory receptor has been identified as a major regulator of T-cell exhaustion during chronic HIV infection, promoting viral persistence [99, 101]. In addition to reducing T-cell receptor signaling, PD1 upregulates the expression of basic leucine transcription factor ATF-like (BATF), which also inhibits T-cell function [99]. Interestingly, it has been described that there is a positive correlation between the mRNA levels of CD300a inhibitory receptor and BATF levels on HIV-specific CD8$^+$ T cells, suggesting that CD300a, along with other inhibitory receptors, may also have a role in T-cell exhaustion during chronic HIV infection [99]. Along these lines, it has been shown that the alpha-herpesvirus porcine pseudorabies virus (PRV) protects infected cells from NK cell-mediated cytotoxicity through a mechanism involving CD300a [98]. NK cell function is crucial against herpesviruses in particular, since these cells limit viral replication and disease progression [102, 103]. PRV induces the exposure of PS and PE to the outer leaflet of the plasma membrane of infected cells. In consequence, the binding of the CD300a inhibitory receptor expressed on NK cells to its ligands PS and PE on the infected cells is increased, leading to an inhibition of NK cell-mediated cytotoxicity [98]. As a matter of fact, NK cells express very high levels of CD300a [13, 24]. The fact that other viruses are also capable of inducing the externalization of PS and PE when infecting host cells [12, 59, 94], suggest that they may also

downregulate immune cell responses by binding to CD300a and blocking activation signals on $CD8^+$ T cells and NK cells (Figure 2).

Altered CD300 receptors expression in viral infection

Altered expression of CD300 molecules have been described during viral infections, potentially suggesting an additional role for this family of receptors in the pathogenesis of viral diseases. This altered expression could be a direct effect of the virus, as, for example, the profound downregulation of CD300b gene expression on cells infected with rabies virus [104], or an indirect effect. Regarding the latter, the CD300a inhibitory receptor was able to diminish BCR-mediated signaling and its expression was significantly downregulated on B cells from HIV-infected patients, suggesting that this receptor could be involved in the B-cell hyper-activation and dysfunction characteristic of HIV infection [39]. The altered expression of CD300a is not corrected by effective combined antiretroviral therapy (cART), suggesting that residual effects of HIV infection are sufficient to maintain low levels of CD300a on B cells [39]. Within $CD4^+$ T cells, CD300a is differentially expressed among different cell subsets [13, 105, 106], and its cell surface levels are also altered during HIV infection, independently of the introduction of cART [107]. Interestingly, a $CD4^+$ T-cell subset coexpressing CD300a, PD1, and CD38 was significantly expanded in HIV infected patients that were not receiving cART, while it was very rare in healthy donors and patients on cART. Furthermore, a correlation between CD300a expression on $CD4^+$ T cells and markers associated with disease progression was found [107]. Other members of the CD300 family have been studied during HIV infection. For example, it has been shown that the expression of the inhibitory receptor CD300f and the activating receptor CD300e on monocytes from HIV-infected patients under cART was correlated with markers of disease progression and immune inflammation [108].

The CD300a inhibitory receptor has been recently related to latent CMV infection. It was observed that on T cells, the frequency of CD300a+ cells increased in young CMV seropositive individuals [109]. Age may also have a role in the increased percentage of CD300a+ cells, although the old subjects that participated in the study were all CMV seropositive [109]. Just as in T cells, latent CMV infection and age induce significant changes in the expression of CD300a on NK cells [110].

Future considerations

The high evolutionary adaptation of viruses over decades has made it considerably difficult to find efficacious treatments against viral infections, which is the reason why searching for new targets has been so important lately. Here, we have reviewed the potential role of CD300 molecules in various mechanisms of viral infection of host cells and viral evasion of host immune responses (Figure 2). We are now at the first step of understanding the involvement of CD300 receptor family during viral infections. However, the recent findings have opened a door to further research that should determine the role of CD300 receptors in infections caused by different families of viruses and to clarify the specific mechanisms in which viruses utilize the capacity of CD300 molecules for their benefit. It is important to keep in mind that, in addition to lymphocytes, CD300a is also expressed on other cell types such as monocytes, dendritic cells, mast cells, basophils, etc. Therefore, it should also be explored how viruses that utilize CD300a as an attachment receptor may affect these cells as well. Furthermore, considering that other CD300 molecules bind PS and PE, they may also have an important role in the apoptotic mimicry mechanism used by many viruses. Clearly, a global strategy to study the CD300 receptors function during viral infections could help in the search of new targets, in order to develop efficient therapies against viruses.

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Abbreviations: **cART**: combined anti-retroviral therapy · **CLM**: CMRF-35-like molecules · **ITAM**: immunoreceptor tyrosine-based activating motif · **ITIM**: immunoreceptor tyrosine-based inhibitory motif · **MDM**: monocyte-derived macrophage · **MNV**: murine norovirus · **PE**: phosphatidylethanolamine · **PS**: phosphatidylserine

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The Biology and Disease Relevance of CD300a, an Inhibitory Receptor for Phosphatidylserine and Phosphatidylethanolamine

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Brief Reviews

The Journal of Immunology

The Biology and Disease Relevance of CD300a, an Inhibitory Receptor for Phosphatidylserine and Phosphatidylethanolamine

Olatz Zenarruzabeitia,* Joana Vitallé,* Cristina Eguizabal,† Venkateswara R. Simhadri,‡ and Francisco Borrego*,§

The CD300a inhibitory receptor belongs to the CD300 family of cell surface molecules that regulate a diverse array of immune cell processes. The inhibitory signal of CD300a depends on the phosphorylation of tyrosine residues embedded in ITIMs of the cytoplasmic tail. CD300a is broadly expressed on myeloid and lymphoid cells, and its expression is differentially regulated depending on the cell type. The finding that CD300a recognizes phosphatidylserine and phosphatidylethanolamine, two aminophospholipids exposed on the outer leaflet of dead and activated cells, has shed new light on its role in the modulation of immune functions and in its participation in the host response to several diseases states, such as infectious diseases, cancer, allergy, and chronic inflammatory diseases. This review summarizes the literature on CD300a expression, regulation, signaling pathways, and ligand interaction, as well as its role in fine tuning immune cell functions and its clinical relevance. ***The Journal of Immunology*****, 2015, 194: 5053–5060.**

To preserve the identity and integrity of the host and, at the same time, be effective against offenses, a complex and delicate balance of stimulating and inhibitory signals is required to efficiently regulate the activation status of the immune system. Among several other mechanisms that accomplish this mission, this balance is achieved by signals that emanate from cell surface receptors with activating and inhibitory capabilities (1–3). Some of them are clustered in the genome as families that consist of paired receptors with activating and inhibitory functions. In general, the inhibitory receptors are characterized by the presence of one or more ITIMs in their cytoplasmic tail (1, 3), whereas the activating receptors have a transmembrane charged residue that allows the association with adaptor proteins carrying ITAMs or a PI3K-binding motif (YxxM) (1, 2).

The human CD300 multigene family has seven members, which are named alphabetically according to their location on chromosome 17. The mouse counterparts, also known as CLM, LMIR, and MAIR, are encoded by nine genes located on mouse chromosome 11, the syntenic region of human chromosome 17 (4, 5). Nonetheless, with the exception of the two ITIM-bearing receptors (CD300a and CD300f), the rest of the CD300 family members are not perfect functional orthologs (4, 5). All of the receptors of the CD300 family have an extracellular IgV-like domain. The activating members have a short intracellular tail, and the transmembrane domain associates with ITAM-containing adaptor proteins, such as DAP12 and FcεRIγ, whereas the inhibitory receptors have a long intracellular tail that carries ITIMs. In addition to the IgV-like domain, CD300g has an extracellular mucin-like domain, but it does not have a known intracellular signaling motif (4, 5). It was shown that CD300 family members have the potential to form homodimers and heterodimers, which implies that, in addition to the signal that emanates from each single receptor, the formation of heterocomplexes adds a new layer of intricacy to the signaling pathways of the CD300 family of receptors (6).

In this review, we focus on the biology and disease relevance of the inhibitory receptor CD300a, whose gene ranks at the top of the human genes that show evidence of positive selection, suggesting a need to maintain some critical function (7).

Regulation of expression

Transcripts encoding human CD300a were detected in cells from both the myeloid and lymphoid lineages (4, 5). Nonetheless, the cell surface expression of CD300a has been difficult to determine because the majority of available mAbs display cross-reactivity and recognize both CD300a and

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Abbreviations used in this article: ALL, acute lymphoblastic leukemia; CLP, cecal ligation and puncture; KIR, killer cell Ig-like receptor; mDC, myeloid dendritic cell; MFG-E8, milk-fat globule EGF-factor VIII; pDC, plasmacytoid dendritic cell; PE, phosphatidylethanolamine; PS, phosphatidylserine; SCF, stem cell factor; SHIP, Src homology region 2 inositol 5′ phosphatase; SHP, Src homology region 2 domain-containing phosphatase; TIM, T cell/transmembrane, Ig, and mucin.

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CD300c on the cell surface (8–10). These are paired receptors with inhibitory and activating properties whose extracellular domains exhibit >80% similarity at the amino acid level. Recently, specific anti-CD300a and anti-CD300c mAbs were generated (11–13).

With regard to the lymphoid lineage, CD300a is expressed on the surface of all human NK cells (9, 14) and in subsets of T and B cells (8, 10, 15–17). Human naive $CD4^+$ T cells express low levels of CD300a, whereas effector/memory cells can be subdivided into $CD300a^+$ and $CD300a^-$ subsets, and regulatory T cells are $CD300a^-$. Memory $CD300a^-$ cells tend to proliferate slightly less than do memory $CD300a^+$ cells (8, 15), and the expression of CD300a is associated with Th1 cells that are more polyfunctional and, after stimulation, upregulate the T-box transcription factor eomesodermin, whereas the IL-17 single-producing $CD4^+$ T cells are mostly $CD300a^-$ (10, 15). In contrast, in human $CD8^+$ T cells, CD300a expression is coupled to a more cytotoxic phenotype, suggesting that CD300a is mostly expressed on effector/memory cells, whereas naive $CD8^+$ T cells express low levels of the receptor (17). Naive B cells express low levels of CD300a, whereas memory B cells and plasma cells express variable levels, and germinal center B cells are negative for CD300a cell surface expression (16). In the myeloid lineage, CD300a is detected on the surface of plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs), monocytes, macrophages, neutrophils, eosinophils, basophils, and mast cells (13, 18–25).

In humans, cell surface expression of CD300a is regulated by multiple stimuli. For example, in $CD4^+$ T cells, stimulation with anti-CD3 plus anti-CD28 mAbs and Th1-differentiation conditions upregulate cell surface expression of CD300a, whereas TGF-β exhibits a negative regulatory effect (8, 15). These in vitro–obtained results are in agreement with the fact that TGF-β1 is required for the development of regulatory T cells, which are $CD300a^-$, and Th17 cells, which tend to be enriched in the $CD300a^-$ subset (10, 15). On naive B cells, BCR and TLR9 stimulation, along with T cell help, failed to upregulate the expression of CD300a in vitro, although TLR9 stimulation alone was sufficient to increase the expression of the receptor in memory B cells (16). The differential effect of TLR9 on CD300a expression on naive versus memory B cells may be explained by the low expression of TLR9 on human naive B cells and/or the fact that it is not coupled to other signaling pathways at this developmental stage. In contrast, IL-4 and TGF-β1 are negative regulators of CD300a expression on memory B cells (16).

LPS, IFN-γ, hypoxia, and the hypoxia-mimetic agent desferrioxamine upregulate the expression of CD300a on monocytes (26–28). However, these results should be re-evaluated in light of the finding that human monocytes significantly express CD300c on the cell surface, and the mAbs used in these studies recognized both CD300a and CD300c (12, 13). In neutrophils, LPS and GM-CSF treatment caused a rapid translocation of an intracellular pool of CD300a to the cell surface, contributing to the observed increased expression in response to those stimuli (18). It is quite conceivable that an intracellular pool of CD300a also exists in basophils, which translocates to the cell surface in <20 min upon cell stimulation through FcεRI (21, 25). In eosinophils, CD300a expression is also upregulated by hypoxia and GM-CSF, and the inhibition of hypoxia-inducible factor 1 abolished this upregulation (24). In pDCs, IFN-α production in response to TLR7 and TLR9 agonists downregulates the expression of CD300a (22), and the eosinophil-derived major basic protein and eosinophil-derived neurotoxin downregulate it on cord blood–derived mast cells (19).

Transcripts encoding mouse CD300a, also known as CLM-8 (29), LMIR-1 (30), and MAIR-I (31), are also found in cells of the lymphoid and myeloid lineages. On the cell surface, it is expressed on the majority of myeloid cells, including macrophages, mast cells, dendritic cells, and granulocytes. On lymphocytes, mouse CD300a is expressed on subsets of B cells, with higher expression on marginal zone B cells (29–32). However, in contrast to human CD300a, it is not detected on the surface of unstimulated NK and T cells (31).

In mice, CD300a cell surface expression was detected on NK cells after stimulation with IL-12 (31), and peroxisome proliferator-activated receptor β/δ directly regulates the expression of the *CD300A* gene in macrophages (33). In vitro, IL-33, a cytokine that has a key role in initiating Th2 responses, upregulates the expression of CD300a and CD300f on eosinophils, and it only upregulates the expression of CD300a on bone marrow–derived mast cells. However, injection of IL-33 in the peritoneal cavity did not induce the expression of CD300a in any of the analyzed cell types in contrast to the IL-33–induced upregulation of CD300f in vivo (34). These results may explain the requirement of CD300f, but not CD300a, for IL-33–induced eosinophil and mast cell activation (34).

Identifying CD300a ligands

Several groups used CD300-Ig fusion proteins as a tool to identify cells expressing ligands for the CD300 molecules (13, 35–38). An interesting finding was that CD300a-Ig binds to apoptotic/dead cells from distant species in a Ca^{2+}-dependent manner, suggesting that this receptor binds evolutionarily conserved ligands (37, 38). Apoptotic/dead cells are characterized by changes in the plasma membrane, including the loss of phospholipid asymmetry (39). From the early stages of apoptosis, cells expose phosphatidylserine (PS) and phosphatidylethanolamine (PE) in the outer leaflet of the plasma membrane (39–43), which act as "eat-me" signals and lead to their engulfment by phagocytes (44, 45). The binding of CD300a-Ig to dead cells was blocked by milk-fat globule EGF-factor VIII (MFG-E8), a ligand for PS, and by duramycin, a ligand for PE (37, 38), suggesting that these two aminophospholipids are ligands for CD300a. Subsequent experiments that included a variety of techniques, such as surface plasmon resonance, ultracentrifugation, ELISA, and immunoblotting, confirmed the direct binding of CD300a-Ig to purified aminophospholipids and to PS- or PE-containing liposomes (37, 38). In addition, human CD300a-Ig exhibited a preference for binding PE over PS (38).

The functional recognition of purified PE was demonstrated in a reporter cell system expressing human CD300a-CD3ζ chimeric receptors (13, 38). However, the functional recognition of PS could not be detected. The apparent discrepancy between the binding results to liposomes and pure lipids and the functional results may be explained by the fact that CD300a has a stronger binding to PE than to PS. Moreover, the particular steric environment of the extracellular part of

CD300a may also affect its binding to these aminophospholipids. To identify the residues that are involved in human CD300a binding to PS and PE, a molecular model was generated based on the crystal structure of T cell/transmembrane, Ig, and mucin (TIM)-4 complexed with PS (38, 46). The metal ion and a molecule of PS or PE were placed in positions corresponding to the PS bound to the TIM-4 structure in the crystal structure of CD300a (38, 47). The model showed that PE and PS interact with CD300a residues that form a cavity into which the hydrophilic heads of the lipids can penetrate (38). The structural model was validated by analyzing the binding of CD300a-Ig mutants to lipids and dead cells. A WLRD motif in human CD300a was shown to be very important for binding (38). A similar motif, WFND, is required for the TIM molecules that bind PS (46, 48, 49). The binding of mouse CD300a-Ig to PS immobilized on a membrane, but not to PE, was observed by some investigators (37), whereas others did not find any binding of mouse CD300a-Ig to any lipid immobilized on a membrane (35). The reason for the discrepancy in these studies is unknown.

Mechanisms of signaling

Human CD300a has four tyrosine residues in its cytoplasmic segment. Three of those tyrosines are within consensus sequences for classical or canonical ITIMs (V/LxYxxL/V), and the fourth is part of a nonclassical or permissive ITIM (SxYxxI) (14, 50). In contrast, the cytoplasmic tail of mouse CD300a possesses two classical ITIMs, and a third tyrosine is within a tyrosine-based sorting motif (YVNL) that was shown to mediate endocytosis of the receptor upon cross-linking with mAbs (30, 31) (Fig. 1).

Tyrosine phosphorylation of ITIMs is required for the transmission of the inhibitory signal (9, 51, 52), and site-directed mutagenesis experiments showed that the four ITIMs in human CD300a were important for the inhibitory function of this receptor, with the third plasma membrane–distal ITIM being the most essential (9, 51). Similarly, tyrosine residues within ITIMs are responsible for the inhibitory signal of murine CD300a (30, 52). The chimeric receptor killer cell Ig-like receptor (KIR)-CD300a, consisting of the extracellular domains of KIR2DL2 fused to the transmembrane and cytoplasmic segments of human CD300a, was used to investigate the kinase that phosphorylates CD300a ITIMs. The interaction of Jurkat cells expressing KIR-CD300a with target cells expressing HLA-Cw3, the KIR2DL2 ligand, resulted in phosphorylation of CD300a ITIMs by the Src tyrosine kinase Lck but not by ZAP-70 (51). It is reasonable to expect that another Src tyrosine kinase might phosphorylate the CD300a ITIMs in cells that do not express Lck.

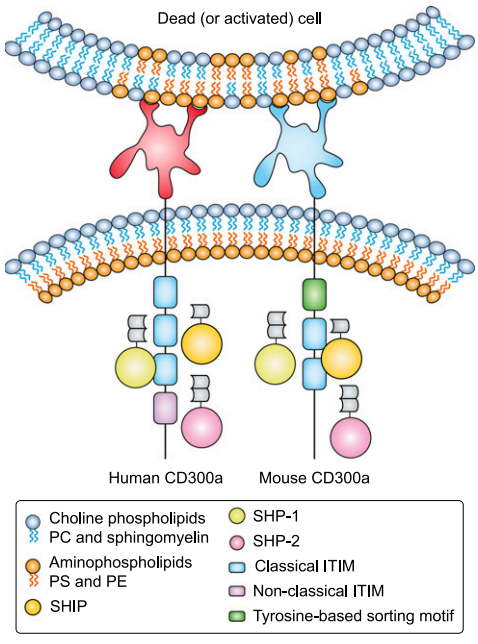

FIGURE 1. Schematic representation of human and mouse CD300a inhibitory receptor. The intracellular signaling motifs for each receptor are indicated, and the described interaction with phosphatases is shown. CD300a recognizes the aminophospholipids PS and PE exposed on the outer leaflet of dead (and activated) cells. PC, phosphatidylcholine.

Phosphorylated CD300a ITIMs are able to recruit different phosphatases, depending on the examined cell type and the method of stimulation. For example, treatment of human NK cells with the tyrosine phosphatase inhibitor sodium pervanadate induced tyrosine phosphorylation of CD300a and the subsequent association with both Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 (14), whereas in IL-5- or eotaxin-activated human eosinophils, cross-linking of the receptor with mAbs recruited SHP-1 but not SHP-2 (23). In coprecipitation experiments, treatment of human cord blood–derived mast cells with pervanadate resulted in SHP-1 and Src homology region 2 inositol 5′ phosphatase (SHIP)-1, but not SHP-2, association with CD300a. Intriguingly, upon cross-linking of the receptor with mAbs, only SHIP-1 associated with CD300a in the same cell type (19). Furthermore, immunoprecipitation of CD300a from human mast cells that were treated with Kit-CD300a, a bispecific Ab fragment linking Kit with CD300a, induced its tyrosine phosphorylation and the recruitment of SHIP but not SHP-1 (53). Triggering of CD300a with mAbs induced weak phosphorylation of SHIP-1 on human basophils, but the phosphorylation status of SHP-1 and SHP-2 was not tested (21). In a mouse B cell line, coligation of the BCR with anti-mouse IgG and Fc-CD300a, a chimeric molecule consisting of the extracellular and transmembrane domains of FcγRIIB and the cytoplasmic domain of mouse CD300a, resulted in the association of the intracellular tail of CD300a with SHP-1, SHP-2, and SHIP (30). The three phosphatases were recruited upon pervanadate treatment in RBL-2H3 cells ectopically expressing mouse CD300a, but only SHP-1 and SHIP were recruited after cross-linking of the receptor with mAbs (52). In bone marrow–derived mast cells, mouse CD300a coimmunoprecipitated with SHP-1, SHP-2, and SHIP upon pervanadate treatment (30, 31). Finally, in a more physiological setting, SHP-1 recruitment to CD300a was observed when bone marrow–derived mast cells and macrophages were mixed with apoptotic cells and treated with LPS (37, 54). The binding to SHP-2 and SHIP was not tested.

In an effort to understand which phosphatase is responsible for the transmission of the inhibitory signal, wild-type and phosphatase-deficient DT40 B cells ectopically expressing human CD300a were used to ascertain the role of each one of the phosphatases previously described to bind CD300a-phosphorylated ITIMs. CD300a in SHP-2– and SHIP-deficient DT40 cells was still able to inhibit BCR-mediated

signals, such as Ca^{2+} mobilization. However, in SHP-1–deficient cells, the CD300a-mediated inhibition was largely abolished, indicating a dominant role for this phosphatase (51). This was further confirmed by the use of Jurkat cells expressing the chimeric receptor KIR-CD300a. Although both SHP-1 and SHP-2 coimmunoprecipitated with KIR-CD300a after interaction with the ligand, only knocking down SHP-1 expression resulted in a decrease in the inhibitory potential of KIR-CD300a (51). The dominant role of SHP-1 was further demonstrated by knocking down SHP-1 expression in bone marrow–derived mast cells from CD300a-deficient mice. Although $CD300a^{-/-}$ mast cells produced significantly more TNF-α than did $CD300a^{+/+}$ mast cells after stimulation with LPS in the presence of apoptotic cells, there was no significant difference in TNF-α production between SHP-1–knocked down $CD300a^{+/+}$ bone marrow–derived mast cells and SHP-1–knocked down $CD300a^{-/-}$ bone marrow–derived mast cells (54).

An important and unresolved question is whether coligation of CD300a with the activating receptor is necessary for the inhibitory signal. Although experiments were performed with (9, 10, 14, 16, 51) and without (22, 23, 55) coligation, few publications have addressed this issue (19, 52). Some investigators demonstrated that coligation of CD300a with FcεRI is essential for the CD300a-mediated inhibitory signal (52). Instead, others showed that there is no requirement for the coligation of CD300a with the activating receptor for CD300a to inhibit the FcεRI-mediated activation signal, despite the fact that coligation increased the inhibitory effect (19, 53). Interestingly, the effect on mast cell survival did not change significantly when CD300a was coligated with Kit (19). Furthermore, it was shown that the bispecific Ab fragment Kit-CD300a was able to inhibit Kit-mediated signals in mast cells stimulated with stem cell factor (SCF), but the Ab fragment control IgG-CD300a did not have any inhibitory effect, suggesting that, at least in these settings, coligation of CD300a and Kit and/or cross-linking of CD300a is required for the inhibitory effect (53).

Immune regulation

Before PS and PE were identified as CD300a ligands, its role in regulating immune functions was studied using mAbs. Hence, its engagement by agonist mAbs decreased NK cell–mediated cytotoxicity and inhibited IgE-dependent Ca^{2+} mobilization and mediator release from mast cells and SCF-mediated mast cell activation, differentiation, and survival, as well as IgE-induced basophil degranulation (9, 14, 19, 21, 25, 31, 53, 56). The mAb-mediated cross-linking of CD300a also reduces FcγRIIa-triggered reactive oxygen species production and Ca^{2+} flux in neutrophils (18), suppresses eosinophil survival, migration, and inflammatory mediator production triggered by eotaxin, IL-15, and GM-CSF (23), and LPS- and CpG-induced IL-8 secretion by myelomonocytic cell lines (55). Cross-linking of CD300a regulated type I IFN and TNF-α secretion by pDCs in response to TLR7 and TLR9 stimulation (22). Also, CD300a was identified as a regulator of transendothelial migration in a transcriptional profiling of human monocytes following their adhesion to and passage through the endothelial monolayer (57). CD300a expression levels were upregulated following transmigration, and engagement of the receptor with mAbs significantly reduced monocyte transendothelial migration. In contrast, small interfering RNA–mediated downregulation of CD300a increased their rate of migration. Upregulation of CD300a following transendothelial migration may prepare monocytes to terminate the actual transmigration after they get in contact with apoptotic cells at the site of inflammation (57). Cross-linking of CD300a with mAbs also regulated TCR-mediated and BCR-mediated signaling (10, 16).

Given that both PS and PE are expressed on dead cells (39–45, 58), the relevance and significance of the interaction between CD300a and PE/PS were demonstrated by the role of this receptor in modulating the engulfment of dead cells (38, 54). In fact, CD300a downregulates the uptake of apoptotic cells by macrophages, and its ectopic expression in $CD300a^{-}$ cell lines also decreased the clearance of dead cells (38). These results somehow alter the existing "eat-me" signal paradigm in that, during the death process when cells expose PE, they may provide a "don't-eat-me-yet" signal after interacting with CD300a. $CD300a^{-/-}$ bone marrow–derived mast cells and macrophages treated with LPS in the presence of apoptotic cells produced higher levels of proinflammatory cytokines, indicating that CD300a acts as an inhibitory receptor in these cell types after interacting with PS and PE on apoptotic cells (54). Furthermore, PS-expressing tumor targets decrease NK cell–mediated cytotoxicity (11), and apoptotic cells and PS-containing liposomes inhibit IgE-induced basophil degranulation in a CD300a-dependent manner (59).

Disease relevance

The role of the CD300 family of receptors in several pathologies, their possible usage as biomarkers, and the potential for targeting these molecules for therapeutic purposes have been well documented (4) over the course of the last years.

Viral infections. To efficiently replicate, viruses, such as HIV, hepatitis C virus, and others, activate the host cells leading to an increase in intracellular calcium, which, in turn, causes externalization of PS and PE, the ligands of CD300a. Moreover, translocation of these two phospholipids to the outer leaflet of the plasma membrane is one of the earliest events associated with apoptosis induced by viruses, such as HIV. Recent reports suggested that exposure of PS, and probably also PE, inhibits inflammation and the immune response, allowing the virus to avoid recognition by the immune system (60). In fact, anti-PS Abs have the ability to inhibit HIV infection in vitro (61) and showed therapeutic potential in in vivo models of CMV and Pichinde virus infection (60). The expression of CD300a on circulating B cells is downregulated during HIV infection (16), suggesting the possibility that this inhibitory receptor may contribute to the B cell hyperactivation and dysfunction observed in HIV-infected patients (62). The decrease in CD300a expression on B cell subsets, with the exception of plasmablasts, was not corrected by effective antiretroviral therapy. In contrast, a significant positive correlation between $CD4^{+}$ T cell count and CD300a expression on memory B cells was observed in patients whose viremia was controlled by antiretroviral therapy. Altogether, these results indicate that the altered CD300a expression on B cells during HIV infection is a complex process involving several factors. Also, a positive correlation between mRNA levels of CD300a and the expression of the transcription factor BATF in HIV-specific

$CD8^+$ T cells was reported (63). BATF expression is very high in $CD8^+$ exhausted T cells, and it inhibits the function of HIV-specific cells through a mechanism that involves the increased expression of inhibitory receptors, such as CD300a (63).

A variety of viruses enclose their capsid in a lipid bilayer that can be obtained during virus budding from plasma membrane (64). Very importantly, this implies the incorporation of PS, and likely PE, into the viral envelope. The presentation of PS and PE on the outer leaflet of these membranes camouflages viruses as apoptotic bodies regulating cell entry through a process termed "apoptotic mimicry" (64). Consequently, viral envelope PS and PE are very important for enveloped viral replication, and it was demonstrated that enveloped viruses, such as dengue, vaccinia, West Nile, Sindbis, and Ebola viruses, use a PS-mediated viral entry mechanism after interacting with PS receptors (65–71). Among the human PS-binding molecules, not all of them enhance virus binding to cells and facilitate their engulfment (64). TIM-1, TIM-4, Protein S, and Gas6 (which bridge PS-containing membranes to cells expressing the receptor tyrosine kinases Tyro3, Axl, and Mer) and MFG-E8 (which bridges PS-containing membranes to cells expressing integrins $\alpha v\beta 3$ and $\alpha v\beta 5$) enhance virus entry. In contrast, other PS-binding receptors, such as TIM-3, stabilin-1, stabilin-2, and BAI1, do not increase binding to pseudotyped lentiviral vectors. Interestingly, human CD300a increases virus binding but does not enhance their transduction (64, 70). This resembles the ability of CD300a to bind apoptotic cells; however, this binding does not induce phagocytosis of dead cells (38, 54). In fact, CD300a suppressed phagocytosis of apoptotic cells by human macrophages (38). Therefore, the resulting signals from the binding of CD300a to PS- (and PE)-containing virus envelope might suppress viral endocytosis, which would lead to abortive virus infection.

Sepsis. Defective removal of dead cells has deleterious consequences for the host (44, 58). The nature of the immune response to cell death depends on which, where, and how cells die, as well as what immune cells interact with them (72). Variations in these parameters determine whether cell death is immunogenic, tolerogenic, or silent (72). It is quite possible that the CD300 family of receptors plays a central role in determining the outcome of the immune response when dead cells interact with different components of the immune system. Indeed, recently published data support this hypothesis (54, 73, 74). A large number of cells undergo apoptosis in the peritoneal cavity during mouse models of cecal ligation and puncture (CLP) peritonitis (75). In this model, mast cells play an important role (76), and the expression of CD300a on their cell surface controls chemokine production, as shown by the fact that CD300a-deficient peritoneal mast cells produced more chemoattractants, leading to increased neutrophil recruitment and better bacterial clearance. As a consequence, $CD300a^{-/-}$ mice showed prolonged survival after CLP peritonitis (54). Ab blockade of CD300a interaction with PS also prolonged survival after CLP in wild-type mice (54), indicating that CD300a regulates mast cell inflammatory responses to microbial infections.

Allergy. The expression of CD300a on cell types, such as mast cells, eosinophils, and basophils, that have an important role in the initiation, regulation, and effector phases of allergic responses, along with its ability to downregulate their activity in response to diverse stimuli, led to the design of bispecific Ab fragments targeting CD300a, along with other receptors, with the goal of downregulating the function of these cells during disease conditions in mouse models. Bispecific Ab fragments specific for CD300a and c-Kit abrogated mast cell degranulation induced by SCF during cutaneous anaphylaxis (53). Another bispecific Ab fragment linking CD300a to IgE bound to FcεRI, and, therefore, specific for FcεRI-expressing cells (i.e., basophils and mast cells), was able to abolish allergic and inflammatory responses in OVA-induced acute experimental asthma and IgE-dependent passive cutaneous anaphylaxis (56). A third bispecific Ab fragment, targeting CD300a to CCR3 and specific for mast cells and eosinophils, was able to reduce eosinophil signaling in vivo, eosinophil and mast cell mediator release, bronchoalveolar lavage fluid inflammation, eosinophil-derived TGF-β1 in the bronchoalveolar lavage fluid, and lung remodeling. Very importantly, this Ab fragment also reversed lung inflammation in a model of chronic established asthma (77). Also, in a model of allergic peritonitis, neutralization of CD300a with specific mAbs resulted in a significant increase in inflammatory mediators and eosinophilic infiltration (19). In humans, it was shown that the basal expression of CD300a on basophils from birch pollen allergic patients was significantly lower than for healthy control individuals (25). Interestingly, apoptotic cells inhibited anti-IgE–mediated basophil degranulation in healthy donors more efficiently than in allergic patients, suggesting that CD300a cell surface levels are very important in regulating the threshold for inhibiting IgE-mediated signals (59). Along these lines, recent studies showing the effects of hypoxia on the expression of CD300a in human eosinophils (24) and monocytes (28) are important for understanding the behavior of these cell types in diseased tissues and shed light on their role in inflammatory conditions.

Autoimmune disorders and chronic inflammatory conditions. The CD300 gene complex has been linked to *PSOR2*, a susceptibility locus for psoriasis, which may also overlap with loci for rheumatoid arthritis and atopic dermatitis (78, 79). A single nucleotide polymorphism that encodes for a nonsynonymous polymorphism (R94Q) within the Ig domain of CD300a was associated with susceptibility to psoriasis (78); however, other studies disputed this linkage. Still, CD300a-Ig with arginine at position 94 binds better to dead cells, PE, and PS than does CD300-Ig with glutamine at position 94 (38), highlighting the relevance of this polymorphism. Interestingly, the surface expression of CD300a on $CD4^+$ T cells is significantly lower in psoriatic patients compared with healthy controls (8); however, the Ab used in this latter study recognized both CD300a and CD300c. Although CD300c is not expressed on the surface of $CD4^+$ T cells of healthy subjects (12, 13), it is not known whether it would be expressed in psoriatic patients.

CD300A was proposed, in combination with three other genes (*KPNA4*, *IL1R2*, and *ELAVL1*), as a biomarker that can help to differentiate ulcerative colitis from Crohn's disease and noninflammatory diarrhea (80). This may benefit the serologic testing for inflammatory bowel disease, which only has sensitivity and specificity ~80%, to differentiate Crohn's disease from ulcerative colitis; it is based on reactivity to bacterial Ags and not host gene expression (81). When fed

a high-fat diet, mice lacking CD300a develop chronic intestinal inflammation with expanded mesenteric lymph nodes and decreased numbers of intestinal capillaries (33). This leads to triglyceride malabsorption and reduced body weight. In CD300a$^{-/-}$ animals that are on high fat diet, peritoneal macrophages are M1 activated and produce higher levels of IL-6 in response to LPS. These results suggest that CD300a-mediated inhibitory signals have the ability to suppress chronic intestinal inflammation.

Cancer. CD300a was identified, along with other markers, to be differentially expressed in acute lymphoblastic leukemia (ALL) compared with normal CD19$^+$CD10$^+$ B cell progenitors (82). Sixteen differentially expressed markers, including CD300a, were validated for minimal residual disease detection by four-color flow cytometry analysis (82). More recently, it also was reported that pre-B cell–derived ALL expresses high levels of CD300a and other ITIM-containing receptors, such as LAIR1 and PECAM1. Importantly, patients who had higher expression levels of these receptors at the time of diagnosis exhibited shorter overall and relapse-free survival, suggesting the potential use of these receptors as biomarkers for the stratification of patients with ALL (83). Furthermore, mouse genetic studies demonstrated that CD300a, PECAM1, and LAIR1 calibrate oncogenic signaling strength through recruitment of SHP-1 and SHIP-1, indicating that targeting of CD300a, as well as other inhibitory receptors, could be a strategy to treat ALL (83).

A recent study reported that the interaction between CD300a and PS inhibits tumor cell killing by NK cells (11). Several studies showed that, in the tumor microenvironment, there is a significant stress imposed on the tumor endothelium by acidity, reactive oxygen species, and transient hypoxia, which result in the redistribution and exposure of PS and PE (84). Indeed, expression of PS was detected in gastric carcinoma (85), ovarian carcinoma (86), and melanoma (87). Recent data support that the binding of CD300a-Ig to tumor cells is reduced when PS is blocked and that the blocking of PS enhances NK cell–mediated cytotoxicity. Blocking of PS partially restored NK cell cytotoxicity, indicating that tumor cells express an additional ligand for CD300a (11). This additional ligand might be PE, which also was shown to be a ligand for CD300a (13, 38). Therefore, a new tumor immune-evasion mechanism was suggested to be mediated through the interaction between PS and PE on tumor cells and CD300a on cytotoxic lymphocytes.

Conclusions

CD300a-mediated signaling is a very complex process that involves many players. More studies are required to define the targets of the phosphatases involved in the CD300a signaling pathway and the unique downstream signaling components. Little is known about the topology of PS and PE exposed on apoptotic cells and how they are engaged by specific receptors, including CD300a. Furthermore, there is no information on whether CD300a recognizes PS and PE as monomers, dimers, or higher-order oligomers and whether it is able to recognize these lipids in *cis*, as well as in *trans*. In a model of thioglycollate-induced peritonitis, it was shown that oxidation products of PE exposed on the plasma membrane of resident macrophages maintain self-tolerance by blocking phagocytosis of apoptotic cells by the freshly recruited inflammatory monocytes (88). Although it was proposed that the PS-binding molecule MFG-E8 has a role in this process (88), it also would be very interesting to study the role of CD300a in this model, as well as to elucidate whether the oxidation status of PE affects its binding to CD300a. Another fascinating question is the physiological relevance of lipid recognition by CD300a expressed on lymphocytes (i.e., NK cells, CTLs, subsets of CD4$^+$ T cells, and memory B cells). So far, studies demonstrated the ability of CD300a to deliver inhibitory signals in lymphocytes, but future studies addressing the role of CD300a in key immune functions, such as cell differentiation, Ag presentation, cytokine production, cell-mediated cytotoxicity, termination of the immunological synapse, and so forth, are warranted. We are at the starting point in understanding the role of CD300a in disease settings and its potential as a therapeutic target. In light of the in vitro clinical data and preclinical data obtained from mouse models, we need to know more about the involvement of CD300a in human diseases, such as cancer, viral infections, sepsis, and autoimmune, inflammatory, and allergic diseases. Through its binding to PS and PE, CD300a is able to recognize the viability and activation status of cells and, consequently, have a significant influence on the final outcome of the immune response.

Disclosures

The authors have no financial conflicts of interest.

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CD300c costimulates IgE-mediated basophil activation, and its expression is increased in patients with cow's milk allergy

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Background: Basophils express high-affinity IgE receptors (FcεRI), which play an essential role in allergic diseases. It is important to characterize new cell-surface receptors that modulate IgE-mediated basophil activation threshold to design promising immunomodulatory therapies.

Objectives: We sought to analyze the expression of CD300 receptors on human basophils and their implication in IgE-mediated basophil activation processes.

Methods: Blood samples from healthy subjects and patients with cow's milk allergy were collected through the Basque Biobank under an institutional review board–approved protocol. PBMCs were obtained by means of density centrifugation, basophils were purified with a specific isolation kit, and phenotypic and functional studies were performed by using flow cytometry.

Results: We demonstrate that basophils express the activating receptor CD300c, which is specifically upregulated in response to IL-3. CD300c works as a costimulatory molecule during IgE-mediated basophil activation, as shown by a significant increase in degranulation and cytokine production when basophils are activated in the presence of CD300c cross-linking compared with activation through the IgE/FcεRI axis alone. Coligation of FcεRI and CD300c increased intracellular calcium mobilization and phosphorylation of signaling intermediates evoked only by FcεRI ligation. We show that the natural ligands of CD300c, phosphatidylserine and phosphatidylethanolamine, modulate IgE-mediated basophil activation. Furthermore, we have observed that CD300c expression in children with cow's milk allergy is increased compared with that in healthy control subjects and that the intensity of expression correlates with the severity of the hypersensitivity symptoms.

Conclusion: CD300c could be considered a biomarker and therapeutic target in patients with IgE-mediated allergic diseases because it seems to be involved in the modulation of IgE-mediated basophil activation. (J Allergy Clin Immunol 2019;143:700-11.)

Key words: *Basophils, CD300, CD300a, CD300c, allergy, IgE-mediated activation, IL-3, activation threshold*

Basophils are major effector cells of IgE-dependent immune and allergic responses.[1-3] They express high levels of the high-affinity IgE receptor (FcεRI) on their surface, and cross-linking of FcεRI-bound IgE by bivalent or multivalent allergens induces the secretion of multiple stored and newly synthesized inflammatory mediators, including vasoactive amines (eg, histamine), lipid metabolites (eg, leukotriene C_4), and type 2 cytokines (eg, IL-4 and IL-13).[4-6] These substances contribute to the development of allergy and other forms of inflammation.

In allergic reactions basophil degranulation and consequent release of various mediators occur after loss of the balance between activating and inhibitory signals. However, the means that govern this equilibrium are not completely elucidated. Among other mechanisms, the balance could be accomplished by signals originating from cell-surface receptors with activating and inhibitory capabilities. Some inhibitory receptors are characterized for having a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIMs),[7-9] whereas many activating receptors are characterized for having a short cytoplasmic tail and a charged amino acid residue that allows their association with immunoreceptor tyrosine-based activating motifs (ITAM)–bearing adaptors, which transduce activation signals and other adaptors that bind to, for example, phosphoinositide 3-kinase.[7-11]

The human CD300 family consists of 8 receptors encoded in chromosome 17, and they are expressed in both myeloid

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Abbreviations used
APC: Allophycocyanin
cIMDM: Complete Iscove modified Dulbecco medium
Erk: Extracellular signal-regulated kinase
fMLP: Formyl-methionyl-leucyl-phenylalanine
FMO: Fluorescence minus one
ITAM: Immunoreceptor tyrosine-based activating motif
ITIM: Immunoreceptor tyrosine-based inhibitory motif
JAK: Janus kinase
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PerCP: Peridinin-chlorophyll-protein
PS: Phosphatidylserine
STAT: Signal transducer and activator of transcription
TSLP: Thymic stromal lymphopoietin

and lymphoid lineages, except CD300g, which is expressed on endothelial cells.[12-16] The CD300 molecules are type I transmembrane proteins with a single IgV-like extracellular domain. CD300a and CD300f contain ITIMs in their cytoplasmic tail and are considered inhibitory receptors, although CD300f is also able to deliver activating signals through motifs reported to bind the p85α regulatory subunit of phosphoinositide 3-kinase.[17] On the other hand, CD300b, CD300c, CD300d, CD300e, and CD300h are activating receptors. The mouse CD300 molecules are encoded by 9 genes located on mouse chromosome 11, the syntenic region of human chromosome 17.[12]

CD300 receptors, including CD300a, CD300b, CD300c, and CD300f, are capable of recognizing phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine (PS), which are exposed on the outer leaflet of the plasma membranes of dead and activated cells.[18-23] In addition, other lipids, such as ceramide and sphingomyelin, have been proved to bind CD300f and CD300e.[24-26] Through its lipid binding, this receptor family is poised to have an important role in host responses in patients with severe pathologic conditions and complex biological processes.[12,15]

Over the last years, the biological and clinical significance of CD300 molecules and their participation in the pathogenesis of several diseases (allergy among others) have been documented both in human and mouse models.[12,15] In fact, the expression and function of several members of the CD300 receptor family have been described on cell types, such as mast cells, eosinophils, and basophils, that have important roles in the initiation, regulation, and effector phases of allergic responses. Thus far, several authors have reported that mast cells constitutively express the inhibitory receptors CD300a[10,27] and CD300f[25,26,28] and the activating members CD300b,[29] CD300c,[23] and CD300d[28] and that at least the inhibitory receptors CD300a[30] and CD300f[31,32] are expressed on eosinophils.

Regarding human basophils, it has been shown that they express the inhibitory receptor CD300a.[33-35] Furthermore, it has been described that basal expression of CD300a is lower in basophils from patients with birch pollen allergy than in basophils from healthy control subjects.[33] Interestingly, apoptotic cells expressing the CD300a ligands PS and PE inhibited anti-IgE–mediated basophil degranulation in healthy donors more efficiently than in allergic patients.[35] Altogether, these data suggest that CD300a could have an important role in the modulation of the activation threshold of basophils during allergic reactions.[33-35]

Despite the relevant role of CD300a in human basophil activation processes, the expression and function of other members of the CD300 family have not been determined. Here we have analyzed the expression of different members of the CD300 family and found that, in addition to the CD300a receptor, human basophils also express the CD300f inhibitory receptor and the CD300c activating receptor, with the latter being the only member of the family that increases its expression levels in response to IL-3. We have demonstrated that CD300c acts as a costimulatory molecule during basophil activation through the IgE/FcεRI axis. Importantly, we show that expression levels of CD300c on basophils from children with cow's milk allergy are greater than those in healthy control children and that there is an association between the severity of the hypersensitivity symptoms and levels of CD300c expression on basophils.

METHODS

Subjects and samples

Blood samples from healthy donors and donors with cow's milk allergy (both adults and children) were collected through the Basque Biobank (http://www.biobancovasco.org). All the patients included in the study presented IgE-mediated allergy symptoms, positive skin prick test responses, and *in vitro*–specific IgE to cow's milk, α-lactalbumin, β-lactoglobulin, and casein in the 6 months before blood extraction (Table I). Hypersensitivity to cow's milk was classified as grade I (local reaction with perioral and/or contact urticaria), grade II (systemic reaction without cardiovascular and/or respiratory involvement with gastrointestinal symptoms), or grade III (severe systemic reaction with cardiovascular and/or respiratory involvement) at the time of diagnosis, according to the criteria described by Niggemann and Beyer.[36] Samples from control children (Table II) were collected from healthy siblings of patients with a diagnosis of celiac disease who participated in a common type of study.

The Basque Biobank complies with the quality management, traceability, and biosecurity set out in the Spanish Law 14/2007 of Biomedical Research and the Royal Decree 1716/2011. All subjects provided written and signed informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Basque Ethics Committee for Clinical Research (PI2015182; version 2; November 18, 2015).

Functional experiments

For functional experiments, nonprimed and IL-3–primed PBMCs, purified basophils, or both were used. For priming, cells were cultured in complete Iscove modified Dulbecco medium (cIMDM) and stimulated with IL-3 for 2 hours. Then, they were washed thoroughly and starved from IL-3 for 22 additional hours. Functional experiments were performed the next day.

The detailed list of antibodies and reagents used for the experiments, as well as experimental protocols for flow cytometric analyses and functional experiments, such as calcium mobilization assay, phosphorylation assay, cross-linking of CD300c, and measurement of activation markers and cytokine production; lipid stimulation experiments; stimulation of basophils in the presence or absence of apoptotic K562 cells; and data analysis are detailed in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Human basophils express CD300a, CD300c, and CD300f receptors

Despite a few studies showing that human basophils express the inhibitory receptor CD300a,[33,34] expression of other members of the CD300 receptor family has not been determined. We analyzed

TABLE I. Clinical data of children with cow's milk allergy

Patient no.	Sex	Age	Hypersensitivity	IgE, milk	IgE, α-lactalbumin	IgE, β-lactoglobulin	IgE, casein
1	Male	18 mo	Grade III	3.37	0.09	0.26	1.06
2	Female	4 y	Grade III	4.43	3.73	1.53	0.99
3	Male	16 mo	Grade I	4.44	5.78	0.06	0.27
4	Female	6 y	Grade II	46.3	10.9	4.9	46
5	Male	5 y	Grade II	0.69	0.79	0.26	0.11
6	Female	17 mo	Grade I	5.13	1.42	6.55	1.06
7	Female	3 y	Grade II	8.91	5.01	1.74	7.18
8	Male	4 mo	Grade I	5.79	0.32	0.47	0.01
9	Female	22 mo	Grade III	0.92	0.92	0.11	0.64
10	Male	7 y	Grade III	62.5	4.48	20.7	64.5
11	Male	3 y	Grade III	22.1	6.93	1.18	7.99
12	Male	6 mo	Grade I	6.72	2.95	2	4.58
13	Male	13 mo	Grade I	0.48	0.3	0.14	0.31
14	Male	15 y	Grade III	15.3	11	3.69	14
15	Female	12 mo	Grade I	2.42	1.18	1.64	3.5
16	Male	12 y	Grade I	3.26	0.3	0.05	0.79
17	Male	2 y	Grade I	3.25	3.25	0.47	1.39

TABLE II. Clinical data of healthy children

Control no.	Sex	Age (y)
1	Male	6
2	Male	3
3	Female	4
4	Female	9
5	Male	12

the expression pattern of CD300a, CD300c, CD300e, and CD300f on circulating basophils from healthy donors' peripheral blood.

To identify basophils among the PBMCs, we used a staining strategy based on expression of the surface receptors FcεRI and CD203c within lineage-negative ($CD3^-CD14^-CD19^-CD20^-$) cells (Fig 1, *A*). We also tested a staining strategy based on surface expression of CD123 and the absence of HLA-DR and concluded that both strategies were equally valid for basophil identification (data not shown). Regarding CD300 receptor expression, we observed that basophils express not only the CD300a inhibitory receptor but also CD300f and the CD300c activating receptor at lower levels, although they do not express CD300e (Fig 1, *A*).

Expression of CD300c on basophils increases in response to IL-3

Next, we studied regulation of the expression of CD300c, CD300e, and CD300f in response to several IgE-dependent and IgE-independent basophil activators, such as anti-IgE mAb, IL-3, thymic stromal lymphopoietin (TSLP), IL-33, and formyl-methionyl-leucyl-phenylalanine (fMLP). We did not study regulation of CD300a expression because it has been described previously by others.[33] Interestingly, except for a very significant upregulation on CD300c expression in response to IL-3 (Fig 1, *B*), stimulation of basophils for 18 hours did not induce any other significant change in expression of CD300c, CD300e, or CD300f. When we tested the effect of IL-3 on CD300c expression over a short time course, we did not see any change in the first 3 hours of stimulation (Fig 1, *C*). This suggests that IL-3–mediated upregulation of CD300c expression involves transcriptional and/or translational mechanisms.

We also tested the effect of IL-3 on purified basophils and found that the expression of CD300c was induced after 18 hours, demonstrating a direct effect of IL-3 on modulation of CD300c expression on basophils (Fig 1, *D*). Nonetheless, IL-3–induced CD300c levels were lower in purified basophils, suggesting that, in addition to a direct influence, IL-3 also has effects on other cell types among PBMCs, which could indirectly affect regulation of CD300c expression on basophils.

IL-3 is a cytokine that signals through the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) pathway.[37,38] Hence we wanted to investigate whether upregulation of CD300c in response to IL-3 was also dependent on the JAK/STAT pathway. To do this, we used the JAK1/2-specific inhibitor ruxolitinib[39] over 18 hours of stimulation with IL-3. We used a concentration (1 μmol/L) that corresponds to intermediate serum levels achieved in treated patients.[40] We observed that not only was the IL-3–dependent activation of basophils inhibited (data not shown) but also upregulation of CD300c was abrogated in the presence of ruxolitinib (Fig 1, *E*), demonstrating that upregulation of CD300c in response to IL-3 is dependent on the JAK/STAT pathway.

CD300c costimulates basophil IgE-dependent degranulation and cytokine production

Once we described the regulation of CD300c expression on basophils, we wanted to study the role of this activating receptor in basophil function. It has been described previously that CD300c acts as an activating receptor in several human immune cells, such as monocytes, mast cells, and natural killer cells.[16,19,23,41] Therefore we hypothesized that CD300c could also have an activating and costimulatory role in basophils.

To determine the activation status of basophils, we analyzed the surface expression of CD63 and CD203c markers associated with anaphylactic degranulation and activation of basophils,

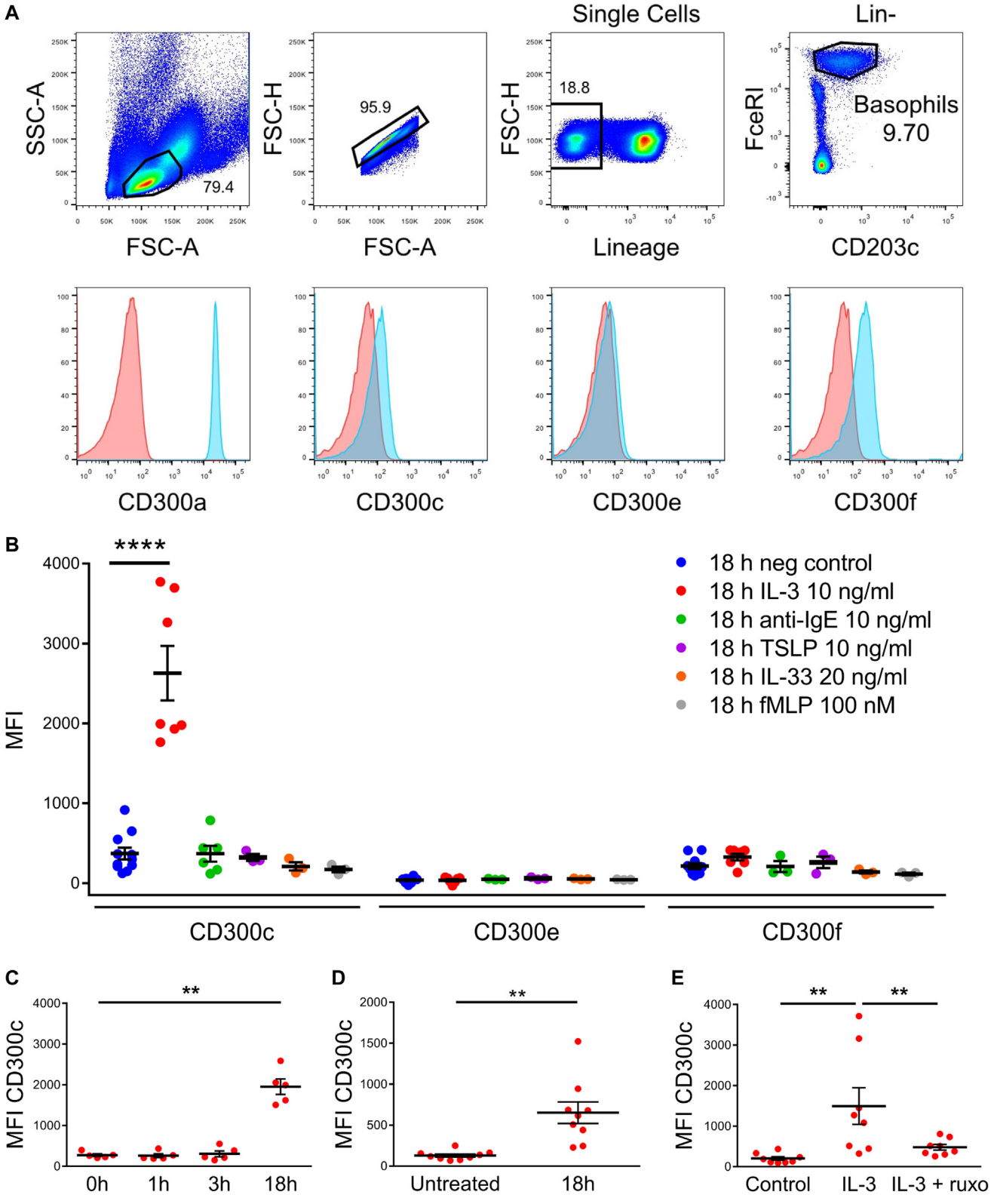

FIG 1. CD300 molecule expression on human basophils. **A,** Expression of CD300 receptors on basophils. Gating strategy is shown. *Blue histograms* represent binding of anti-CD300 mAbs, and *red histograms* represent FMO control. A representative example from data obtained from 12 to 15 healthy donors is shown. *FSC*, Forward scatter; *SSC*, side scatter. **B,** Median fluorescence intensity *(MFI)* of CD300c, CD300e, and CD300f expression on basophils in response to IL-3, anti-IgE mAb, TSLP, IL-33, and fMLP during 18 hours. Each *dot* represents a different donor, and means ± SEMs are represented. **C,** MFI of CD300c expression on basophils before and after stimulation with IL-3 (10 ng/mL) during 1, 3, and 18 hours. Each *dot* represents a different donor, and means ± SEMs are represented. **D,** MFI of CD300c expression on untreated and IL-3–treated purified basophils. Basophils were treated with 10 ng/mL IL-3 for 18 hours. Means ± SEMs are represented. Data obtained from 9 independent experiments are represented. **E,** MFI of CD300c expression on basophils before and after stimulation with IL-3 (10 ng/mL) during 18 hours in the absence and presence of ruxolitinib (*ruxo*; 1 μmol/L). Each *dot* represents a different donor. Means ± SEMs are represented. $^{**}P < .01$ and $^{****}P < .0001$.

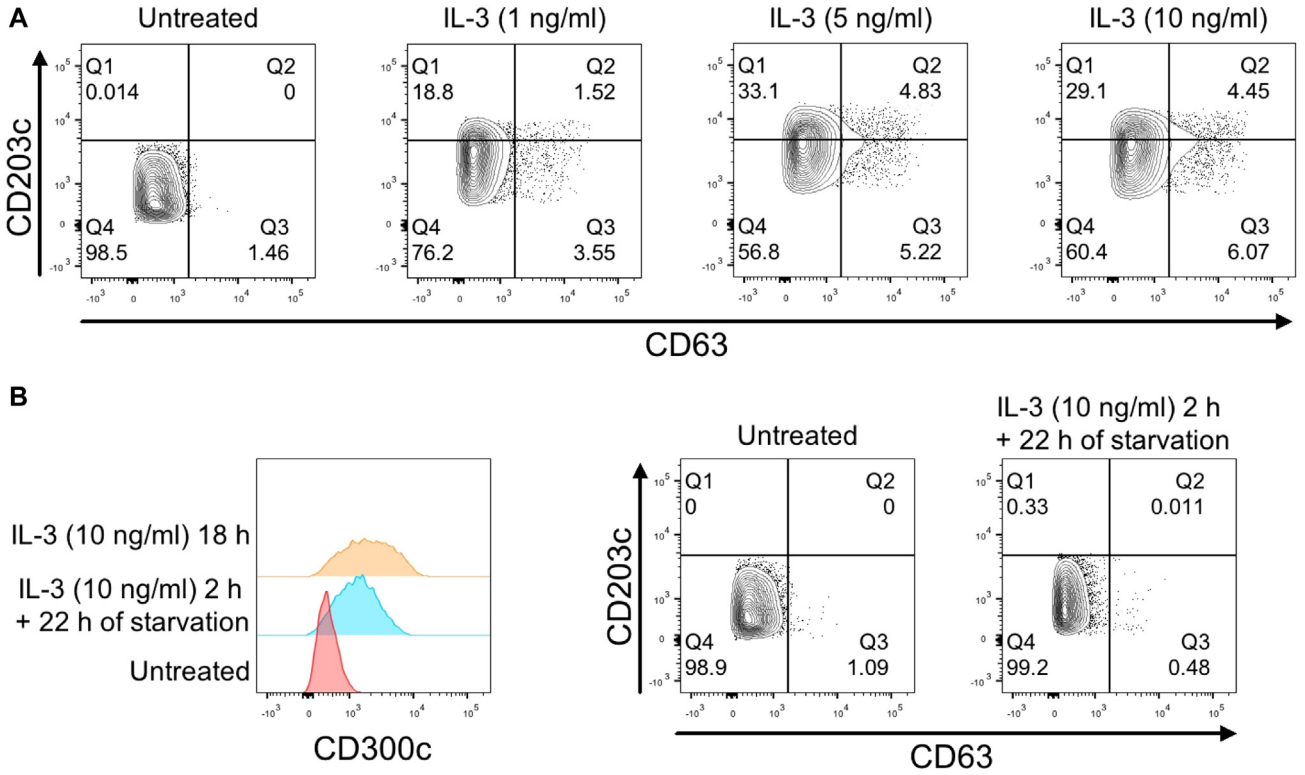

FIG 2. Priming basophils with IL-3 for 2 hours induces late CD300c expression upregulation. **A,** Contour plots representing expression of the basophil activation markers CD63 and CD203c in response to different concentrations of IL-3 during 18 hours. Contour plots are representative of data obtained from 6 independent experiments. **B,** Histograms *(left)* showing expression of CD300c on untreated *(red histogram)* and IL-3–treated basophils. *Blue histogram* shows expression of CD300c on basophils treated with 10 ng/mL IL-3 for only 2 hours, washed, and cultured for 22 hours in the absence of IL-3 (IL-3–primed basophils), and *orange histogram* shows expression of CD300c on basophils treated with 10 ng/mL IL-3 for 18 hours. Contour plots *(right)* representing expression of CD63 and CD203c of untreated and IL-3–primed basophils. Contour plots and histograms are representative of data obtained from 6 independent experiments.

respectively.[42] First, we wanted to design an experimental protocol to study the role of CD300c in basophils. As shown in Fig 1, *A* and *B*, basal expression of CD300c on basophils is relatively low and increases in response to 18 hours of stimulation with 10 ng/mL IL-3. However, that stimulus also activates basophils, as indicated by upregulation of the expression of surface molecules that are not or are barely expressed on resting basophils (ie, CD63 and CD203c; Fig 2, *A*). Our objective was to obtain high levels of CD300c while avoiding basophil activation. Thus, we first checked the effect of different concentrations of IL-3 during 18 hours of culture. Despite lower doses of IL-3 reducing the percentage of activated basophils, they did not return to a basal resting status (Fig 2, *A*).

Next, we stimulated PBMCs with 10 ng/mL IL-3 only for 2 hours, washed them thoroughly, and cultured them for 22 additional hours in the absence of IL-3. We could see that when using the latter protocol, expression levels of CD300c on basophils were very similar to those obtained when cells were stimulated with IL-3 for 18 hours, and additionally, expression of the activation markers CD203c and CD63 did not change (Fig 2, *B*). From here on, we used this protocol in which cells were first primed for 2 hours with IL-3 and then washed thoroughly and starved from IL-3 for 22 hours to study CD300c function in IL-3–primed basophils. Notwithstanding the above, on some occasions, experiments with non–IL-3–primed basophils were also performed as controls.

To analyze whether CD300c receptor contributes to the IgE-dependent basophil activation, we measured upregulation in the expression of CD63 and CD203c, as well as production of the cytokines IL-4 and IL-13, 2 interleukins produced by basophils in response to IgE-dependent activation, after stimulation of basophils with anti-IgE or anti-IgE plus IL-3 in the presence and absence of CD300c cross-linking with specific mAbs. We observed that the frequency of $CD63^+$ basophils in response to IgE-mediated stimulation was significantly greater when CD300c was cross-linked than when an isotype control mAb was used (Fig 3, *A*, upper line, and see Fig E1, A, in this article's Online Repository at www.jacionline.org), indicating a costimulatory role of CD300c in response to IgE-dependent basophil activation. We also observed an increase in CD203c expression on basophils, although it was not statistically significant, when they were stimulated through FcεRI in the presence of CD300c cross-linking (Fig 3, *A*, lower line, and see Fig E1, *B*). When we activated IL-3–primed purified basophils in an IgE-dependent manner in the presence of anti-CD300c antibodies we could still see the CD300c costimulatory role (see Fig E1, *C*). Yet, the results were not as significant as those obtained with PBMCs (Fig 3, *A*), probably because of the lower

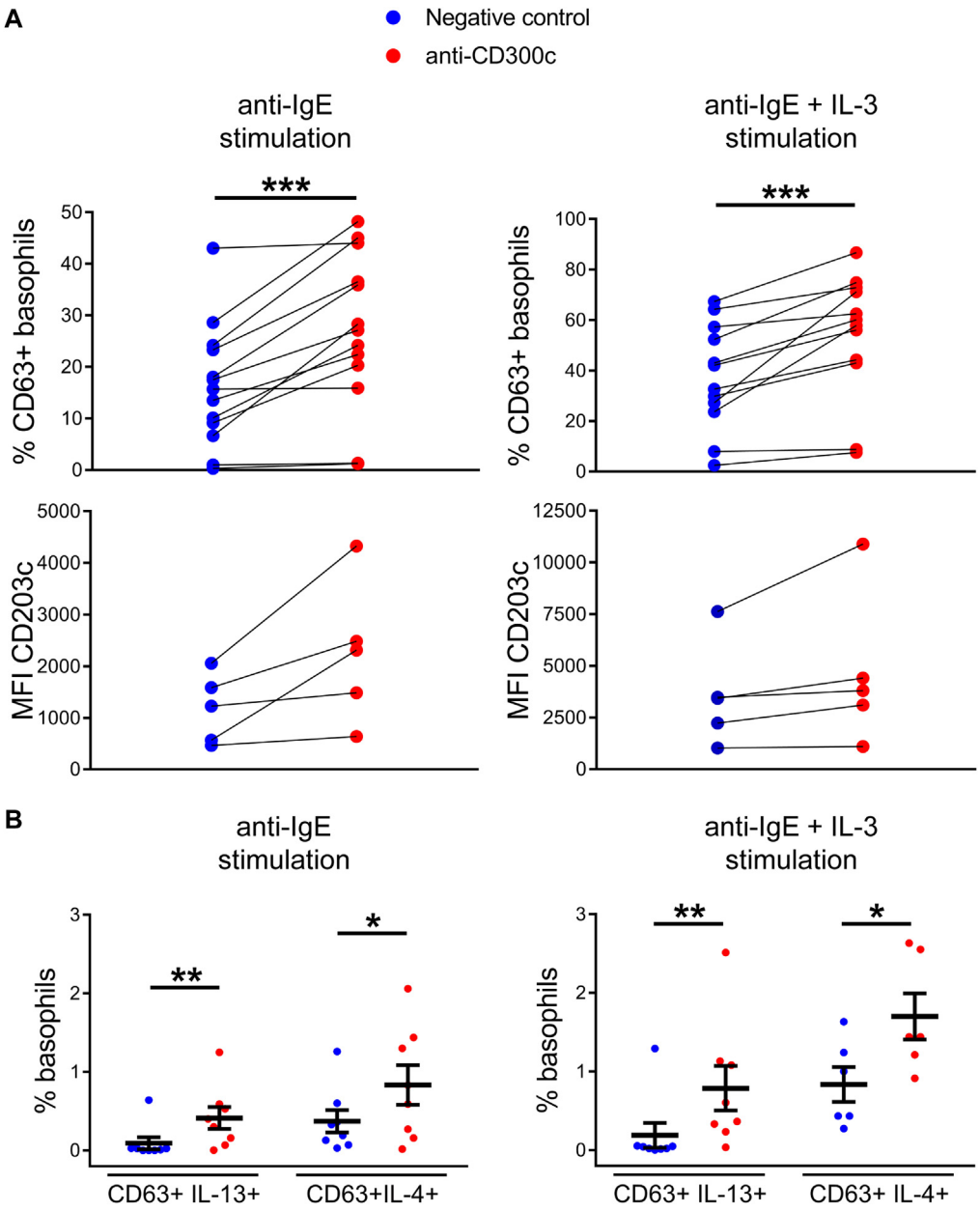

FIG 3. CD300c cross-linking enhances IgE-dependent basophil activation marker expression and cytokine production. **A,** Dot graphs show percentages of $CD63^+$ basophils *(upper line)* and median fluorescence intensity *(MFI)* of CD203c *(lower line)* in the presence *(red dots)* and absence *(blue dots)* of cross-linking of CD300c after stimulation with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). Each *pair of dots* represents a different experiment. Data obtained from 12 independent experiments are represented. **B,** Dot graphs show percentages of $CD63^+IL\text{-}13^+$ and $CD63^+IL\text{-}4^+$ basophils in the presence *(red dots)* and absence *(blue dots)* of CD300c cross-linking after stimulation with anti-IgE (10 ng/mL; *left graph*) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL; *right graph*). Each *pair of dots* represents a different experiment. Data obtained from 8 independent experiments and means ± SEMs are represented. $*P < .05$, $**P < .01$, and $***P < .001$.

levels of CD300c expression on IL-3–primed purified basophils (Fig 1, *D*, and see Fig E1, *D*). In addition, a higher percentage of $CD63^+IL\text{-}13^+$ and $CD63^+IL\text{-}4^+$ basophils was observed in response to IgE-mediated stimulation in the presence of cross-linking of CD300c (Fig 3, *B*). Taken together, these results indicate that the CD300c receptor acts as a costimulator in IgE-dependent basophil activation processes, inducing not only the degranulation of basophils but also the production of at least 2 key cytokines for development of allergic inflammation.

CD300c costimulation strengthens FcεRI-mediated activation of signaling intermediates in basophils

Once we demonstrated that CD300c has a costimulatory role in the FcεRI-mediated activation of basophils, we studied the molecular basis of this influence. We analyzed whether CD300c cross-linking was able to modulate the IgE/FcεRI-mediated signaling pathway in human basophils from peripheral blood.

During the first steps of the FcεRI signaling cascade, there is recruitment of the protein tyrosine kinase Syk to the

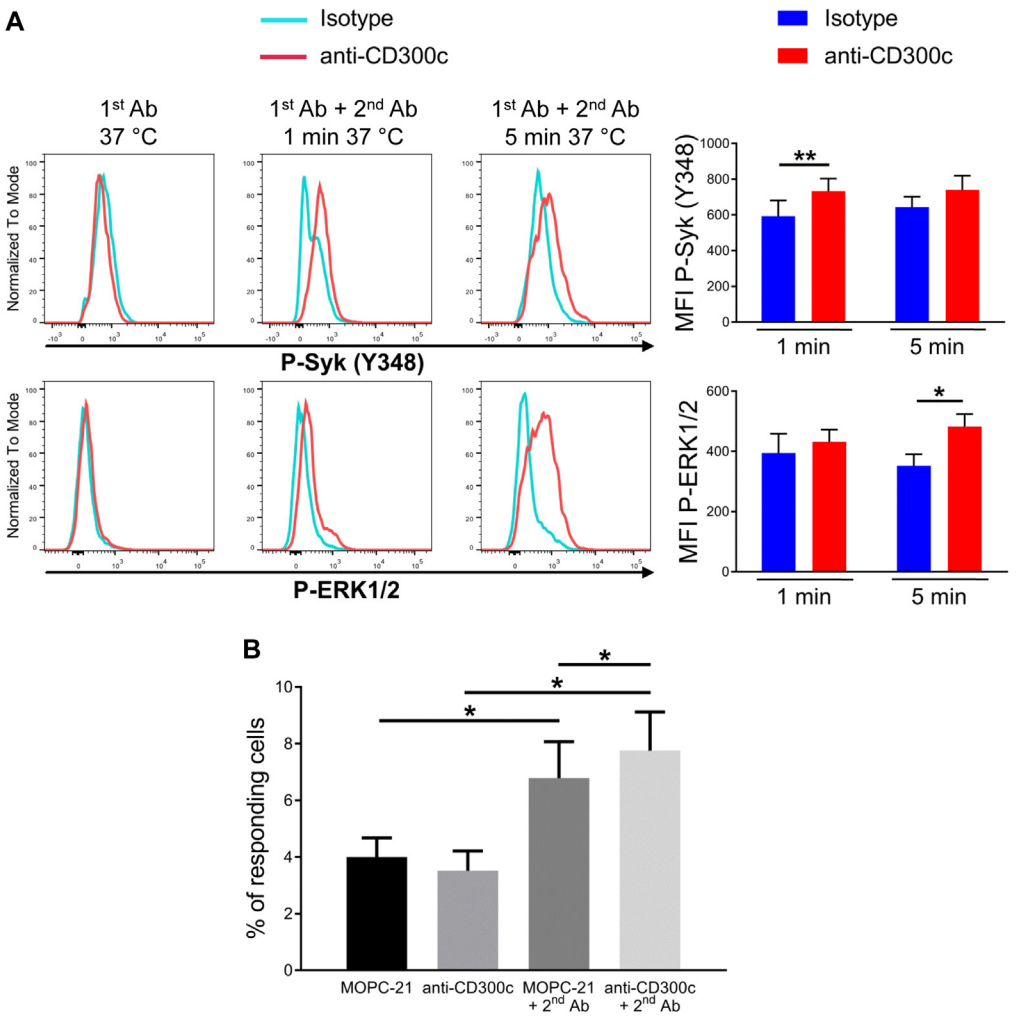

FIG 4. CD300c stimulation strengthens FcεRI-mediated activation of signaling intermediates. **A,** Representative histograms showing phosphorylation levels of Syk (*P-Syk*; *upper line*) and phosphorylation levels of Erk (*P-ERK1/2*; *lower line*) in basophils in which the primary mAbs anti-CD300c (in red) or isotype-matched control MOPC-21 (in blue) were added and then not cross-linked *(left column)* or cross-linked with secondary antibody *(Ab)* for 1 *(middle column)* or 5 *(right column)* minutes. Bar graphs show MFIs of P-Syk *(upper graph)* and P-ERK1/2 *(lower graph)* in basophils in which the primary mAbs anti-CD300c (in red) or isotype-matched control (in blue) were added and then cross-linked with secondary antibody for 1 or 5 minutes. Data are from 9 independent experiments, and means ± SEM are represented. *MFI*, Median fluorescence intensity. $*P < .05$ and $**P < .01$. **B,** Basophil Ca^{++} mobilization. FcεRI-positive cells were first acquired for 30 seconds, at which point the primary mAb anti-CD300c or isotype-matched control were added. Then, the primary mAbs were cross-linked with secondary antibody *(Ab)*, and fluorescence was measured. Percentages of responding cells from 60 to 210 seconds were measured. Data are from 6 independent experiments, and means ± SEMs are represented. $*P < .05$.

phosphorylated tyrosine residues in the ITAMs of the β and γ subunits of FcεRI. Then Syk becomes activated and initiates the signaling cascade that involves phosphorylation and activation of several downstream proteins, such as mitogen-activated protein kinases, and release of Ca^{2+} from internal stores.[43-45]

To have an idea of the kinetics of the FcεRI signaling pathway in the presence and absence of CD300c cross-linking, we measured phosphorylation levels of Syk, which is an early event in the FcεRI signaling cascade, and phosphorylation of extracellular signal-regulated kinase (Erk) 1/2, which appears more downstream in the pathway. We saw that phosphorylation levels of the intermediates Syk and Erk1/2 were significantly greater in the presence of CD300c cross-linking after 1 and 5 minutes of FcεRI-mediated stimulation, respectively (Fig 4, *A*).

We also investigated the ability of CD300c to modulate intracellular calcium mobilization. In basophils that are activated through FcεRI, engagement of CD300c with soluble anti-CD300c mAbs, followed by cross-linking with anti-mouse IgG $F(ab')_2$, induced a transient and rapid increase in intracellular calcium levels that was significantly greater than that observed when cells were stimulated with an isotype-matched control (Fig 4, *B*).

Altogether, these results demonstrate that CD300c has a costimulatory role in the activation of the FcεRI downstream signaling pathway, which is mediated in part by an increase in

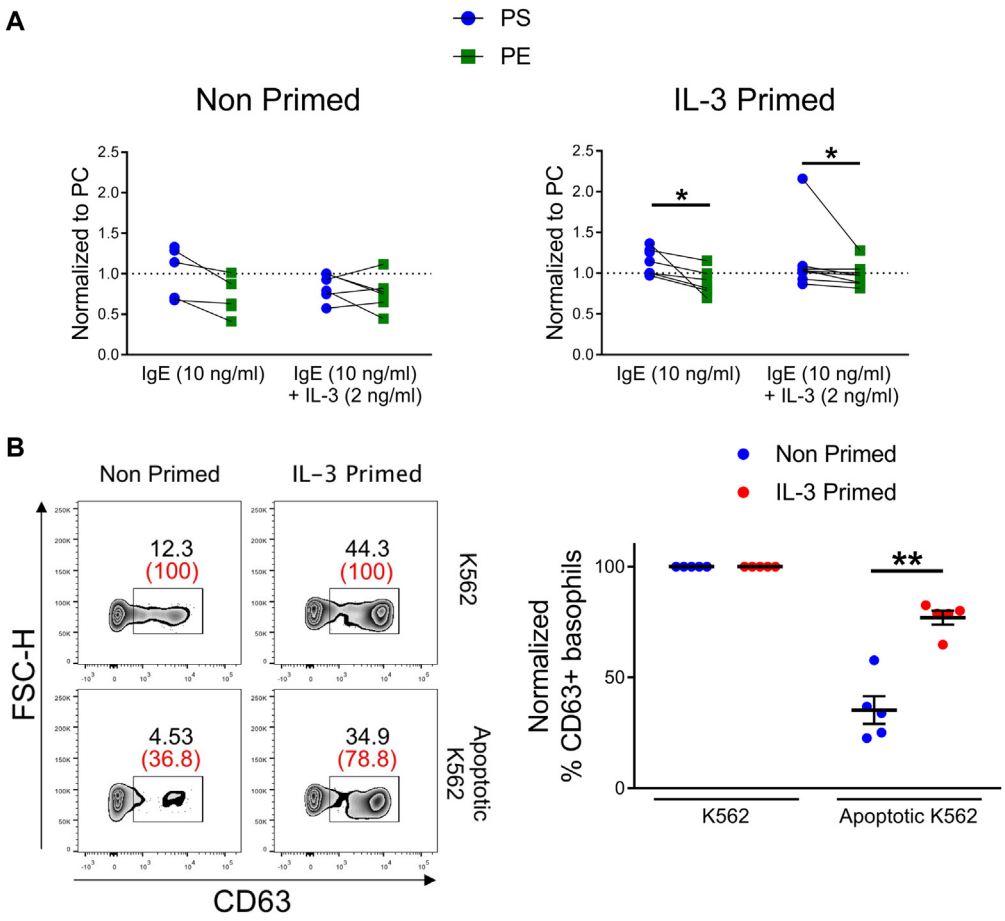

FIG 5. PS and PE modulate IgE-dependent basophil activation. **A,** Relative values of basophil activation in response to anti-IgE and anti-IgE plus IL-3 in the presence of PS *(blue dots)* and PE *(green dots)*. Values of relative activation were measured by dividing the percentage of $CD63^+$ cells in the presence of PS and PE by the percentage of $CD63^+$ cells in the presence of the control PC. Results of non–IL-3–primed *(left panel)* and IL-3–primed *(right panel)* basophils are shown. *Values above and below the dotted line* indicate that the percentage of $CD63^+$ basophils was higher and lower, respectively, than the percentage obtained in the presence of the PC phospholipid control. **B,** *Left,* Zebra plot showing a representative experiment of percentage of $CD63^+$ basophils (numbers in black) in response to anti-IgE plus IL-3 in the presence of nonapoptotic and apoptotic K562 cells. Normalized values are shown in red and parentheses. *Right,* Dot graph showing normalized percentage of $CD63^+$ basophils. Results of non–IL-3–primed (blue) and IL-3–primed (red) basophils are shown. Results were normalized to the percentage of $CD63^+$ basophils obtained in the presence of nonapoptotic K562 cells. Means ± SEMs are represented. $*P < .05$ and $**P < .01$.

FcεRI-mediated intracellular calcium mobilization and Syk and Erk1/2 activation.

PS and PE binding to CD300c modulate IgE-dependent basophil activation

As it has been demonstrated by us and others,[19,23] the binding affinity of CD300a and CD300c to PS is very similar, whereas CD300a has a higher affinity for PE than CD300c. We wanted to explore the IgE-dependent costimulatory role of CD300c on basophils by stimulating them in the presence of CD300c natural ligands (ie, PS and PE). The phospholipid phosphatidylcholine (PC) served as a negative control. On the one hand, IL-3–primed and nonprimed basophils were incubated in lipid-coated plates and were also stimulated with anti-IgE or anti-IgE plus IL-3. After 2 to 3 hours, cells were stained with the appropriate mAbs to measure activation levels of basophils. We saw that in non–IL-3–primed cells IgE/FcεRI-mediated activation of basophils was lower, especially when activated in the presence of PE, compared with activation levels observed in the presence of control PC (Fig 5, *A*, and see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). These results suggest that in the absence of high levels of CD300c, the ligands PS and PE generally induced an inhibitory effect on IgE/FcεRI-dependent basophil degranulation, probably because of their binding mostly to the CD300a inhibitory receptor. Interestingly, in IL-3–primed basophils, in which CD300c expression was increased significantly (Fig 2, *B*), the decrease in the percentage of $CD63^+$ basophils after FcεRI-mediated activation in the presence of PS and PE was not so marked compared with that in the presence of PC (Fig 5, *A*, and see Fig E2, *B*). This suggests that greater levels of CD300c counteract the inhibitory role of CD300a. Moreover, it was very interesting to see that in IL-3–primed basophils costimulation with PS resulted in greater

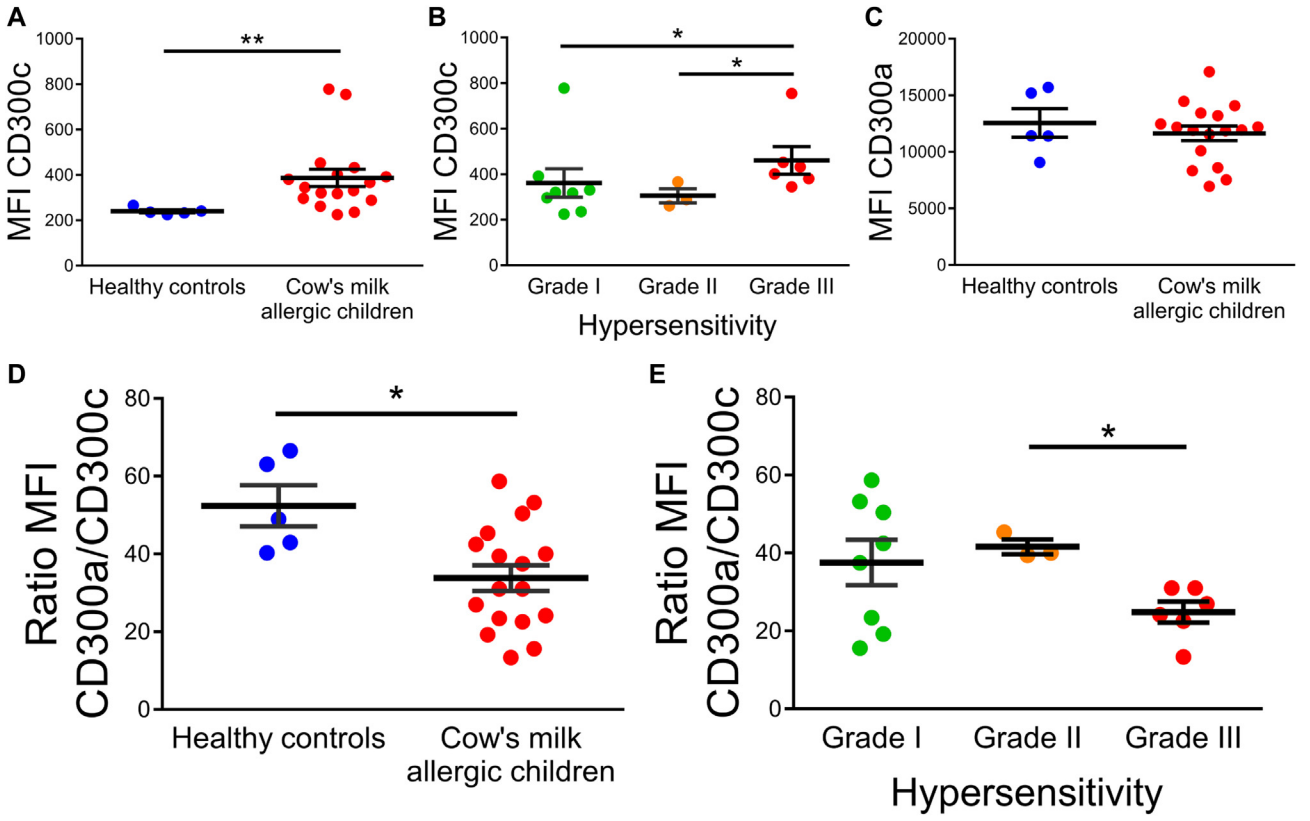

FIG 6. Basophils from children with cow's milk allergy exhibit greater expression of CD300c. **A,** Dot graph showing basal median fluorescence intensity *(MFI)* of CD300c on basophils from healthy children (blue) and children with cow's milk allergy (red). **B,** Dot graph showing basal MFI of CD300c on basophils in children with cow's milk allergy classified by the severity of the symptoms of hypersensitivity as grade I (green), grade II (orange), and grade III (red). **C,** Dot graph showing basal MFI of CD300a on basophils from healthy children (blue) and children with cow's milk allergy (red). **D,** Dot graphs representing MFI CD300a/CD300c ratio on basophils from healthy children and children with cow's milk allergy. **E,** Dot graphs representing the MFI CD300a/CD300c ratio on basophils from children with cow's milk allergy classified by the severity of the symptoms of hypersensitivity as grade I (green), grade II (orange), and grade III (red). Each *dot* represents a donor, and means ± SEMs are shown. $*P < .05$ and $**P < .01$.

basophil activation compared with activation levels obtained with the control lipid PC, whereas in the presence of PE, which has a greater affinity for the CD300a inhibitory receptor, there was still an inhibitory effect on basophil activation, although at lower levels than in nonprimed cells (Fig 5, *A*).

We also stimulated basophils in the presence of nonapoptotic or apoptotic K562 cells, which expose PS and PE in the outer leaflet of the plasma membrane. We saw, as has been described previously by Sabato et al,[35] a significant decrease in the frequency of $CD63^+$ cells in nonprimed IgE/FcεRI-activated basophils activated in the presence of apoptotic cells. However, in IL-3–primed basophils expressing greater levels of CD300c, the decrease in the frequency of $CD63^+$ basophils in the presence of apoptotic cells was significantly lower (Fig 5, *B*).

Taken together, these results led us to conclude that the interaction of PE with CD300a is predominant over the interaction with CD300c, which leads to an inhibitory signal. However, importantly, cross-linking of CD300c by PS on activated basophils counteracts and even overrides cross-linking of CD300a, resulting several times in a net positive signal and therefore decreasing the threshold of basophils FcεRI-mediated activation.

Allergic subjects displayed a significantly greater expression of CD300c

It has been described before that basal expression of the CD300a inhibitory receptor is lower in basophils of patients with birch pollen allergy than in healthy donors.[33] Here we compared the expression of CD300c on basophils in healthy children with those from children with cow's milk allergy. Clinical data of allergic patients, which consist mainly of the degree of hypersensitivity symptoms and levels of specific IgE, are shown in Table I. In Table II data of healthy children are shown. We saw that basal expression of CD300c is significantly greater in allergic children than in healthy control subjects (Fig 6, *A*). Importantly, we observed that CD300c expression increases with the severity of the symptoms of hypersensitivity in allergic children, especially in children with more severe symptomatology (Fig 6, *B*). We verified the costimulatory role of CD300c in fresh and nonprimed basophils from allergic patients (see Fig E3 in this

article's Online Repository at www.jacionline.org). We did not see significant differences between healthy and allergic children in the CD300a inhibitory receptor expression on basophils (Fig 6, *C*). However, if we calculate the CD300a/CD300c ratio, we can observe a significant decrease in this ratio in patients with more severe symptoms (Fig 6, *D* and *E*). A lower CD300a/CD300c ratio could be interpreted as a lower basophil activation threshold. Altogether, these results suggest that the CD300c molecule might have a role in modulating the activation threshold of basophils in allergic patients and possibly in the clinical manifestations of hypersensitivity.

DISCUSSION

Basophils are one of the target cells that have been shown to participate in allergic diseases through their ability to bind IgE. Because human basophils express high levels of FcεRI, blocking their interaction with IgE has been a main goal in antiallergic therapy. In this context, clinical responses after administration of the anti-IgE mAb omalizumab to patients with food allergy that correlated with basophil suppression have been described, as measured by using the allergen-induced basophil activation test. Clinical trials with omalizumab in patients with food allergy, including milk allergy, resulted in achieving tolerance to higher amounts of the allergen in some patients.[46-48] In addition, cell-surface activating and inhibitory receptors, such as CD48 and CD300a, respectively, hold promising therapeutic possibilities based on preclinical studies.[11,49] In this line inhibition of activating receptors, such as CD300c, can prevent development of allergic reactions by increasing the threshold of IgE-mediated basophil activation.

Several *in vivo* mouse models have demonstrated the role of certain CD300 molecules (ie, CD300a and CD300f) in the regulation of activation of key effector cells, such as mast cells, in allergic responses. Furthermore, genetic approaches and use of bispecific antibody fragments against CD300a have shown the potential of these molecules as therapeutic targets.[26,49-51] However, less is known about the role of human CD300 molecules in controlling activation status of basophils, eosinophils, and mast cells during allergic responses. Specifically for basophils, only the ability of the CD300a inhibitory receptor to downregulate IgE-mediated activation of these cells has been described.[33-35] Here, for the first time, we show that basophils express not only the CD300a inhibitory receptor but also CD300f and the CD300c activating receptor at lower levels. Our results differ from those reported by Takahashi et al.[23] We believe that modifications in the experimental setup, including staining strategies and using different mAbs, could explain the differences between our results and those published by Takahashi et al.

Apart from the phenotypic analysis, the present study has addressed the question of whether CD300c activating receptor expressed in human basophils works as a costimulatory receptor for FcεRI-mediated signals. Our results have provided evidence that CD300c increases degranulation, cytokine production, intracellular calcium mobilization, and phosphorylation of signaling intermediates induced by FcεRI ligation alone. The costimulatory role of CD300c was demonstrated by ligation with specific mAbs and by the ability of its 2 natural ligands, PS and PE, to modulate IgE-mediated basophil activation. Supporting the costimulatory role of CD300c, we have also found that its expression in children with cow's milk allergy is significantly increased compared with that in healthy control subjects and that it correlates with symptom severity.

IL-3 is a very important cytokine for the development, maturation, and survival of basophils.[52-55] Furthermore, IL-3 markedly increases the activation and release of mediators from basophils in response to cross-linking of FcεRIα through IgE[54-56] and also activates them in an IgE-independent manner.[57,58] We have shown that IL-3 is the only tested stimulus able to upregulate CD300c expression on human basophils. IL-3–induced expression of CD300c was apparent after 18 to 24 hours of stimulation, somehow suggesting that it involves transcriptional and translational mechanisms. Other tested stimuli, including anti-IgE mAb, did not have any effect on CD300c cell-surface levels. This is in contrast to the fast upregulation (3 minutes) of CD300a inhibitory receptor expression in IgE/FcεRI-activated basophils,[33] which probably indicates that there is an intracellular pool of CD300a ready to go to the cell surface after stimulation, in a similar manner as observed in human neutrophils.[59] Given our results, we propose that one of the mechanisms through which IL-3 increases basophil activation is by inducing CD300c activating receptor expression, which has a costimulatory role during IgE-mediated activation.

Signaling cascades initiated from cell-surface receptors modulate the activation threshold of basophils. Previously, it was described that CD300a inhibits IgE-mediated basophil activation,[33-35] and here we have shown that CD300c has a costimulatory role. Human CD300a and CD300c are paired receptors that possess highly homologous sequences in the extracellular domain and have opposing functions in immune regulation, which is explained by the presence of ITIM motifs in the intracellular tail of CD300a and by association of CD300c with adaptor proteins containing ITAM motifs.[12,15]

We and others have demonstrated previously the recognition of the same ligands (ie, PS and PE) by CD300a and CD300c receptors, although with different affinities.[19,22,23] CD300a exhibits a stronger binding to PE than CD300c. On the other hand, both CD300a and CD300c exhibit similar binding affinity to PS. In human basophils our results indicate that in the presence of the natural ligands PS and PE, the CD300a-mediated inhibitory signal is predominant, although the degree of this preponderance depends on the expression levels of CD300c and priming of cells with IL-3. This is particularly evident when IL-3–primed basophils were IgE stimulated in the presence of PS, resulting in a greater activation when compared with the control lipid PC. Even in the presence of PE, we observed that the inhibition was lower when basophils were primed with IL-3 and expressed higher levels of CD300c compared with non–IL-3–primed cells. Similar results were observed when basophils were stimulated in the presence of apoptotic cells. This led us to propose that the interplay between the paired receptors CD300a and CD300c has an important role in determining the threshold of IgE-mediated activation of basophils. This notion might have relevance in disease situations, where levels of the CD300a inhibitory receptor are diminished and levels of the CD300c activating receptor are increased. Good examples of such clinical conditions are the description of low levels of CD300a inhibitory receptor expression on basophils from patients allergic to birch pollen[33] and high levels of CD300c activating receptor on

basophils from children with cow's milk allergy (Fig 6). The relationship between the inhibitory and activating capabilities of CD300a and CD300c, respectively, might also be important in the success of allergen-specific immunotherapy because previous studies have shown that there is an increase in the number of apoptotic monocytes expressing the ligands of CD300a and CD300c (ie, PS and PE) early in the induction of protective and tolerogenic allergen-specific immunotherapy.[60]

Food allergy is characterized by altered responses and the development of immediate hypersensitivity reactions to ingested foods. IgE, basophils, and mast cells have an important role in driving immediate hypersensitivity reactions, with the consequent release of vasoactive mediators in response to food allergens.[61] Here we demonstrate that CD300c increases IgE-mediated basophil degranulation. Importantly, our data with a cohort of 17 children allergic to cow's milk have shown that there is a correlation between expression levels of CD300c on basophils and the severity of hypersensitivity symptoms,[36] especially in children with more severe symptomatology. These symptoms are the clinical indicator of granule release, suggesting that CD300c might have a role in the clinical manifestations of allergic reactions by decreasing the activation threshold of basophils. However, it would be very important to study a larger cohort of patients allergic to cow's milk and other allergens to obtain a definitive conclusion on the role of CD300c receptor in allergy. Moreover, it would be interesting to analyze IL-3 levels in sera of allergic subjects. They can be increased in allergic patients, which could be the reason for which the expression of CD300c appears increased in basophils from allergic children.

In addition, basophil activation through IgE/FcεRI also leads to a secondary late-phase reaction characterized by cytokine production. Basophils are a main source of the T_H2 cytokines IL-4 and IL-13, which are crucial for isotype class-switching to IgE and amplification of T_H2 responses that in turn aggravate chronic allergic inflammation.[6,62] Also, IL-13 produced by basophils in response to IgE/FcεRI-mediated activation might have a role in the development of intestinal inflammation.[63] Our results indicate that CD300c-mediated signals are able to diminish the threshold of basophils activation through IgE/FcεRI, increasing the production of IL-4 and IL-13 and indicating a possible role of CD300c in the maintenance of allergic inflammation.

Taken together, our results led us to conclude that the interaction between PS and PE with CD300c costimulates IgE/FcεRI-dependent basophil degranulation. Nonetheless, more studies, especially in the context of allergic and inflammatory diseases, are required to completely understand the biological relevance of these findings. The clinical relevance is shown by the high expression of CD300c on basophils from children with cow's milk allergy, although a larger cohort of patients is required to confirm these results. Our data provide interesting contributions to study the role of phospholipid recognition in the modulation of basophil responses and endorse the possible role of CD300c as a biomarker of disease activity and as a pharmacologic target for the treatment of allergic diseases and possibly for other pathologic situations, such as autoimmunity.

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Key message

- **CD300c decreases the IgE-mediated activation threshold, and its expression is increased in children with cow's milk allergy.**

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METHODS

Cell isolation, purification, and culture

Adult PBMCs were obtained by using Ficoll density centrifugation from buffy coats. Isolated PBMCs and purified basophils (>95% of basophils) were cultured in Iscove modified Dulbecco medium (Thermo Fisher Scientific, Waltham, Mass) supplemented with 10% human AB serum (Invitrogen, Carlsbad, Calif) and 1% GlutaMAX (Thermo Fisher Scientific), hereafter referred to as cIMDM. Basophil enrichment was carried out by using a negative selection method with the Human Basophil Isolation Kit II from Miltenyi Biotec (Bergisch Gladbach, Germany). Fresh whole blood samples from children were collected in sodium citrate–containing tubes and used only for determination of surface expression of CD300c on basophils.

Antibodies and reagents

The following anti-human mAbs were used for flow cytometric analysis: fluorescein isothiocyanate anti-CD63 (clone H5C6), fluorescein isothiocyanate Lineage Cocktail 3 (lin 3; CD3, CD14, CD19, and CD20), Brilliant Violet 412 anti-CD123 (clone 9F5), and peridinin-chlorophyll-protein (PerCP)–Cy5.5 anti–HLA-DR (clone G46-6) from BD Biosciences (San Jose, Calif); phycoerythrin anti-CD203c (clone 97A6) and phycoerythrin anti-CD300a (clone E59.126) from Beckman Coulter (Fullerton, Calif); allophycocyanin (APC) anti-CD300e (clone UP-H2) and phycoerythrin and APC anti-CD300f (clone UP-D2) from Miltenyi Biotec; eFluor660 anti-CD300c (clone TX45), phycoerythrin-Cy7 anti-FcεRI (clone AER-37), PerCP-eFluor710 anti–phosphorylated ERK1/2 (clone MILAN8R), and APC anti-P-Syk (Y348; clone moch1ct) from eBioscience (San Diego, Calif); and Brilliant Violet 421 anti-human IL-4 (clone MP4-25D2), PerCP-Cy5.5 anti-human IL-13 (clone JES10-5A2), purified anti-CD300c (clone TX45), and purified isotype control (clone MOPC-21) from BioLegend (San Diego, Calif).

The following reagents were also used: human recombinant IL-3, human TSLP, and human recombinant IL-33 from Miltenyi Biotec; human anti-IgE from Abbiotec (San Diego, Calif); fMLP from Sigma (St Louis, Mo); 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS or PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE or PE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC or PC) from Avanti Polar Lipids (Alabaster, Ala); BD GolgiStop Protein Transport Inhibitor (monensin), BD GolgiPlug Protein Transport Inhibitor (brefeldin A), BD Cytofix/Cytoperm Plus Kit, BD Cytofix Fixation Buffer, BD Phosflow Perm Buffer III, and BD Lysing buffer from BD Biosciences; Fluo-4 cell permeant from Thermo Fisher Scientific; and ruxolitinib and camptothecin from Selleckchem (Houston, Tex).

Flow cytometric analyses

Adult PBMCs and purified basophils were washed with staining buffer containing PBS and 1% BSA to block Fc receptors and stained with the respective fluorochrome-conjugated mAbs for 30 minutes on ice. For intracellular staining, cells were permeabilized after extracellular staining with the BD Cytofix/Cytoperm Plus Kit, according to the manufacturer's protocol, and incubated with different labeled mAbs for the detection of intracellular cytokines. Then, cells were washed to remove unbound mAbs and further acquired in a FACSCanto II Flow cytometer (BD Biosciences). Whole blood from healthy children and children with cow's milk allergy was used to determine CD300c expression. Briefly, 100 μL of whole blood was stained with the respective fluorochrome-conjugated mAbs for 20 minutes at room temperature. Next, red blood cells were lysed with BD FACS Lysing buffer for 15 minutes at room temperature. Then, cells were washed to remove unbound mAbs and further acquired in a FACSCanto II Flow cytometer (BD Biosciences). We used fluorescence minus one (FMO) control, which contains all the fluorochrome-conjugated mAbs in a panel, except for the one that is being measured. FMO control is used to identify and gate cells in the context of data spread caused by the multiple fluorochromes in a given panel. Flow cytometric data were analyzed with FlowJo software, version 10.0.7 (TreeStar, Ashland, Ore).

Functional experiments

For functional experiments, nonprimed and IL-3–primed PBMCs and purified basophils were used. For priming cells with IL-3, freshly isolated PBMCs were cultured in cIMDM and stimulated with IL-3 for 2 hours. Then, they were washed thoroughly and starved from IL-3 for 22 hours. Then, functional experiments were performed.

Calcium mobilization assay. IL-3–primed cells were washed and resuspended in RPMI 1640 containing 10% FBS (5×10^6 cells/mL). Next, cells were labeled with Fluo-4 (2 μg/mL) from Life Technologies (Grand Island, NY) for 30 minutes at 30°C while protected from light, adding the PE-Cy7–conjugated anti-FcεRI mAb during the last 15 minutes for basophil identification. Then, cells were washed twice and resuspended at 2×10^6 cell/mL. Aliquots of 1 mL were warmed at 37°C for 5 minutes, followed by acquisition in a FACSCanto II flow cytometer.

To establish a baseline, cells were first acquired for 30 seconds without stimuli, and then 5 μg of anti-CD300c mAb or isotype control was added and acquired for 30 seconds, followed by cross-linking with the addition of 8.5 μg of goat anti-mouse IgG $F(ab')_2$. Cells were further acquired for 6 more minutes. Basophils were electronically gated based on their forward- and side-scatters properties and FcεRI expression. The percentage of responding cells was determined by electronically gating basophils that had higher Fluo-4 fluorescence intensity than the baseline between 60 seconds (after cross-linking of receptors) and 210 seconds once cytoplasmic Ca^{++} levels reached basal values. Data were analyzed with FlowJo software, version 7.6.5 and 10.0.7 (TreeStar).

Phosphorylation assay. IL-3–primed PBMCs were washed with PBS and stained for the surface markers FcεRI and CD203c for 15 minutes in cIMDM. Then, cells were resuspended at 10^6 cells/mL and cross-linked with either 5 μg of anti-CD300c or isotype control, followed by addition of 8.5 μg of goat anti-mouse IgG $F(ab')^2$ for 1 and 5 minutes at 37°C. After the incubation time, cells were harvested and washed with PBS before fixing them with the BD Cytofix buffer at 37°C for 10 minutes. Cells were washed again 2 times and permeabilized with the BD Phosflow Perm buffer III for 30 minutes on ice, and then they were incubated for 30 minutes on ice with specific labelled phospho-mAbs for detection of phosphorylated intermediates. Finally, cells were washed to remove unbound mAbs and further acquired in a FACSCanto II Flow cytometer. FMO control was determined. Data were analyzed with FlowJo software.

Cross-linking of CD300c and measurement of activation markers and cytokine production. Culture plates (24 wells) were coated with 5 μg of either anti-human CD300c or isotype control at 10 μg/mL concentrations for 2 to 3 hours at 37°C. Adult nonprimed and IL-3–primed PBMCs and purified basophils were then added to the mAb-coated plates (2×10^6 cells/mL) in cIMDM. PBMCs and purified basophils were also stimulated with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). For measurement of activation markers, after 2 to 3 hours of stimulation, cells were stained with appropriate mAbs against CD203, CD63, and FcεRI surface receptors. To measure cytokine production, PBMCs were incubated at 37°C for 6 hours in the presence of BD GolgiStop (monensin) and BD GolgiPlug (Brefeldin A), and cells were harvested and stained with different labeled mAbs for the detection of activation markers and cytokine production, as described above. Sample acquisition and data analysis were carried out, as described previously.

Lipid stimulation experiments. For lipid stimulation experiments, the lipids PC, PS, and PE were diluted in 100% methanol, plated in 24-well plates at 5 μg/mL, and air-dried for 2 to 3 hours. Later, the IL-3–primed and nonprimed PBMCs (2×10^6 cells/mL) were added to the lipid-coated plates and also stimulated with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). After 2 to 3 hours, cells were stained with appropriate mAbs against CD203, CD63, and FcεRI surface receptors to measure basophil activation.

Stimulation of basophils in the presence or absence of apoptotic K562 cells. K562 human erythroleukemic cells (5×10^5 cells/mL) were cultured overnight at 37°C in the presence of

3 μmol/L camptothecin to induce apoptosis in K562 cells. Induction of apoptosis was determined by using Annexin V staining with a flow cytometer. The next day, IL-3–primed and nonprimed PBMCs (5×10^5 cells/well) were cocultured with normal or apoptotic K562 cells (10^7 cells/well; ratio 1:20) in 48-well plates and stimulated with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL) in a final volume of 1 mL. After 2 to 3 hours, cells were stained with appropriate mAbs against surface receptors, CD63, and FcεRI to measure basophil activation.

Statistical analysis and graphic representation

GraphPad Prism software (version 6.01; GraphPad Software, La Jolla, Calif) was used for graphic representation and statistical analysis. Data were represented in dot plot graphs and bar graphs showing means ± SEMs. Values obtained from different experiments were compared with the nonparametric Wilcoxon matched-pairs signed-rank test. For comparison between healthy and allergic children, we removed from the analysis the outliers identified with the robust regression and outlier removal (ROUT) method, and then the nonparametric unpaired Mann-Whitney rank test was used.

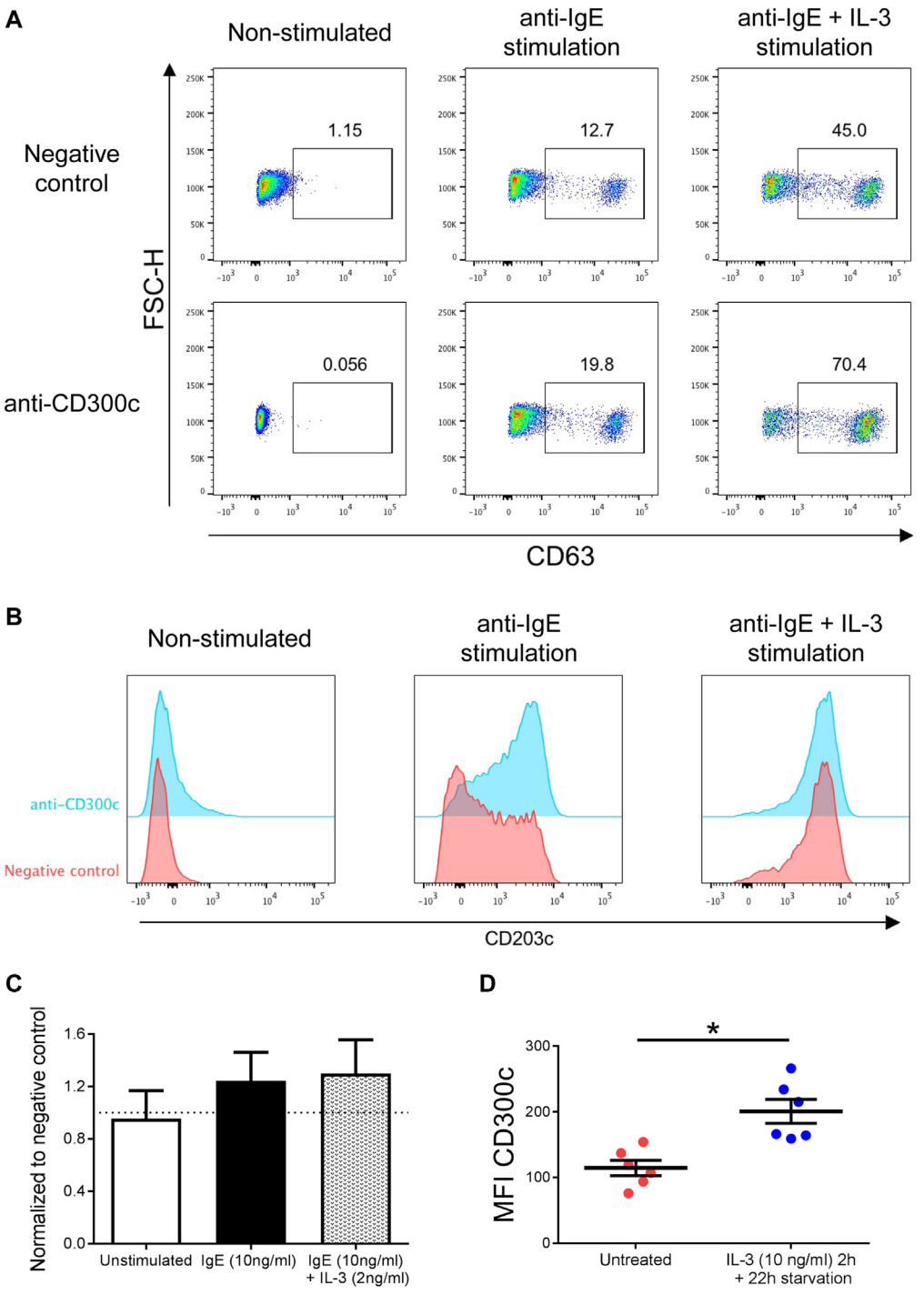

FIG E1. CD300c cross-linking enhances IgE-dependent basophil activation marker expression. **A,** Percentages of $CD63^+$ basophils in the presence and absence of cross-linking of CD300c in nonstimulated conditions and after stimulation with anti-IgE or anti-IgE plus IL-3. *FSC*, Forward scatter. **B,** Representative histograms showing expression of CD203c in the presence (blue) and absence (red) of cross-linking of CD300c in nonstimulated conditions and after stimulation with anti-IgE or anti-IgE plus IL-3. **C,** Bars graph showing relative values of basophils activation in response to anti-IgE and anti-IgE plus IL-3 in the presence of anti-CD300c mAb. Values of relative activation were measured by dividing the percentage of $CD63^+$ cells in the presence of anti-CD300c mAb by the percentage of $CD63^+$ cells in the presence of the isotype control. Results using IL-3–primed purified basophils are shown. *Values above and below the dotted line* indicate that the percentage of $CD63^+$ basophils was higher and lower, respectively, than the percentage obtained in the presence of the isotype control. Means ± SEMs are represented. **D,** Dot graph showing the median fluorescence intensity *(MFI)* of CD300c on untreated (red) and IL-3–primed (blue) purified basophils. Means ± SEMs are shown. $*P < .05$.

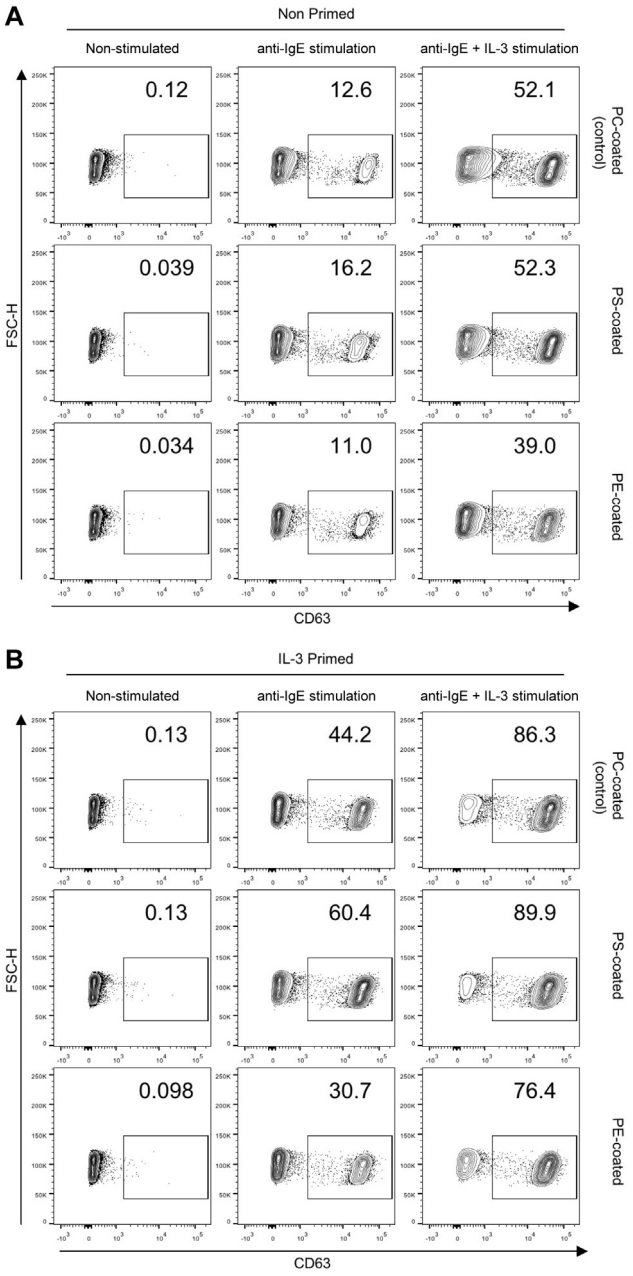

FIG E2. PS and PE modulate IgE-dependent basophil activation. Representative contour plots showing percentages of $CD63^+$ basophils in non–IL-3–primed **(A)** and IL-3–primed **(B)** unstimulated *(left column)* or stimulated with anti-IgE (10 ng/mL; *middle column*) and anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL; *right column*) in the presence of the phospholipids PC (control), PS, and PE. *FSC*, Forward scatter.

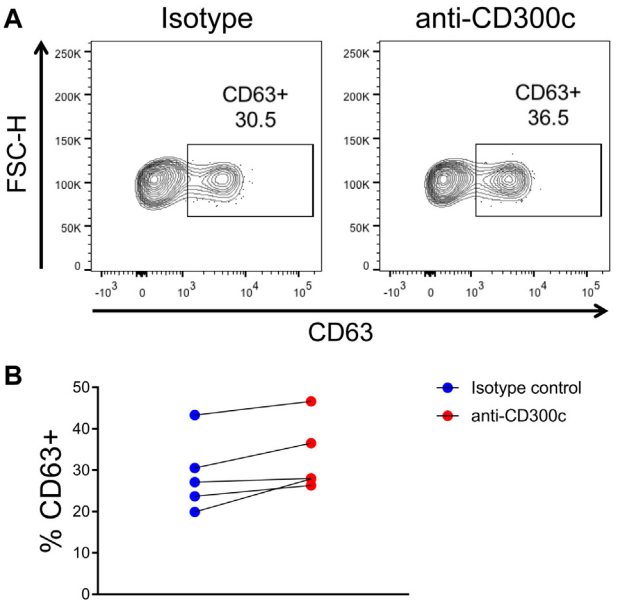

FIG E3. CD300c cross-linking enhances IgE-dependent basophil activation marker expression in basophils from allergic patients. **A,** Contour plots showing percentages of $CD63^+$ basophils in the presence and absence of cross-linking of CD300c after stimulation with anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). Contour plots are representative of data obtained from 5 independent experiments. **B,** Dot graphs showing percentages of $CD63^+$ basophils in the presence *(red dots)* and absence *(blue dots)* of cross-linking of CD300c after stimulation with anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). Each *pair of dots* represents a different experiment.

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LETTER TO THE EDITOR

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Increased expression levels of CD300c on basophils from allergic individuals

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TO THE EDITOR:

Immunoglobulin E (IgE)-mediated allergy refers to the adverse reaction in some patients caused by the crosslinking of the high-affinity IgE receptor (FcεRI) on basophils and mast cells by allergen-specific IgEs. Once the contact with allergens is established, immunomodulatory therapeutic agents to prevent the onset of allergic symptoms are currently quite limited. Blocking the interaction of specific IgE with the FcεRI that is present in basophils and mast cells is an important target in anti-allergic therapy. Clinical responses have been observed after the administration of omalizumab, a humanized anti-IgE antibody, to patients with food allergy, which correlated with the suppression of degranulation of basophils.[1,2] These results indicate that actions aimed to blocking or modulating the IgE/FcεRI axis represent a promising strategy for the treatment of allergy and anaphylaxis. Therefore, in order to develop new immunomodulatory therapies, it is very important to characterize cell surface receptors capable of modulating IgE-mediated activation threshold in basophils and mast cells.[3]

The human CD300 family of receptors consists of 8 receptors expressed in both myeloid and lymphoid lineages.[4] It has been recently demonstrated that the phosphatidylserine and phosphatidylethanolamine binding CD300c receptor acts as a co-stimulatory molecule during basophil activation through the IgE/FcεRI axis. CD300c cross-linking significantly augmented IgE-mediated basophils degranulation and cytokine production in a process involving increased calcium mobilization and phosphorylation of signaling intermediates such as protein tyrosine kinase (Syk) and extracellular signal–regulated kinases (ERK). Moreover, it was observed that basal expression levels of CD300c on basophils from IgE-dependent cow's milk allergic children are higher than those from healthy control children, suggesting that CD300c could be used as a biomarker in the diagnosis of the IgE-dependent allergic pathology.[5]

In order to further assess the clinical relevance of those findings, we have studied the expression of CD300c on basophils from patients with two IgE-dependent allergies. Clinical features from patients are shown in Table 1. We collected peripheral blood samples from 3 different cohorts: 1) adults with dust mites allergy (n = 36), 2) adults with grass pollen allergy (n = 22), and 3) non-allergic control individuals (n = 26).

To identify basophils among the peripheral blood mononuclear cells (PBMCs), we have used a staining strategy based on the expression of the surface receptor CD123 and the absence of human leukocyte antigen - DR isotype (HLA-DR) (shown in the article's online supplementary Figure S1). First, we analyzed the expression of CD63, a basophil activation marker which is

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Patient	Gender	Age (years)	Symptoms	Severity	Total IgE (kU/L)	IgE Der p (kU/L)	IgE Phl pe (kU/L)
HCAC_001	Female	60	ASTHMA + RHINITIS	Mild	3526	>100	-
HCAC_002	Male	19	ASTHMA + RHINITIS	Mild	215	27.6	-
HCAC_003	Male	34	ASTHMA + RHINITIS	Severe	168	18.4	-
HCAC_004	Female	15	ASTHMA + RHINITIS	Moderate	1012	>100	-
HCAC_005	Female	43	RHINITIS	Mild	133	39.8	-
HCAC_006	Male	20	RHINITIS	Mild	526	>100	-
HCAC_007	Female	31	ASTHMA + RHINITIS	Mild	254	39.2	-
HCAC_008	Male	28	ASTHMA + RHINITIS	Mild	618	>100	-
HCAC_009	Female	36	RHINITIS	Mild	175	18.5	-
HCAC_010	Female	20	ASTHMA + RHINITIS	Moderate	362	81.8	-
HCAC_011	Female	31	ASTHMA + RHINITIS	Mild	52	nd	-
HCAC_012	Male	41	ASTHMA + RHINITIS	Severe	168	4.46	-
HCAC_013	Female	40	ASTHMA + RHINITIS	Mild	1247	>100	-
HCAC_014	Female	36	ASTHMA + RHINITIS	Severe	416	59.2	-
HCAC_015	Female	78	ASTHMA + RHINITIS	Mild	384	17.3	-
HCAC_016	Female	26	ASTHMA + RHINITIS	Mild	445	49.6	-
HCAC_017	Female	78	ASTHMA + RHINITIS	Mild	361	5.47	-
HCAC_018	Female	46	RHINITIS	Moderate	199	11.6	-
HCAC_019	Female	15	RHINITIS	Mild	ND	80.1	-
HCAC_020	Female	25	ASTHMA + RHINITIS	Mild	666	nd	-
HCAC_021	Female	41	ASTHMA + RHINITIS	Mild	260	40.8	-
HCAC_022	Male	18	ASTHMA + RHINITIS	Mild	213	27.3	-
HCAC_023	Female	32	ASTHMA + RHINITIS	Mild	458	34.8	-
HCAC_024	Female	20	RHINITIS	Mild	37	10.6	-
HCAC_025	Female	49	ASTHMA + RHINITIS	Mild	18	4.46	-
HCAC_026	Female	23	RHINITIS	Mild	38	1.76	-
HCAC_027	Male	27	RHINITIS	Mild	44	13.4	-
HCAC_028	Female	31	RHINITIS	Mild	67	8.02	-
HCACGR_001	Male	20	RHINITIS	Mild	782	56.7	-
HCACGR_002	Female	15	ASTHMA + RHINITIS	Mild	449	69.2	-
HCACGR_003	Male	17	RHINITIS	Mild	721	44.4	-
HCACGR_004	Female	16	ASTHMA + RHINITIS	Mild	2882	>100	-

(continued)

Patient	Gender	Age (years)	Symptoms	Severity	Total IgE (kU/L)	IgE Der p (kU/L)	IgE Phl pe (kU/L)
HCACGR_006	Male	21	ASTHMA + RHINITIS	Moderate	1553	>100	-
HCACGR_007	Female	30	ASTHMA + RHINITIS	Mild	691	35.8	-
HCACGR_008	Female	47	RHINITIS	Mild	481	7.2	-
HCACGR_009	Female	29	RHINITIS	Mild	302	nd	12.6
HCACGR_010	Female	45	ASTHMA + RHINITIS	Mild	301	39.8	-
HCGR_001	Female	36	RHINITIS	Mild	493	-	73.2
HCGR_002	Male	44	RHINITIS	Mild	14	-	1.23
HCGR_003	Male	28	ASTHMA + RHINITIS	Mild	324	-	51.8
HCGR_004	Female	34	RHINITIS	Mild	1237	-	15.9
HCGR_005	Male	28	RHINITIS	Mild	924	-	28.4
HCGR_006	Female	37	RHINITIS	Mild	Nd	-	Nd
HCGR_007	Male	31	RHINITIS	Mild	17,3	-	5.35
HCGR_008	Male	43	RHINITIS	Mild	148	-	2.54
HCGR_009	Female	30	ASTHMA + RHINITIS	Mild	228	-	12.5
HCGR_010	Female	56	ASTHMA + RHINITIS	Mild	11,01	-	2.1
HCGR_011	Female	11	RHINITIS	Mild	140	-	26.8
HCGR_012	Female	24	ASTHMA + RHINITIS	Mild	988	-	89.6
HCGR_013	Male	46	RHINITIS	Mild	242	-	29
HCGR_014	Female	36	RHINITIS	Mild	374	-	23.6
HCGR_015	Male	48	RHINITIS	Mild	107	-	21.2
HCGR_016	Female	54	RHINITIS	Mild	44,6	-	7.32
HCGR_017	Male	44	RHINITIS	Mild	34,7	-	6.04
HCGR_018	Male	25	RHINITIS	Mild	136	-	16.3
HCGR_020	Male	57	RHINITIS	Mild	409	-	57.3
HCGR_021	Female	16	RHINITIS	Mild	436	-	>100
HCGR_022	Male	33	RHINITIS	Mild	194	-	72.2

Table 1. (Continued) Clinical data of allergic individuals. *Dr p:* Dermatophagoides pteronyssinus*; Phl p:* Phleum pratense

rapidly mobilized to the cell surface by polyclonal anti-IgE and allergens, as well as other degranulation stimuli.[6] In agreement with previous publications,[7] we observed that subjects with dust mites and/or grass pollen allergy show a significant increase in the median fluorescence intensity (MFI) of CD63 compared with basophils from non-allergic subjects, indicating that they are activated *in vivo* (Fig. 1A and online supplementary Figure S2). We cannot confirm that the increased expression on CD63 is exclusively due to a higher expression on basophils, and that other possibilities, as for example a greater adhesion of platelets, have

some role in it. However, in several clinical studies it has been demonstrated that the presence of platelets in the cluster of CD63-positive basophils is minor.[8–10]

Next, we analyzed cell surface expression levels of the inhibitory and activating CD300a and CD300c receptors, respectively. We observed that surface CD300c expression levels are significantly higher in basophils from individuals with an IgE-dependent allergy (dust mites and grass pollen) than in those from non-allergic individuals (Fig. 1B). This increased expression of CD300c could diminish basophils FcεRI-mediated activation threshold.[5] It has been previously described that the basal expression of the CD300a inhibitory receptor is lower in basophils from patients with birch pollen allergy than in basophils from healthy control subjects.[11] However, we have not seen significant differences between healthy and allergic individuals related to CD300a expression (Fig. 1C), which is in agreement with previous results in cow's milk allergic children.[5]

We performed correlation analysis between the intensity of CD300c and CD63 expression, but we did not find any significant result (data not shown). As it has been mentioned before, an association between the severity of the hypersensitivity symptoms and the levels of CD300c expression on basophils in cow's milk allergic children has been described.[5] However, in this study the vast majority of the recruited patients (88%) were classified to have mild symptomatology (Table 1), and therefore it was not adequate to perform this analysis.

Unlike mite allergy, grass pollen allergy is a seasonal affection, and the exposure of patients to the allergen varies throughout the year. Although the great majority of samples have been collected during the grass season, we have two samples of the same individual collected at different times of the year, one in grass season and the other not. We observed that the expression of CD300c is higher during the grass pollen season than out of the season (MFI 492 vs 376, respectively). This may suggest that the exposure of allergic patients to higher amounts of allergen could induce an up-regulation of basophil activating receptors such as CD300c. The specific mechanism regulating CD300c

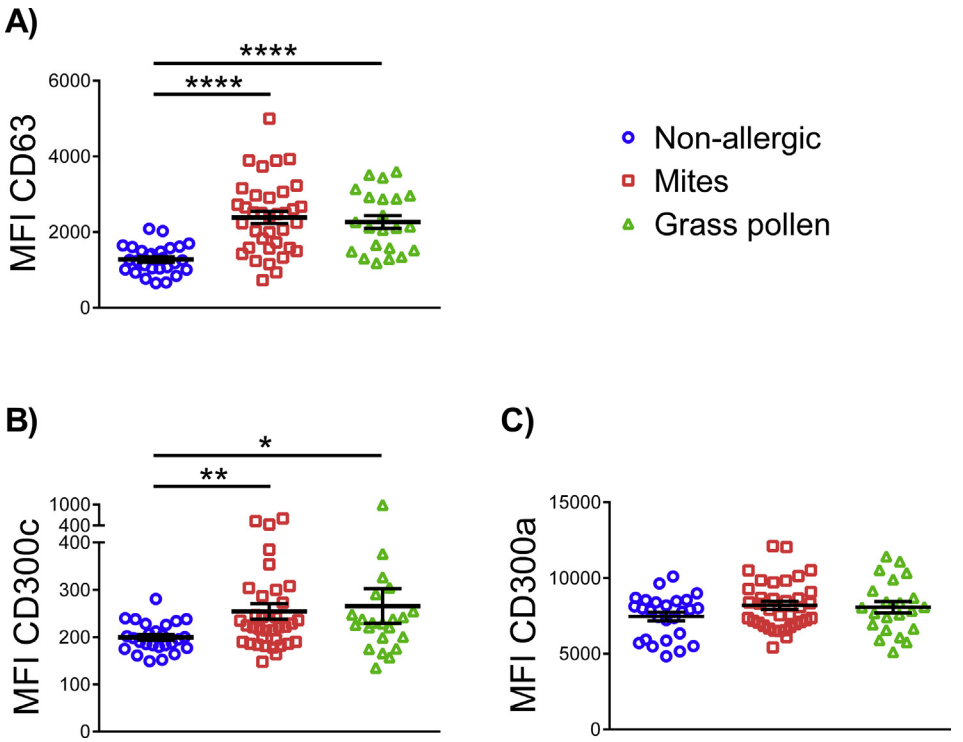

Fig. 1 Basophils from allergic individuals exhibit greater expression of CD63 and CD300c. Dot graph showing basal median fluorescence intensity (MFI) of CD63 (A), CD300c (B) and CD300a (C) on basophils from non-allergic (blue), allergic to dust mites (red) and allergic to grass pollen (green) individuals. Each dot represents a donor, means ± SEMs are shown. *P < .05, **P < .01, ****P < .0001

expression in these allergic patients is still unknown and deserves further studies.

To date, the only tested stimulus able to upregulate CD300c expression is IL-3.[5] This is a very important cytokine for the development, maturation, and survival of basophils.[12] This cytokine is known to markedly increase the activation and release of mediators from basophils in IgE-dependent responses,[12] and the autocrine priming with IL-3 has been described as an important mechanism behind the hyperreactive nature of basophils in the allergic disease.[13] We analyzed IL-3 in plasma from allergic subjects, but the levels of this cytokine were mostly undetectable. It is possible that the mild symptoms exhibited by the majority of patients may be related to the observed results. Based on our data, we propose that baseline expression levels of CD300c, together with CD63 expression, on human basophils could be helpful for the diagnosis of IgE-dependent allergies. Furthermore, considering that CD300c is capable of modulating the threshold of IgE-mediated activation in human basophils,[5] we believe that, as previously demonstrated,[5] an increased expression of CD300c decreases the IgE-dependent activation threshold of basophils in allergic individuals.

Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for publication and ethics approval

Blood samples from healthy donors and allergic patients were collected through the Basque Biobank (http://www.biobancovasco.org). The Basque Biobank complies with the quality management, traceability, and biosecurity set out in the Spanish Law 14/2007 of Biomedical Research and the Royal Decree 1716/2011. All subjects provided written and signed informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Basque Ethics Committee for Clinical Research (PI2015182; 15–56; version 3; March 23, 2017).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2019.100060.

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