PhD
Program in Translational
and Molecular Medicine
DIMET

University of Milano-Bicocca
School of Medicine and School of Science

Unravelling the Fingerprint and Regulatory Functions of Human Tolerogenic DC-10

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XXVIII cycle,
Academic year 2015-2016

The research presented in this thesis was performed at the San Raffaele Telethon Institute for Gene therapy (SR-TIGET), Department of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, in the laboratory of Dr. Silvia Gregori.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Antigen Presenting Cells

Antigen presenting cells (APC) are a heterogeneous population of leukocytes whose aim is to capture and transfer information from the outside to the cells of the adaptive immune system. Thanks to the expression of major histocompatibility complex (MHC) class I and II and costimulatory molecules, APC are indeed fundamental in the promoting functional activity to T cells. Endogenous peptides, derived from intracellular sources such as replicative viruses, are presented on MHC class I molecules to $CD8^+$ T cells, while exogenous peptides, derived from extracellular sources such as microbes, are presented on MHC class II molecules to $CD4^+$ T cells.

In lymphoid organs, the three main populations of APC are dendritic cells (DC), macrophages, and B cells. DC are the most effective cells in priming naïve T cells, partly because macrophages and B cells express appropriate costimulatory molecules only upon infection or contact with microbial products. On the contrary, if antigen (Ag) concentrations are very low, B cells baring high affinity receptors (Immunoglobulin (Ig)M or IgD) for Ags are the most effective APC because the other APC cannot capture enough Ag. There are also non-professional APC, which express MHC class II and costimulatory molecules for short periods of time throughout sustained

inflammatory responses. Fibroblasts, glial cells, pancreatic β cells, epithelial and vascular endothelial cells belong all to this second group of APC. Because most of the abovementioned cells are not efficient in processing proteins into MHC-binding peptides, it is unlikely that they contribute significantly to induction of T cell responses. Exceptions are the thymic epithelial cells, which constitutively express MHC molecules and play a critical role in presenting MHC-peptide (MHCp) complexes to maturing T cells within the thymus.

Often the term APC is used synonymously with DC, but the concepts ad terms, as Steinman precised in one of his review[1], they are different: the term APC best refers to antigenicity, which means the use of MHC products to present peptides, while DC couple antigenicity with the immunogenicity (or tolerogenicity). DC not only present Ags, but have also a number of other features that are required for promoting specific immune responses to T cells.

Dendritic cells (DC)

Through their capacity to discriminate among self, altered-self, and non-self, DC are suited to organize the immune defence to intruders, to prevent tissue damage and excessive inflammation even when a powerful immune response is ongoing, and to induce immunological tolerance when required. DC act as adjuvants with their unique ability to induce primary immune

responses, but their characteristics enable them not only to regulate T-cell activation, but also to maintain T-cell tolerance in the periphery. Moreover, thanks to their ability to migrate, DC display unique properties that allow them to form a link between the periphery and lymphoid organs, where immune responses are generally initiated.

Ralph Steinman discovered in the mouse spleen this rare population of cells characterized by stellate morphology and extended veils[2] and named them DC. From this milestone study his group then demonstrated that: i) DC, which prominently express both MHC class I and II molecules[3], were unrivalled stimulators of T cells in primary mixed leukocytes reactions[4]; ii) DC process protein Ags and initiate Ag-specific cellular immune responses[5]. Starting from Steinman's work, DC were then studied extensively, and there is now clear evidences that multiple subsets of DC exist with specialized phenotypes and functions. Nevertheless, DC display unifying features, such as morphology, ability to process and present Ags, capacity to migrate from body barriers to the T cell zones in lymphoid organs, and competence in cross-talking with T cells.

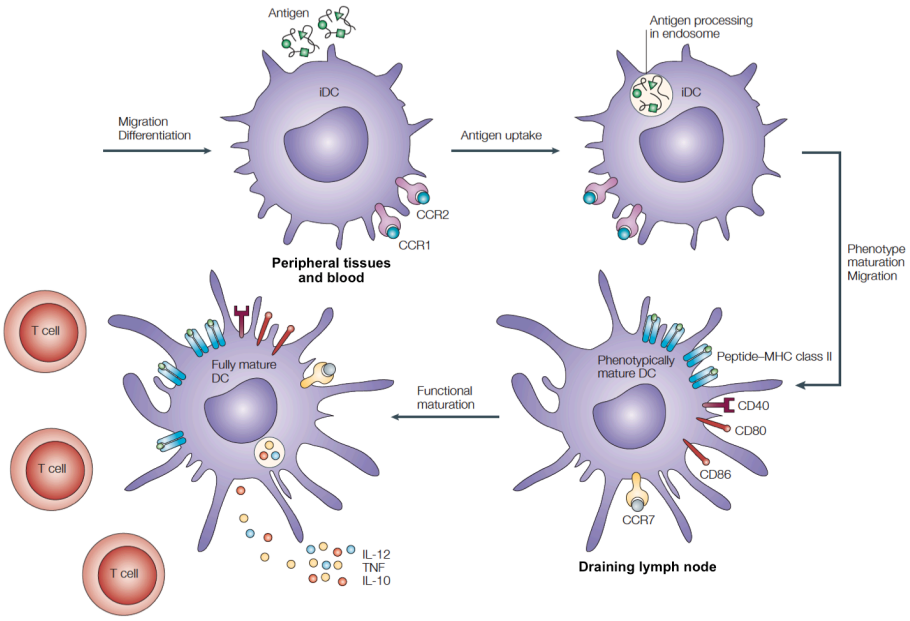

Figure 1. Checkpoints of dendritic cell immunology. Immature dendritic cells (iDC) are circulating in the blood or recruited to peripheral tissues, where they continuously internalize antigens that can be processed by an endosomal, MHC class-II-restricted pathway. After antigen capture and depending on the nature of the antigen, DC migrate to the draining lymphoid tissue and mature phenotypically, upregulating the expression of costimulatory and MHC class II molecules. They present peptide–MHC class II complexes on the cell surface, interact with antigen-specific lymphocytes and mature functionally, activating T cells and producing pro-inflammatory cytokines, such as interleukin-12 (IL-12) and tumour-necrosis factor-α (TNF-α) (modified from Hackstein & Thomson 2004)

Dendritic cell features

Antigens processing and presentation

DC are specialized in Ag processing and can efficiently present endogenous and exogenous Ags in both MHC class I and II contexts. Other cells, like macrophages, can present Ags in the MHC II context efficiently, but the Ag processing is different. While macrophages endocytose and rapidly digest Ags, DC sequester and preserve capture Ags for later presentation. This preservation, critical for immunogenicity, is due to two peculiar DC characteristics: i) low lysosomal protease activity, resulting in a limited capacity for lysosomal degradation (peptides presented by DC are 8-27 amminoacids long); ii) low acidity of DC lysosomes that results in decreased Ag digestion rate, ultimately leading to an increase availability of partially processed peptides for MHC loading.

DC capture Ags from the environment through two different mechanisms: micropinocytosis and receptor-mediated phagocytosis. The pinocytic vesicles allow DC to sample a large volume of extracellular fluid and soluble proteins that are present at low concentrations. Phagocytosis and DC activation is mediated by Pathogen Associated Molecular Patterns (PAMP), like bacterial carbohydrates or nucleic acids, which engage specific receptors: Toll Like Receptors (TLR), C-type Lectins

Receptors (CLR), and receptors from the Specific Intracellular adhesion molecule-3-Grabbing Non-integrin (SIGN) family. In addition, DC also express Fc receptors that mediate ingestion of opsonized particles and can be activating or inhibitory. The latter maintain DC in an immature tolerogenic state.

Captured soluble and particulate Ags are targeted to MHC class II compartments in order to be presented to $CD4^+$ T cells. In immature DC, MHC class II molecules are rapidly internalized and have a short half-life. Maturation of DC lead to increased class II synthesis and powered translocation of MHC II-peptide complexes to cell surface, where they remain stable for days.

To generate $CD8^+$ cytotoxic T cells (CTLs), DC process Ags in the cytosol, where they undergo ubiquitin conjugation. The ubiquitinylated proteins are directed to the proteasome, which cleaves proteins into peptides that are then translocated into Endoplasmatic Reticulum (ER), trimmed into 8-10 amminoacids and loaded on MHC class I. Another interesting pathway leading to $CD8^+$ T cell responses is the cross-presentation, an alternative MHC class I pathway that can present peptides derived from extracellular Ags.

Maturation and migration

DC exist in two functionally distinct and phenotypically different stages: steady-state and activated. Steady-state DC

capture and present Ags to T cells, but the outcome of this antigen presentation is tolerance and not immunity. These DC express high levels of pattern recognition and activation receptors, allowing them to sense changes in the environment, like pathogens and inflammatory cytokines (i.e. Tumor Necrosis Factor α (TNF- α) and Interleukin (IL-)1β).

Upon receipt of an activation stimulus, DC undergo phenotypic and functional changes that culminate in the complete transition from Ag-capturing cell to APC. DC loss adhesive structure, reorganize cytoskeleton, and acquire high cellular motility, that lead to DC migration from the periphery to draining secondary lymphoid organs. Mature DC entrance in the draining lymph nodes is driven to the paracortical area by a CCL19 and CCL21 chemokine gradient[7]. Upon encounters T cells, DC receive additional maturation signals from CD40 ligand (CD40L), RANK, CD137 and OX40 ligand (OX40L) molecules, which induce the release of chemokines that attract lymphocytes[8]. These DC-T cell interactions in the lymph nodes have been confirmed in the living state with two-photon microscopy[9,10].

DC- T cell cross-talk

DC play a central role both in initiation of naïve T cell responses and in effective re-stimulation of memory T cells. An essential step in the initiation of an immune response is the formation of

the immunological synapse between different APC, including DC, and T cells via cell surface receptors and secreted molecules. Along with presenting an Ag in the context of MHC class I and II, DC also provide additional signals to T cells by expressing an array of co-stimulatory molecules (i.e. CD80, CD86, CD40) and by secreting different cytokines (i.e. IL-12, Interferon (IFN)-γ, IL-10). All these components are required for productive T cell priming to occur and the different signals will determine the outcome of a naïve T cell polarization. Thus, through different cytokine secretion, DC orchestrate Ag-specific T cell differentiation toward T helper (Th)1, Th2, Th17, T follicular helper (Tfh) or Treg pathways. A key molecule expressed on DC suitable for T cell clonal expansion, effector and memory cell formation and maintenance is CD70, the receptor for CD27, expressed on T cells[11,12]. When T cell encounter MHCp complex on DC in absence of costimulatory molecules, the outcome of the immune response is the induction of tolerance: DCs induce transient antigen-specific T cell activation followed by T cell deletion and unresponsiveness[13]. In addition to enforce tolerance to self and harmless environmental Ags, DC can silence self-reactive T cells by inducing anergy or deletion: these mechanisms involve ligation of inhibitory molecules, such as Programmed cell Death Ligand 1 (PDL-1)[14,15]. Notably, DC are also involved in promoting tolerance through the induction of T regulatory (Treg) cells. *In vitro*

differentiated DC subsets specialized in tolerance induction will be better described at the end of this chapter.

Subsets of human dendritic cells

DC lineage

There is a classical division of DC in two main lineages: myeloid (myDC) and plasmacytoid DC (pDC).

MyDC express typical myeloid markers, such as CD11c, CD13, CD33 and CD11b. CD11c is shared between monocytes and DC, but the use of other markers, like CD1c (BDCA-1) and CD141 (BDCA-3), allows the identification of different myeloid DC subsets[16]. Moreover, a third subset of myDC is present in tissue and lymph nodes, characterized by the expression of $CD14^+$ with a monocyte-like or macrophage-like phenotype. These cells, initially called interstitial DC, may arise from classical monocytes.

pDC express CD123, CD303 (BDCA-2) and CD304 (BDCA-4) and lack myeloid markers[16]. Their name is linked to their morphology, which is similar to that of plasma cells.

In addition, there are two specialized self-renewing DC populations: Langerhans cells (LC), present in stratified

squamous epithelium and capable of differentiating into migratory DC, and microglia, found in the parenchyma of the brain. The latter is not unanimously considered a DC type, due to some shared features with macrophages.

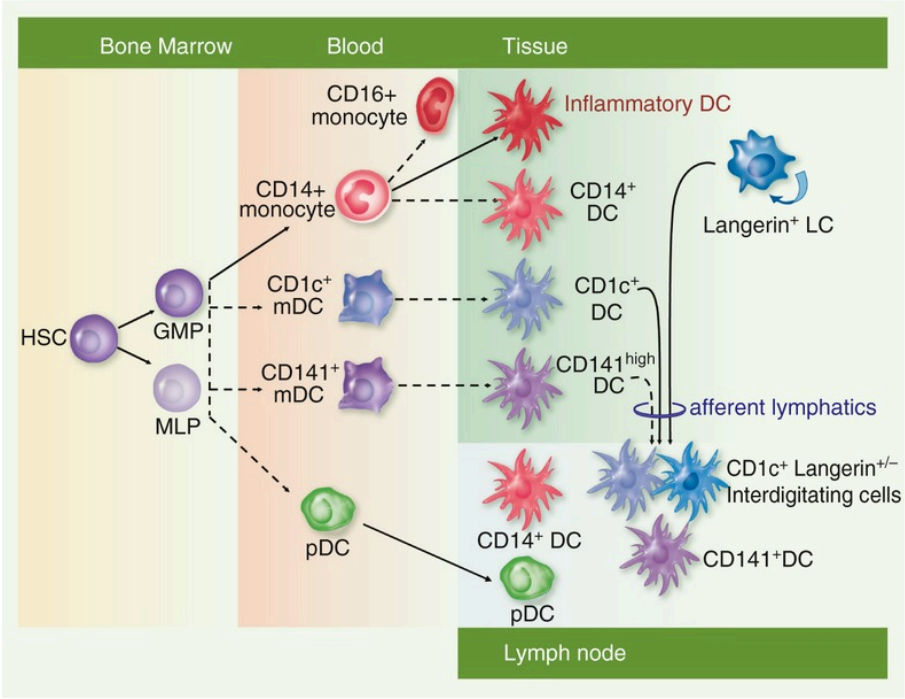

Figure 2. Distribution of major human dendritic cell (DC) subsets in blood, epithelial tissues and lymph nodes. Broken arrows indicate relationships that require further confirmation in humans. Classical monocytes, blood myeloid DC (mDC) and plasmacytoid DC (pDC) are derived from bone marrow progenitors and are putative precursors of tissue and lymphoid DCs (from Collin et al. 2013)

Blood DC subsets

Peripheral blood is easily accessible from the human body, and because of limited access to human tissue, much of early work in humans has focused on blood DC. For this reason, blood DC are well defined in humans, and are likely to be precursors of tissue and lymphoid organ DC. Indeed, blood pDC, $CD1c^+$ and $CD141^+$ myDC are immature forms of their tissue counterparts[18–20]. The nomenclature of different DC population in blood was discussed extensively among scientists, and now there is a classification approved by the Nomenclature Committee of the International Union of Immunological Societies[21].

$CD1c^+$ myDC are the major population of human myDC in blood. They express a variety of TLR and other PAMP receptors that stimulate them to secrete TNF- α, IL-8 and IL-10, and, upon TLR7/8 ligation, IL-12[19]. $CD1c^+$ myDC are good stimulators of naïve $CD4^+$ T cells, but have inferior capacity to cross-present Ag to $CD8^+$ T cells compared to $CD141^+$ myDC[22].

$CD141^+$ myDC represent 10% of human blood myDC. The wide expression of CD141 on other cells, like migratory $CD14^+$ and $CD1c^+$ myDC, make it difficult to identify them[22], even though they show a lower expression of $CD11b^+$ and $CD11c^+$ compare to $CD1c^+$ myDC[23]. $CD141^+$ myDC are considered the prominent cross-presenting DC subset, because their ability to stimulate

$CD8^+$ T cells with exogenous Ag is higher compared to any other DC population[22,24]. $CD141^+$ myDC have enhanced ability to take up dead or necrotic cells via CLEC9A and sense viral nucleic acids with TLR3 and TLR8[25]. Conversely, $CD141^+$ myDC did not express Fc receptors and thus lack the ability to uptake Ag-antibody complexes[16]. $CD141^+$ myDC secrete IL-12, TNF- α, IFN-β and IFN-λ upon different TLR stimulation[19,22,26]. In literature there are conflicting results about the comparison of cytokine profile between $CD141^+$ and $CD1c^+$ myDC, probably due to different combination of TLR stimuli[19,27].

$CD303^+$ pDC are the most abundant DC subset in the blood, and are characterized by their production of type I IFN in response to viruses[28]. To identify and discriminate them against myDC, CD68 marker can be used in combination with CD123 and CD304[29]. To sense viral and self nucleic acids, these DC express very high levels of TLR7 and TLR9. Upon TLR stimulation, $CD303^+$ pDC secrete high levels of IFN-α/β but no IL-10 or IL-12[27]. Freshly isolated $CD303^+$ pDC do not prime naïve T cells efficiently and appear less mature compared to myDC until[28]. They are able to induce Treg cells and tolerance, an ability probably linked to the uptake of DNA released from apoptotic cells through TLR7[30,31].

Macrophages

Macrophages play different roles in maintaining the organism's integrity, by directly participating in pathogen elimination and repairing tissue under sterile inflammatory conditions. Macrophages capture both extra- and intracellular pathogens, eliminate or deliver them to appropriate sub-compartments of lymphoid organs. While DC are uniquely suited for stimulating naïve T cells in secondary lymphoid organs, macrophages usually present Ag in the periphery to activated, already primed, T cells. This interaction makes macrophages important effector cells during adaptive immunity. Moreover, macrophages act as regulator of immune responses, thanks to their ability to limit the induction of effector specific responses.

Macrophages as APC

Macrophages, like DC, have all the machinery required for Ag processing and presentation of exogenous peptides and endogenous peptides in the context of MHC class II and class I, respectively. Macrophages can recognize foreign or modified-self Ags through different pattern recognition receptors that mediates either phagocytosis or endocytosis. In the former case, Ags are opsonized by antibodies or complement and their recognition occurs through Fc or complement receptors (FcR or

CR, respectively). The direct recognition of Ags by macrophages occurs via different receptors: lectins (Mannose Receptor (MR), β-glucan receptor (or dectin-1), scavenger receptors (SR)-A, and TLRs. As indicated above, Ag processing in macrophages is different compared to that of DC, since it usually leads to complete neutralization of up-taken proteins or microorganisms.

Subsets of macrophages

There are different subpopulations of macrophages, each one displaying its own characteristics. In tissues, macrophages acquire unique and distinct morphological and functional properties, becoming highly specialized cells, like Kupffer cells in the liver. Based on the stimuli used to induce their activation and polarization, the conventional macrophage nomenclature indicates M1 as classical activated macrophages and M2 as alternatively activated macrophages, mirroring the Th1 and Th2 subdivision of T helper cells[32]. In the 1980s, the effect of IFN-γ on macrophages was demonstrated, showing an increase release of peroxide and subsequent enhanced ability to kill intracellular pathogens[33]. Ten years later, IL-4 was found to induce alternative macrophage activation[34].

Due to the fact that macrophages are probably the most plastic cell among cells of hematopoietic origin, this schematic

subdivision in M1/M2 subsets can be too restrictive. While in some pathological conditions, such as allergic reaction, parasite infections, and many tumours, the functional phenotypes of macrophages *in vivo* mirror those of canonical *in vitro* defined M1- or M2- polarized states, in others, like degenerative disorders, macrophage populations express mixed or unique phenotypes[35]. Therefore, this dichotomy should be interpreted in a more elastic way.

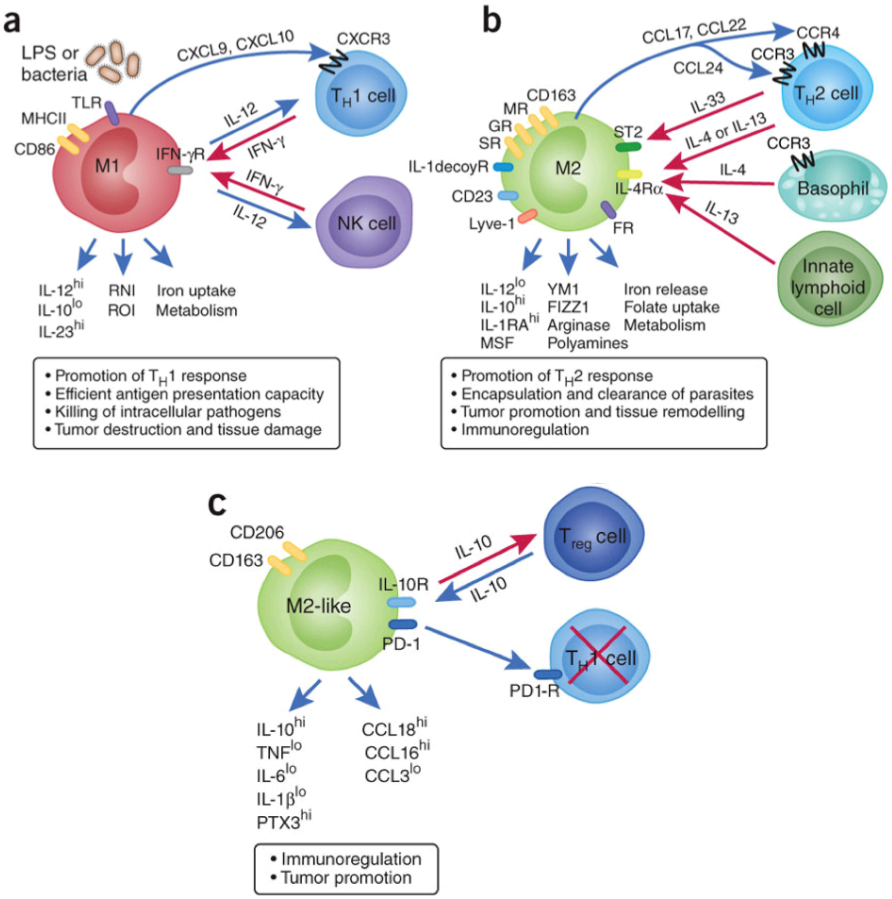

Figure 3. Macrophage subsets and their interaction with different Th cells. (**A**) M1 macrophages crosstalk with T_H1 and NK cells. (**B**) M2 induction of macrophages is driven by T_H2 cells, basophils and innate lymphoid cells through their secretion of IL-4, IL-13 or IL-33. (**B**) M2-like macrophages are polarized by interaction with T_{reg} cells. (modified from Biswas & Mantovani 2010)

M1 macrophages

M1 are the classical activated macrophages. M1 activation can be mediated by IFN-γ or GM-CSF derived by other cell types, including Th1, CTL $CD8^+$ T, or NK cells[37,38], or by other stimuli, like LPS derived by Gram-negative bacteria[39].

M1 macrophages produce inflammatory cytokines, like IL-12, IL-23, IL-6, IL1β, and TNFα, with low or no secretion of IL-10. They express high levels of MHC class II, CD68, CD80 and CD86 molecules, phagocyte large numbers of pathogens and kill intracellular organisms. Once activated, M1 macrophages exhibit strong microbicidial properties by synthesis of Nitrogen Oxide (NO) and acidify the phagosome[40,41].

In general, classically activated M1 macrophages are potent effector cells that kill microorganisms and tumor cells, and produce copious amounts of pro-inflammatory cytokines.

M2 macrophages

Macrophages activated through alternative pathways are referred as M2. M2 activation is initially described dependent on IL-4 and IL-13 secreted by other cell types, including Th2 $CD4^+$ T, innate lymphoid (ILC) cells, and basophils. Subsequently, IL-33 and IL-21 have been showed to drive M2 activation[42,43]. Moreover, other stimuli, including glucocorticoids, vitamin D_3,

Transforming Growth Factor (TGF-)β, and IL-10 promote M2-like functional phenotypes, displaying some but not all properties of IL-4/IL-13 activated macrophages[44].

M2 macrophages produce low amounts of pro-inflammatory cytokines (IL-12, IL-1, TNF and IL-6), secrete IL-10, IL-1 receptor antagonist (IL-1ra), and a variable set of other cytokines, depending on the signal used for their activation. M2 macrophages are characterized by the expression of the macrophage mannose receptor, CD206, and the up-regulation of CD200R glycoprotein[45]. CD163 is another marker of M2 macrophages, but its expression is not highly specific[46]. Alternatively activated macrophages show enhanced phagocytosis, but not enhanced killing functions towards microbes. NO production, that characterized M1 macrophages, is counteracted by enhanced expression of arginase, competing with NO synthases for L-arginine as its substrate[47]. It has been demonstrated that M2 macrophages actively inhibit proliferation of peripheral blood lymphocytes, and $CD4^+$ T cells *in vitro*[48].

In general, M2 cells participate in polarized Th2 responses, parasite clearance, and dampening inflammation, and promoting tissue remodelling, angiogenesis, tumor progression, and immune-regulation.

In vitro differentiated monocyte-derived dendritic cells

DC can be differentiated *in vitro* by culturing peripheral blood $CD14^+$ monocytes with GM-CSF and IL-4[49] or $CD34^+$ hematopoietic progenitors cells with GM-CSF and TNF-α[50]. A large array of mediators has been used to modulate *in vitro* differentiated DC, to induce tolerogenic cells[51]. Among the others, the most studied and well-characterized to generate human tolerogenic DC are 1alpha,25-dihydroxyvitamin D3 (VitD3), dexamethasone (Dexa), rapamycin (RAPA), and IL-10. Treatment of DC with these agents prevents their maturation and/or activation, or impairs their capacity to produce IL-12 while maintains IL-10 production.

Vitamin D3-induced tolerogenic dendritic cells

DC differentiated in the presence of VitD3 (VitD3-DC) induce cells that express low levels of HLA class II and co-stimulatory molecules and produce IL-10, in absence of IL-12[52,53]. VitD3-DC displayed increased levels of membrane-bound TNF-α and PDL-1, both critical for induction of IL-10-expressing Treg cells [54,55]. Several reports described also the effect of VitD3 on *ex vivo* isolated human DC, with different outcome depending on the

treated DC subset. Circulating $CD1c^+$ myDC cultured in the presence of VitD3 become $CD141^+CD14^+ILT3^+$ IL-10 producing DC, with phenotype and function similar to $CD141^+$ dermal DC. These modified DC induce development of $Foxp3^+$Treg cells[56]. Conversely, VitD3 treatment of dermal DC up-regulates the expression of IL-10 and promote $IL\text{-}10^+Foxp3^-$ T cells[57].

Dexamethasone-induced tolerogenic dendritic cells

Dexamethasone-induced tolerogenic DC (Dexa-DC) are characterized by low expression of MHC II and costimulatory molecules and enhanced levels of ILT2, ILT3 expression, and IL-10 secretion[58,59]. As VitD3-DC, Dexa-DC acquire the ability to induce contact-dependent Treg cells that suppress T cell responses in an Ag-dependent manner[55]. Moreover, Dexa-DC maintain their immunosuppressive phenotype even upon TLR4 stimulation[60].

VitD3 and Dexa-induced tolerogenic dendritic cells

VitD3 and Dexa singularly induce tolerogenic phenotype in DC, but they also synergize in promoting human tolerogenic DC. VitD3/Dexa-DC produce higher levels of IL-10 compared to DC

treated with VitD3 or Dexa[61], and inhibit T cell proliferation and induce suppressor T cells[62]. A phase I study in rheumatoid arthritis (RA) has been performed with VitD3/Dexa-DC pulsed with autologous synovial fluid injected in the joint of patients[63]. None of the patients experienced flares in the treated knee in the first 5 days after DC injection, demonstrating VitD3/Dexa-DC stability. If the inflamed joint milieu had modified VitD3/Dexa-DC, pathogenic T cells would be activated, worsening RA symptoms. Based on this phase I trial, authors consider that tolDC therapy is safe and worthy of further investigation. Two new trials are planned, one from the same group in RA and the other by Martines-Caceres group in multiple sclerosis (S.Gregori, personal communication). In the latter, VitD3/Dexa-DC will be pulsed with Myelin Oligodendrocyte Glycoprotein (MOG) Peptides.

Rapamycin-induced tolerogenic dendritic cells

Rapamycin acts through binding to the serine/threonine protein kinase Mammalian Target of RAPA (mTOR) and it is currently used to prevent allo-graft rejection, due to its broad-ranging effects on immune system. Addition of RAPA during DC differentiation (RAPA-DC) suppressed immunostimulatory molecule expression, but paradoxically also decreased the

expression of PDL-1[64]. RAPA-DC are poorly stimulatory, induce T-cell hyporesponsiveness[65], and promote of Foxp3$^+$ T cells[66,67]. However, following TLR ligation with LPS, RAPA-DC display enhanced IL-12p70 secretion, with concomitant decreased production of IL-10, thus acquiring a potential to promote activation of T cell immunity, rather than immune regulation[68].

IL-10-induced tolerogenic dendritic cells

In vitro differentiated DC treated with IL-10 in the last two days of culture show lower levels of HLA class II expression and fail the upregulation of co-stimulatory molecules, but if a strong maturation stimulus by IL-1β, TNF-α, and IL-6 cytokine mixture is added together with IL-10, this modulation of surface molecules is not observed[69]. Moreover, IL-10 treatment upregulates ILT3, ILT4, PDL-1, PDL-2 and HLA-G expression[70,71]. The profile of cytokine release of IL-10 treated DC differs according to protocols used for DC generation. Nevertheless, the amount of pro-inflammatory cytokines like IL-6 and TNF-α is similar as compared to classically matured DC. In contrast, IL-10 production is dramatically increased and the release of IL-12 and IL-23 is abolished[70,72]. IL-10 treated DC dampen CD4$^+$ and CD8$^+$ T cell immune responses[73,74]. However,

the ability to induce T regulatory cells with suppressive ability is acquired only if IL-10 is given to DC together with activating cytokine cocktail[70,75]. With this differentiation protocol, the resulting DC are consisting of two distinct subpopulations: immature $CCR7^{-}CD83^{-}HLA\text{-}DR^{low}$ cells and mature $CCR7^{+}CD83^{+}HLA\text{-}DR^{high}$ cells[76]. Despite both populations express ILT-3 and ILT-4 co-inhibitory molecules and are able to induce Treg, $CCR7^{+}CD83^{+}HLA\text{-}DR^{high}$ DC promote a Treg population with stronger suppressive capacity, showing an activated phenotype. Moreover, when re-stimulated with IL-1β, IL-6, and TNF-α, $CCR7^{+}CD83^{+}HLA\text{-}DR^{high}$ DC maintain their phenotype, revealing resistance to further inflammatory stimuli. These characteristics made $CCR7^{+}CD83^{+}HLA\text{-}DR^{high}$ IL-10-DC an interesting tolerogenic DC population for cell therapy.

DC-10

DC-10 are a distinct population of tolerogenic dendritic cells, which can be generated from peripheral blood monocytes by addition of IL-10 from the beginning of differentiation culture [77]. DC-10 are not only generated *in vitro*, but are also present *in vivo* in blood, spleen and in the decidua of pregnant women[77,78]. DC-10 are $CD11c^{+}CD11b^{+}CD40^{+}$ DC, express CD14 and CD16 but not CD1a; although not activated, display a mature

phenotype, being $CD80^+$ $CD86^+$ and $HLA\text{-}DR^+$. DC-10 spontaneously secrete high amounts of IL-10, in the absence of IL-12, and produce IL-6 and low levels of TNFα. DC-10 induce the differentiation of Ag-specific T regulatory type 1 (Tr1) cells *in vitro*[77]. Differently from other *in vitro* differentiated tolerogenic DC, DC-10 maintain their phenotype and functions also upon LPS stimulation, and showed increased IL-10 production[79]. To efficiently induce Tr1 cells, the three key molecules are IL-10, HLA-G and ILT4. HLA-G expression levels vary among donors, and are genetically defined. DC-10 expressing low levels of HLA-G are less potent in induce Tr1 cell differentiation compared to DC-10 with high HLA-G expression[80]. A single stimulation of allogeneic naïve T cells with DC-10 is sufficient to generate a population of T cells comprising up to 15% of allo-specific Tr1 cells[81,82]. DC-10 can induced not only allo-specific, but also Ag-specific Tr1 cells: DC-10 differentiated from monocytes of allergic patients and pulsed with allergen efficiently promote the generation of allergen-specific Tr1 cells able to suppress cytokine production by effector Th2 cells *in vitro*[83]. We set up a clinical grade protocol to induce DC-10 *in vitro* and indeed they are currently used to generate IL-10-producing T Regulatory Type 1 (Tr1) cells for cell therapy[79,81]. This strategy has been applied in two different clinical setting: DC-10 have been differentiated from patients underwent hematopoietic stem cell transplantation, in

order to induce host-specific Tr1 cells of the donor[84], and kidney donors, to generate donor-specific Tr1 cells of patients[85].

SCOPE OF THE THESIS

The group I worked with during my PhD identified a subset of tolerogenic dendritic cells, called DC-10, characterized by the ability to produce high levels of IL-10. DC-10 can be generated *in vitro* from monocytes in the presence of IL-10 and are potent inducers of adaptive IL-10-producing Tr1 cells. This ability is dependent on both IL-10 secretion and the co-expression at high levels of HLA-G, a non-classical HLA class I molecule, and its receptor immunoglobulin-like transcript 4 (ILT-4). *In vitro* generated DC-10 are currently used for generating Tr1 cells for a Treg-based cell therapy, but we are interested in using them also as DC-based cell therapy.

The aim of my thesis was to further characterized DC-10, both phenotypically and functionally. Specifically, two major lines of research have been investigated:

- **Molecular profile of DC-10 (Chapter 2 and 3):**
  To identify specific DC-10 markers, we performed DNA microarray-based global transcriptional profiling of *in vitro* differentiated DC-10, comparing them to mature DC (mDC), and differentially expressed genes (DEGs) were then validated in comparison to both mDC and immature DC (iDC). Identification of specific markers will allow *in vivo*

tracking of DC-10 in both physiological and pathological setting in the clinic.

Moreover, DC-10 miRNome profile was assessed by RT-PCR. Analysing together DEGs identified by DNA microarray and specifically up-regulated and down-regulated miRNAs will help in better defining the molecular profile of DC-10, highlighting which pathways are fundamental for their tolerogenic role.

- **Modulation of *in vitro* and *in vivo* allogeneic T cell responses by DC-10 (Chapter 4):**

  We investigated whether tolerogenic ability of DC-10 was also exerted on $CD8^+$ T cells, with co-culture experiments in allogeneic setting and we evaluated DC-10 immune-modulatory effects on both $CD4^+$ and $CD8^+$ allogeneic T cells in NSG mouse model. Results from this study will provide a rationale for the use of DC-10 as DC-based cell therapy in allogeneic context.

REFERENCES

1. Steinman, R. & Idoyaga, J. Features of the dendritic cell lineage. *Immunol Rev* **234,** 5–17 (2010).
2. Steinman, R. & Cohn, Z. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137,** 1142–1162 (1973).
3. Steinman, R. M., Kaplan, G., Witmer, M. D. & Cohn, Z. A. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J. Exp. Med.* **149,** 1–16 (1979).
4. Steinman, R. M. & Witmer, M. D. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice Immunology : *PNAS* **75,** 5132–5136 (1978).
5. Nussenzweig, M. & Steinman, R. Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* **151,** 1196–1212 (1980).
6. Hackstein, H. & Thomson, A. W. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat. Rev. Immunol.* **4,** 24–34 (2004).
7. Dieu, M. C. *et al.* Selective recruitment of immature and

mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* **188,** 373–386 (1998).

8. Tang, H. L. & Cyster, J. C. Chemokine Up-Regulation and Activated T Cell Attraction by Maturing Dendritic Cells. *Science (80-.).* **284,** 819–822 (2007).

9. Bousso, P. & Robey, E. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat. Immunol.* **4,** 579–585 (2003).

10. Miller, M. J. Imaging the Single Cell Dynamics of CD4+ T Cell Activation by Dendritic Cells in Lymph Nodes. *J. Exp. Med.* **200,** 847–856 (2004).

11. Hendriks, J. *et al.* CD27 is required for generation and long-term maintenance of T cell immunity. *Nat. Immunol.* **1,** 433–40 (2000).

12. Keller, A. M., Schildknecht, A., Xiao, Y., van den Broek, M. & Borst, J. Expression of Costimulatory Ligand CD70 on Steady-State Dendritic Cells Breaks CD8+ T Cell Tolerance and Permits Effective Immunity. *Immunity* **29,** 934–946 (2008).

13. Hawiger, D. *et al.* Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* **194,** 769–779 (2001).

14. Carter, L. L. *et al.* PD-1:PD-L inhibitory pathway affects

both CD4+ and CD8+ T cells and is overcome by IL-2. *Eur. J. Immunol.* **32,** 634–643 (2002).

15. Sharpe, A. H., Wherry, E. J., Ahmed, R. & Freeman, G. J. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat. Immunol.* **8,** 239–45 (2007).

16. Dzionek, A. *et al.* BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* **165,** 6037–46 (2000).

17. Collin, M., Mcgovern, N. & Haniffa, M. Human dendritic cell subsets. *Immunology* **140,** 22–30 (2013).

18. Macdonald, K. P. a *et al.* Characterization of human blood dendritic cell subsets. *Blood* **100,** 4512–4520 (2002).

19. Mittag, D. *et al.* Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status. *J. Immunol.* **186,** 6207–17 (2011).

20. Segura, E. *et al.* Characterization of resident and migratory dendritic cells in human lymph nodes. *J. Exp. Med.* **209,** 653–660 (2012).

21. Ziegler-Heitbrock, L. *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* **116,** e74-80 (2010).

22. Jongbloed, S. L. *et al.* Human CD141+ (BDCA-3)+

dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* **207,** 1247–60 (2010).

23. Poulin, L. F. *et al.* DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues. *Blood* **119,** 6052–6062 (2012).

24. Joffre, O. P., Segura, E., Savina, A. & Amigorena, S. Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* **12,** 557–569 (2012).

25. Bachem, A. *et al.* Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J. Exp. Med.* **207,** 1273–1281 (2010).

26. Lauterbach, H. *et al.* Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *J. Exp. Med.* **207,** 2703–17 (2010).

27. Sittig, S., Bakdash, G., Weiden, J. & Sköld, A. A comparative study of the T cell stimulatory and polarizing capacity of human primary blood dendritic cell subsets. *Mediators Inflamm.* (2016).

28. Siegal, F. P. *et al.* The Nature of the Principal Type 1 Interferon – Producing Cells in Human Blood. *Science (80-.*

). **284,** 1835–1838 (1999).

29. Strobl, H. *et al*. Identification of CD68+lin- peripheral blood cells with dendritic precursor characteristics. *J. Immunol*. **161,** 740–8 (1998).

30. Moseman, E. A. *et al*. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J. Immunol*. **173,** 4433–4442 (2004).

31. Ito, T. *et al*. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J. Exp. Med*. **204,** 105–15 (2007).

32. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. & Hill, A. M. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol*. **164,** 6166–73 (2000).

33. Nathan, B. Y. C. F., Murray, H. W., Wlebe, I. E. & Rubin, B. Y. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med*. **158,** 670–689 (1983).

34. Stein, M., Keshav, S., Harris, N. & Gordon, S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med*. **176,** 287–92 (1992).

35. Sica, A., Saccani, A. & Mantovani, A. Tumor-associated

macrophages: A molecular perspective. *Int. Immunopharmacol.* **2,** 1045–1054 (2002).

36. Biswas, S. K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* **11,** 889–896 (2010).

37. Billiau, A. & Matthys, P. Interferon-gamma: A historical perspective. *Cytokine Growth Factor Rev.* **20,** 97–113 (2009).

38. Fleetwood, A. J., Lawrence, T., Hamilton, J. A. & Cook, A. D. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J. Immunol.* **178,** 5245–5252 (2007).

39. Guha, M. & Mackman, N. LPS induction of gene expression in human monocytes. *Cell. Signal.* **13,** 85–94 (2001).

40. Nairz, M. *et al.* Nitric oxide-mediated regulation of ferroportin-1 controls macrophage iron homeostasis and immune function in Salmonella infection. *J. Exp. Med.* **210,** 855–73 (2013).

41. Podinovskaia, M., Lee, W., Caldwell, S. & Russell, D. G. Infection of macrophages with Mycobacterium tuberculosis induces global modifications to phagosomal function. *Cell.*

Microbiol. **15,** 843–859 (2013).

42. Kurowska-Stolarska, M. *et al*. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J. Immunol*. **183,** 6469–77 (2009).

43. Pesce, J. *et al*. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J. Clin. Invest*. **116,** 2044–55 (2006).

44. Mantovani, A., Vecchi, A. & Allavena, P. Pharmacological modulation of monocytes and macrophages. *Curr. Opin. Pharmacol*. **17,** 38–44 (2014).

45. Jaguin, M., Houlbert, N., Fardel, O. & Lecureur, V. Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell. Immunol*. **281,** 51–61 (2013).

46. Barros, M. H. M., Hauck, F., Dreyer, J. H., Kempkes, B. & Niedobitek, G. Macrophage polarisation: An immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS One* **8,** 1–11 (2013).

47. Munder, M., Eichmann, K. & Modollel, M. Alternative Metabolic States in Murine Macrophages Reflected by the Nitric Oxide Synthase/Arginase Balance: Competitive

Regulation by CD4 + T Cells Correlates with Th1/Th2 Phenotype. *J. Immunol.* **160,** 5347–5354 (1998).

48. Schebesch, C. *et al.* Alternatively activated macrophages actively inhibit proliferation of peripheral blood lymphocytes and CD4+ T cells in vitro. *Immunology* **92,** 478–86 (1997).

49. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–18 (1994).

50. Caux, C. *et al.* CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. *J. Exp. Med.* **184,** 695–706 (1996).

51. Gordon, J. R., Ma, Y., Churchman, L., Gordon, S. A. & Dawicki, W. Regulatory dendritic cells for immunotherapy in immunologic diseases. *Front. Immunol.* **5,** 7 (2014).

52. Penna, G. & Adorini, L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol.* **164,** 2405–11 (2000).

53. Chamorro, S. *et al.* TLR triggering on tolerogenic dendritic

cells results in TLR2 up-regulation and a reduced proinflammatory immune program. *J. Immunol.* **183,** 2984–94 (2009).

54. Kleijwegt, F. S. *et al.* Critical role for TNF in the induction of human antigen-specific regulatory T cells by tolerogenic dendritic cells. *J. Immunol.* **185,** 1412–8 (2010).

55. Unger, W. W. J., Laban, S., Kleijwegt, F. S., van der Slik, A. R. & Roep, B. O. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur. J. Immunol.* **39,** 3147–59 (2009).

56. Chu, C.-C. *et al.* Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *J. Exp. Med.* **209,** 935–45 (2012).

57. van der Aar, A. M. G. *et al.* Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. *J. Allergy Clin. Immunol.* **127,** 1532–40.e7 (2011).

58. Piemonti, L. *et al.* Glucocorticoids affect human dendritic cell differentiation and maturation. *J. Immunol.* **162,** 6473–81 (1999).

59. Xia, C.-Q., Peng, R., Beato, F. & Clare-Salzler, M. J. Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity. *Scand. J. Immunol.*

62, 45–54 (2005).

60. Bosma, B. M. *et al*. Dexamethasone transforms lipopolysaccharide-stimulated human blood myeloid dendritic cells into myeloid dendritic cells that prime interleukin-10 production in T cells. *Immunology* **125,** 91–100 (2008).

61. Volchenkov, R., Karlsen, M., Jonsson, R. & Appel, S. Type 1 regulatory T cells and regulatory B cells induced by tolerogenic dendritic cells. *Scand. J. Immunol.* **77,** 246–54 (2013).

62. Ferreira, G. B. *et al*. Differential protein pathways in 1,25-dihydroxyvitamin d(3) and dexamethasone modulated tolerogenic human dendritic cells. *J. Proteome Res.* **11,** 941–71 (2012).

63. Bell, G. M. *et al*. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann. Rheum. Dis.* **73,** no pagination (2016).

64. Haidinger, M. *et al*. A versatile role of mammalian target of rapamycin in human dendritic cell function and differentiation. *J. Immunol.* **185,** 3919–31 (2010).

65. Turnquist, H. R. *et al*. mTOR and GSK-3 shape the CD4+ T-cell stimulatory and differentiation capacity of myeloid DCs after exposure to LPS. *Blood* **115,** 4758–69 (2010).

66. Naranjo-Gómez, M. *et al*. Comparative study of clinical grade human tolerogenic dendritic cells. *J. Transl. Med*. **9,** 89 (2011).

67. Macedo, C., Turquist, H., Metes, D. & Thomson, A. W. Immunoregulatory properties of rapamycin-conditioned monocyte-derived dendritic cells and their role in transplantation. *Transplant. Res*. **1,** 16 (2012).

68. Macedo, C. *et al*. Rapamycin augments human DC IL-12p70 and IL-27 secretion to promote allogeneic Type 1 polarization modulated by NK cells. *Am. J. Transplant* **13,** 2322–33 (2013).

69. Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J. & Enk, A. H. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol*. **159,** 4772–80 (1997).

70. Boks, M. A. *et al*. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction--a comparative study of human clinical-applicable DC. *Clin. Immunol*. **142,** 332–42 (2012).

71. Moreau, P. *et al*. IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. *Int. Immunol*. **11,** 803–11 (1999).

72. Bellinghausen, I. *et al*. Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from

hydrocortisone-treated dendritic cells. *J. Allergy Clin. Immunol.* **108,** 242–9 (2001).

73. Steinbrink, K., Graulich, E., Kubsch, S., Knop, J. & Enk, A. H. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* **99,** 2468–76 (2002).

74. Zheng, Z. *et al.* Induction of T cell anergy by the treatment with IL-10-treated dendritic cells. *Comp. Immunol. Microbiol. Infect. Dis.* **27,** 93–103 (2004).

75. Kubsch, S., Graulich, E., Knop, J. & Steinbrink, K. Suppressor activity of anergic T cells induced by IL-10-treated human dendritic cells: association with IL-2- and CTLA-4-dependent G1 arrest of the cell cycle regulated by p27Kip1. *Eur. J. Immunol.* **33,** 1988–97 (2003).

76. Kryczanowsky, F., Raker, V., Graulich, E., Domogalla, M. P. & Steinbrink, K. IL-10-Modulated Human Dendritic Cells for Clinical Use: Identification of a Stable and Migratory Subset with Improved Tolerogenic Activity. *J. Immunol.* **197,** 3607–3617 (2016).

77. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–44 (2010).

78. Amodio, G. *et al.* HLA-G expressing DC-10 and CD4(+) T

cells accumulate in human decidua during pregnancy. *Hum. Immunol.* **74,** 406–11 (2013).

79. Amodio, G. & Gregori, S. Human tolerogenic DC-10: perspectives for clinical applications. *Transplant. Res.* **1,** 14 (2012).

80. Amodio, G. *et al.* HLA-G expression levels influence the tolerogenic activity of human DC-10. *Haematologica* **100,** 548–57 (2015).

81. Bacchetta, R. *et al.* Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–43 (2010).

82. Gregori, S., Roncarolo, M. G. & Bacchetta, R. in *Methods in Molecular Biology* **677,** 31- (2011).

83. Pacciani, V. *et al.* Induction of anergic allergen-specific suppressor T cells using tolerogenic dendritic cells derived from children with allergies to house dust mites. *J. Allergy Clin. Immunol.* **125,** 727–36 (2010).

84. Bacchetta, R. *et al.* Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. *Front. Immunol.* **5,** 16 (2014).

85. Petrelli, A. *et al.* Generation of Donor-specific T Regulatory Type 1 Cells From Patients on Dialysis for Cell Therapy

After Kidney Transplantation. *Transplantation* **99,** 1582–9 (2015).

CHAPTER 2

Co-expression of CD163 and CD141 identifies CD14^{+}CD16^{+} human circulating IL-10-producing dendritic cells

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ABSTRACT

DC-10 are a tolerogenic population of human IL-10-producing dendritic cells (DC), characterized by the expression of HLA-G and ILT-4, that efficiently induce antigen-specific T regulatory type 1 (Tr1) cells *in vitro*. DC-10 can be differentiated *in vitro* by culturing peripheral blood monocytes with GM-CSF, IL-4 and IL-10, are present *in vivo* in peripheral blood and secondary lymphoid organs, and are enriched in human decidua during pregnancy. Thus far, DC-10 have been identified by the co-expression of CD14, CD11c and CD83; however, these markers are not specific and the cell activation influences CD83 expression. To define specific markers improving the identification and selection of *in vivo* occurring DC-10, we performed gene expression profile of *in vitro* differentiated DC-10. We identified CD141 (BDCA-3) and CD163 being specifically expressed by *in vitro* differentiated DC-10 and we showed the specificity of these markers in peripheral blood of healthy volunteers. The co-expression of CD141 and CD163 in combination with CD14 and CD16 allowed the isolation of *in vivo* occurring DC-10 and their identification in peripheral blood. As well as being an important finding for the biology of DC-10, the use of these biomarkers makes it feasible to track DC-10 *in vivo* to further dissect their role in different physiological and pathological settings.

INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells with a unique ability to prime immune responses[1]. DC not only control immunity but also maintain homeostasis and regulate responses to self and non-self antigens[2]. This dual function, which ensure the integrity of the organism, is possible thanks to the presence of different DC subsets with specialized phenotype and functions: pro-inflammatory and tolerogenic DC. In human blood there are two major classes of DC, myeloid DC (myDC) and plasmacytoid DC (pDC)[3]. The former secrete IL-12 and TNF- α and are efficient in priming naïve $CD4^+$ and $CD8^+$ T cells[4,5]. In steady state conditions, circulating myDC have an immature phenotype and can induce tolerance. pDC are the major producers of type I IFN in response to viruses, have a less mature phenotype compared to myDC and can promote T regulatory (Treg) cells, an ability probably linked to the uptake of DNA released from apoptotic cells through TLR7[6–8].

In 2010 a new subset of DC was described in human blood and spleen [9]: the DC-10, a population of human tolerogenic DC. DC-10 has been then identified also in the decidua of pregnant women[10] and at higher frequency in blood of AML patients[11]. DC-10 can be differentiated *in vitro* by culturing peripheral

monocytes in the presence of IL-10[9] and are currently used to generate IL-10-producing T regulatory type 1 (Tr1) cells from cell-based therapies[12–14]. DC-10 express CD11c, CD14 and CD16, and have a mature myeloid phenotype, as confirmed by the expression of CD80, CD86, and HLA-DR. DC-10 produce high levels of IL-10 in the absence of IL-12, and this characteristic, together with the expression of HLA-G and ILT4 on cell membrane, is a critical factor involved in DC-10-mediated induction of Tr1 cells[9,15]. DC-10 were originally identified in blood and secondary lymphoid organs according to the co-expression of CD11c, CD14, and CD83[9]; however these markers are not specific for DC-10 and the expression of CD83 is highly influenced by the activation of the cells.

In the present study, we showed that *in vitro* and *in vivo* DC-10 expressed high levels of CD141 and CD163, in combination with CD14 and CD16. *Ex vivo* isolated $CD14^+CD16^+CD141^+CD163^+$ cells are functionally superimposable to *in vitro* differentiated DC-10: FACS-sorted DC-10 secrete IL-10, whose production is further increased by LPS stimulation, in absence of IL-12, DC-10 prime allogeneic naïve $CD4^+$ T cells, and induce an allo-specific IL-10-producing T cell population.

MATERIALS AND METHODS

Cell preparation

Human peripheral blood was obtained from healthy donors in accordance with local committee approval, and informed consent was provided for the use of blood samples according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated *via* density gradient centrifugation over Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Monocyte fraction of PBMC was enriched by a Percoll® Gradient (GE Healthcare, USA) as described previously[16].

DC differentiation

$CD14^+$ monocytes were isolated from PBMC by positive selection using CD14 MicroBeads (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Cells were cultured in RPMI 1640 (Lonza, Switzerland) supplemented with 10% FCS (Euroclone, Italy), 100 U/ml penicillin/streptomycin (Lonza, Switzerland), 2 mM L-glutamine (Lonza, Switzerland), supplemented with rhGM-CSF (Miltenyi Biotech, Germany) at 100 ng/ml, rhIL-4 (Miltenyi Biotech, Germany) at 10 ng/ml, and

rhIL-10 (CellGenix, Germany) at 10 ng/ml for 7 days to obtained DC-10. On day 3, half of differentiation medium was added to wells. On day 5, DC differentiated in the absence of rhIL-10 were matured by addition of 1 μg/ml of LPS (Sigma Aldrich, CA, USA) for additional 2 days to obtain mature DC (mDC) or left un-stimulated to generate immature DC (iDC). For microarray analysis, 1 μg/ml of LPS (Sigma Aldrich, CA, USA) was added at day 5 of DC-10 differentiation to generate "DC-10_LPS" cells from 4 donors. Resulting DC were recovered on day 7 and their phenotype was evaluated by flow cytometry.

Flow cytometry

DC were stained with antibodies against CD1a, CD14, CD16, HLA-DR, CD11c (Becton Dickinson, NJ, USA), CD141, (Miltenyi Biotech, German), CD163, CLEC4G (R&D system, MN, USA), CD35, FPR1(Becton Dickinson, NJ, USA), ENG (Biolegend, CA, USA). A two steps staining was performed for KITLG (Abcam, OR, USA) and LILRB5 (R&D system, MN, USA), with a secondary antibody donkey anti-rabbit (Biolegend, CA, USA) and anti-goat (R&D system, MN, USA), respectively. FcR Blocking Reagent (Miltenyi Biotec, German) is used to avoid not specific staining. Samples were acquired using a FACS Canto II Flow Cytometer (Becton Dickinson, CA, USA),

and data were analysed with FlowJo software (FlowJo, LLC Oregon, USA).

For sorting, cells were stained with anti-CD1c, anti-CD141 (Miltenyi Biotech, German), anti-CD11c, anti-CD163, anti-CD16 (Becton Dickinson, NJ, USA) and anti-CD14 (Biolegend, CA, USA) antibodies and sorted using a FACS Aria II Flow Cytometer (Becton Dickinson, CA, USA) with a 100 μm nozzle.

Microarray analysis

We differentiated mDC and DC-10 from peripheral blood monocytes of seven distinct healthy donors, and DC-10_LPS from four of them. We extracted total RNA with an RNeasy Mini kit (QIAGEN, CA, USA) according to manufacturer's instructions. We carried out the preparation of terminal-labeled complementary DNA (cDNA), hybridization to the whole-transcript GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) and scanning of the arrays according to the manufacturer's protocols (https://www. affymetrix.com). We preprocessed raw data with the robust multichip analysis (RMA) algorithm. We considered genes differentially expressed if $\log_2$ fold change values was >1 or <-1, with $P<0.05$. We performed all these steps using R and Bioconductor

RT-PCR

Cells were thawed as dry pellet, total RNA were extracted using RNeasy Kit (QIAGEN, CA, USA) and cDNA was synthesized with the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. cDNA from mDC, iDC and DC-10 was loaded in Low Density Taqman® cards with TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA) and PCR was performed on ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, CA, USA) following manufacturer's instructions. SDS 2.2.1 software was used to analyse data, using RPL0 as endogenous control. Quantification relative to endogenous control was carried out using the following formula: $\Delta CT = CT_{gene} - CT_{RPL0}$; relative gene expression = $2^{-\Delta CT}$.

T cell purification and culture

$CD4^+$ T cells were purified from PBMC by negative selection using the untouched $CD4^+$ T cell Isolation kit II (Miltenyi Biotech, Germany) according to the manufacturer's instructions. The recovered $CD4^+$ T cells were depleted of $CD45RO^+$ cells using anti-CD45RO-coupled magnetic beads (Miltenyi Biotech,

Germany). 10^4 DC were cultured with 10^5 allogeneic $CD4^+CD45RO^-$ T cells in 200 µl of X-VIVO 15 medium (Lonza, Switzerland), supplemented with 5% human serum (Sigma Aldrich, CA, USA), and 100 U/ml penicillin/streptomycin (Lonza, Switzerland). After 5 days, T cells were collected, washed, and analysed for their functions. For secondary mixed leucocyte reaction (MLR), PBMC autologous to sorted DC-10 and myDC were depleted of $CD3^+$ cells by Dynabeads® CD3 (Thermofisher, MA, USA) and used as stimulator at 1:1 ratio with allogeneic primed $CD4^+$ T cells.

Proliferation and activation of T cells

To analyse their proliferation, $CD4^+$ T cells were labelled with Cell Proliferation Dye eFluor® 670 (eBioscience, CA, USA) and stimulated with allogeneic sorted DC-10 or myeloid DC (10:1, T:DC) for 5 days. Co-culture supernatants were frozen for cytokine evaluation and T cells were collected and stained with anti-CD25 and anti-HLA-DR antibodies (Becton Dickinson, CA, USA). To measure $CD4^+$ T cell proliferation, dilution of the proliferation dye was evaluated by flow cytometry.

Cytokine determination

To measure IL-6, IL-10, IL-12, and TNF-α produced by sorted populations, 10^5 cells were plated per well in 100 µl final volume. Cells were left un-stimulated or activated with 200 ng/ml of LPS (Sigma, CA, USA) and supernatants were collected after 48h. Levels of IL-6, IL-10, IL-12 and TNF-α were determined by 4-plex Bio-Plex system according to the manufacturer's instructions (Bio-Rad, CA, USA).

To measure cytokine production by activated $CD4^+$ T cells, IFN-γ, GM-CSF and IL-10 levels were determined by BD OptEIA™ ELISA kit (Becton Dickinson, CA, USA).

Statistical analysis

Non-parametric Mann-Whitney U test for continuous variable or Wilcoxon matched pairs test (two-tailed) were used for statistical analysis. All results are presented as mean values±SD. Differences were regarded as significant at *P<0.05, **P<0.01, ***P<0.001 and ****P<0.001. Results were analysed using GraphPad Prism 5.0 (GraphPad Software, CA, USA).

RESULTS

DC-10 specific transcriptome profile is maintained upon LPS activation

We performed a microarray analysis of RNA from *in vitro* differentiated DC-10 on Affymetrix GeneChip® Human Whole Transcript, monitoring the expression of 28869 human genes. As control, mature DC (mDC) from the same healthy donors (HDs) were used. Results showed that the transcriptome profile of DC-10 is consistent among the 7 donors and is different from that of mDC (Fig.1A). Global hierarchical clustering analysis reveals two distinct clusters, corresponding to DC-10 and mDC. According to a $\log_2$ fold change ($\log_2$FC) and P values, we identified 1971 differentially expressed genes (DEGs) in DC-10 as compared to mDC, 932 up-regulated and 1039 down-regulated (Fig.1B). The higher expression of CD14, FCGR3A (encoding CD16), LILRB2 (encoding ILT-4), and IL-10, and the lower expression of IL-12B (encoding IL-12p40) (Suppl. Fig. 1) in DC-10 compared to mDC validated the microarray expression data. To study DC-10 stability, we also analysed the transcriptome profile of DC-10 activated with LPS. Results showed only 505 DEGs between DC-10 un-stimulated and LPS-activated, while the differences in the transcriptome of DC-10 with mDC were maintained (1791 DEGs) (Fig.1B). Thus, DC-10

are a stable population of DC with a defined transcriptional profile that differ from that of classical mDC.

CD141 and CD163 allows *in vivo* identification of DC-10

To identify DC-10 specific markers, among the 1971 DEGs, we focused on genes encoding for protein expressed on the cytoplasmatic membrane. We selected 17 genes that were over-expressed in *in vitro* differentiated DC-10 and showed the highest P value ($p<0.01$) compared to mDC (Suppl Fig.2A) The expression of these genes was validated in RT-PCR on *in vitro* differentiated DC-10 and mDC from additional 4 HDs. Results showed that the expression of the selected genes was consistent among donors and, with the exclusion of PLXNA2 and ABCC2, all genes were confirmed to be differentially expressed between *in vitro* differentiated DC-10 and mDC (Fig.2A). To further select specific marker of DC-10, we investigated the expression of the validated 15 genes in *in vitro* differentiated immature DC (iDC) from the same donors, since iDC are known to be tolerogenic cells[17]. Results indicated that all these 15 genes were differentially expressed in DC-10 compared to iDC (Fig. 2B). We selected genes with a mean fold change expression in DC-10 compared to iDC higher than 10 for further validation (Table 1).

We evaluated the protein expression of the eight top ranking genes on *in vitro* differentiated DC-10 from additional new HDs. DC-10 significantly expressed higher levels of TBHD(CD141) (89.91±5.85%, mean±SD, n=9, p=0.004), CD163 (86.59±5.24%, mean±SD, n=9, p=0.004), and CLEC4G (90.75±9.29%, mean±SD, n=7, p=0.031 and p=0.016) compared to both iDC and mDC differentiated *in vitro* from the same donors (Fig.3A). DC-10 expressed CR1 (CD35) (54.36±32.04%, mean±SD, n=7), FPR1 (66.53±32.68%, mean±SD, n=7), and LILRB5 (LIR8) (60.48±16.55%, mean±SD, n=5) at variable levels, which resulted significantly higher compared to mDC for all markers and to iDC for FPR1 and LIR8. Notably, DC-10 are a very homogenous population of cells at the end of differentiation, therefore the latter three proteins were excluded for being DC-10 specific markers for the variability of the expression levels observed among donors tested (Fig.3A). Although the expression level of ENG (CD105) on DC-10 (91.40±12.93%, mean±SD, n=6) was statistically different compared to both iDC and mDC (p=0.03), this protein could not be considered specific for DC-10, since it was present at lower levels on both mDC (35.28±27.55%, mean±SD, n=6) and iDC (29.33±26.24%, mean±SD, n=6) (Fig.3A). Finally, KITLG was not validated at protein level. These results indicate that *in vitro* differentiated DC-10 specifically expressed high levels of CD141, CD163, and

CLEC4G.

We next investigated the expression of these selected biomarkers on peripheral blood mononuclear cells (PBMC), focusing on the $CD14^{+}CD16^{+}$ population that contains DC-10 [9], $CD14^{+}CD16^{-}$ classical monocytes, and $CD14^{low}CD16^{+}$ non-classical monocytes (Fig.3B). Results showed that $CD14^{+}CD16^{+}$ expressed CD141 at significantly higher levels (57.66±12.80%, mean±SD, n=9, p=0.004) compared to both $CD14^{+}CD16^{-}$ and $CD14^{low}CD16^{+}$ cells (36.99±17.52%, and 19.08±9.49%, mean±SD, n=9, respectively). CD163 was highly expressed on both $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{-}$ cells (92.65±10.07%, and 94.06±5.01%, mean±SD, n=8, p=0.008), while its expression levels on $CD14^{low}CD16^{+}$ monocytes were significantly lower (44.63±21.90%, mean±SD, n=8). CLEC4G was equally expressed in $CD14^{+}CD16^{-}$ (20.46±14.18%, mean±SD, n=7), $CD14^{low}CD16^{+}$ (16.34±5.20%, mean±SD, n=7) and $CD14^{+}CD16^{+}$ (19.85±8.18%, mean±SD, n=7) populations. Therefore, none of the above markers are selective for *in vivo* occurring DC-10, however the combination of CD141 and CD163 with CD14 and CD16 allow DC-10 identification. DC-10 and classical monocytes could be discriminated by the expression of CD16, but activated monocytes up-regulate CD16 expression. On the contrary, CD141 is not up-regulated upon activation in classical monocytes and thus it can be used to

distinguish DC-10 from classical monocytes (Fig.3C). CD163 instead discriminates DC-10 from non-classical monocytes. Based on these analyses we concluded that the co-expression of CD14, CD16, CD141 and CD163 allow the identification of DC-10 in peripheral blood.

$CD14^+CD16^+CD141^+CD163^+$ DC-10 produce IL-10 in absence of IL-12

DC-10 were sorted according to the co-expression of CD14, CD16, CD141, and CD163 from PBMC of HDs. After gating according to physical parameters, we selected $CD16^+$ cells, regardless of CD14 expression levels, and $CD141^+CD163^+$ cells were FACS-sorted (suppl. Fig. 3S). As control, $CD14^+CD16^-$ classical monocytes and $CD16^+CD163^-$ non-classical monocytes were isolated from PBMC of the same HDs. DC-10 were the only cell population that at steady state secreted IL-10 (526.5±476.3 pg/ml, mean±SD, n=6), and its levels increased upon LPS activation (880.9±667 pg/ml, mean±SD, n=6) (Fig.4B). As expected, classical monocytes secreted IL-10 upon LPS activation at levels comparable to that of DC-10 (752.6±297.8 pg/ml, mean±SD, n=6), whereas non-classical monocytes did not. DC-10 did not secreted IL-12 in steady state and upon LPS stimulation, with the exception of one out of six

donors tested. Moreover, DC-10 produced IL-6 at similar levels of classical monocytes at steady state (33.03±4.20 and 29.90±13.22 ng/ml, mean±SD, n=6, respectively), and upon activation, IL-6 production by DC-10 was comparable to that of classical and non-classical monocytes stimulated with LPS (34.58±3.25, 39.17±2.82, and 29.06±8.18 ng/ml, mean±SD, n=6). Finally, DC-10 secreted higher levels of TNF-α compared to classical monocytes, both at steady state (3.13±0.31 and 0.6±0.67 ng/ml, mean±SD, n=6, respectively) and upon activation (10.79±8.07 and 3.23±1.13 ng/ml mean±SD, n=6, respectively). Instead, TNF-α secretion by non-classical monocytes is lower at steady state (0.96±0.86 ng/ml, mean±SD, n=6) and higher upon LPS stimulation (22.59±26.21 ng/ml, mean±SD, n=6,) compared to DC-10. Overall, these data indicate that $CD14^+CD16^+CD141^+CD163^+$ DC-10 display a cytokine profile characterized by a high IL-10/IL-12 ratio, superimposable to that of *in vitro* differentiated DC-10[9,15].

$CD14^+CD16^+CD141^+CD163^+$ DC-10 induce low activation and proliferation of allogeneic naïve $CD4^+$ T cells

FACS-sorted $CD14^+CD16^+CD141^+CD163^+$ DC-10 were then used to stimulate allogeneic naïve $CD4^+$ T cells. As control, we used myeloid DC (myDC) sorted based on the co-expression of CD1c and CD11c. After 5 days of culture, significant lower levels of CD25 and HLA-DR were observed on DC-10-stimulated $CD4^+$ T cells (10.34±9.56% and 6.84±5.88%, mean±SD, n=6, respectively) compared to cells primed with myDC from the same donor (35.97±19.16% and 25.75±8.49%, mean±SD, n=6, respectively) (Fig.5A). A significant reduction in proliferation of 82.41±11.76% (mean±SD, n=8, p=0.0078) was observed in DC-10-stimulated $CD4^+$ T cells (5.82±5.86%, mean±SD, n=8) compared to myDC-stimulated cells (31.64±10.64%, mean±SD, n=8) (Fig.5B). Moreover, the secretion of IFN-γ and GM-CSF by activated T cells were also significantly lower in culture with DC-10 (1367±1117 pg/ml and 299.5±131.9 pg/ml, mean±SD, n=8, respectively) compared to those with myDC (580.4±128.0 pg/ml and 3100±1635 pg/ml, mean±SD, n=8) (Fig.5C). These findings indicate that FACS-sorted $CD14^+CD16^+CD141^+CD163^+$ DC-10, similarly to *in vitro* differentiated DC-10[9] poorly activated allogeneic naïve $CD4^+$ T cells and hypo-stimulated their proliferation and cytokine production compared to myDC. Notably, when $CD4^+$ T cells primed with DC-10 were re-stimulated with the same allogeneic antigen used for their priming, produce IL-10 (798.3±255.2 pg/ml, mean±SD, n=3), at higher levels compared to T cells

primed with myDC (261.0±299.4 pg/ml, mean±SD, n=3). These findings indicate that FACS-sorted $CD14^+CD16^+CD141^+CD163^+$ DC-10 induce a $CD4^+$ T cell differentiation towards an IL-10 producing population.

DISCUSSION

We showed that the combined expression of CD141 and CD163 with CD14 and CD16 identifies human tolerogenic DC-10. $CD14^+CD16^+CD141^+CD163^+$ cells secrete spontaneously, and upon activation, IL-10 in the absence of IL-12, stimulate allogeneic naïve $CD4^+$ T cells at lower levels compared to pro-inflammatory myeloid DC, and induce IL-10-producing $CD4^+$ T cells. The co-expression of CD14, CD16, CD141 and CD163 is specific for DC-10 since classical and non-classical monocytes, as well as myeloid DC do not co-express these markers. The combined expression of CD14, CD16, CD141 and CD163 can be used to purify DC-10 and track these cells in different physiological and pathological conditions in humans.

The expression of CD141 characterized a population of circulating myeloid DC, the classical BDCA-3^+ myDC[5,18]. Despite sharing CD141 expression, DC-10 and classical BDCA-3^+ myDC differ in several aspects. Classical BDCA-3^+ myDC are lineage negative cells, thus they do not express either CD14 or CD16; display a cytokine profile mainly characterized by the expression of IL-12[4], with an IL-10/IL-12 ratio opposite to that of DC-10[19]; stimulate high proliferation and IFN-γ secretion by allogeneic $CD4^+$ T cells, and IFN-γ levels are even higher compared to that induced by classical $CD1c^+$ myDC[5]. In the

dermis of human skin, it has been described a population of $CD14^+CD141^+$ DC that share some features with DC-10, including the constitutive IL-10 secretion and the ability to induce T cell hypo-responsiveness. Both skin $CD14^+CD141^+$ DC and DC-10 promote T regulatory (Treg) cells differentiation, but, while the former induce $FOXP_3$ Treg cells, the latter promote T regulatory T type 1 (Tr1) cells. Chu et al. suggested that skin $CD14^+CD141^+$ DC have an essential role in the maintenance of skin homeostasis and in the regulation of both systemic and tumor alloimmunity[20].

The scavenger receptor CD163 is a molecule strictly linked to IL-10: its expression can be induced by IL-10[21], and the engagement of CD163 elicited potent IL-10 secretion[22]. Therefore, CD163 expression on DC-10 is not surprising and it has been previously shown[9]. CD163 is commonly found at high levels on various tissue resident macrophages that are polarized towards M2-like phenotype. This is not the only characteristic that DC-10 shared with M2 macrophages: DC-10 showed up-regulation in anti-inflammatory and pro-angiogenetic factors, and have an interesting matrix remodeling signature (data not shown) However, DC-10 are blood circulating cells, with a classical DC morphology, express TLR3, which can be found only on DC[23], and are able to prime efficiently naïve $CD4^+$ T cells, even though in a tolerogenic fashion[9]. Interestingly,

$CD14^+CD163^{high}$ $CD160^{high}$ human myeloid cells has been recently identified in intestinal lamina propria: these cells produce high levels of IL-10 and suppress effector T cell proliferation via a $Foxp3^+$Treg-independent mechanism. These DC are reduced in number and impaired in their suppressive activity in ulcerative colitis, suggesting an immune-regulating role in gut homeostasis[24].

We have previously identified DC-10 in blood and secondary lymphoid organ as a subset of $CD11c^+CD14^+$ monocytes that expressed CD83[9]. In the present study we selected DC-10 as $CD14^+CD16^+CD141^+CD163^+$ cells, which are superimposable to $CD11c^+CD14^+CD83^+$ DC-10 in term of phenotype and stimulatory ability. $CD14^+CD16^+CD141^+CD163^+$ cells were indeed $CD11c^+$, HLA-G^+ and ILT-4^+ and induced naïve $CD4^+$ T cell proliferation at similar levels compared to $CD11c^+CD14^+CD83^+$ DC-10 (data not shown). The advantage of CD14, CD16, CD141, and CD163 is to avoid the use CD83, whose expression is variable among donors and is influenced by activation status of the cells: classical monocytes can indeed up-regulate CD83 expression upon stimulation. Conversely, CD141 and CD163 are stably expressed on DC-10 and is neither express nor can be induced on classical and non-classical monocytes, respectively.

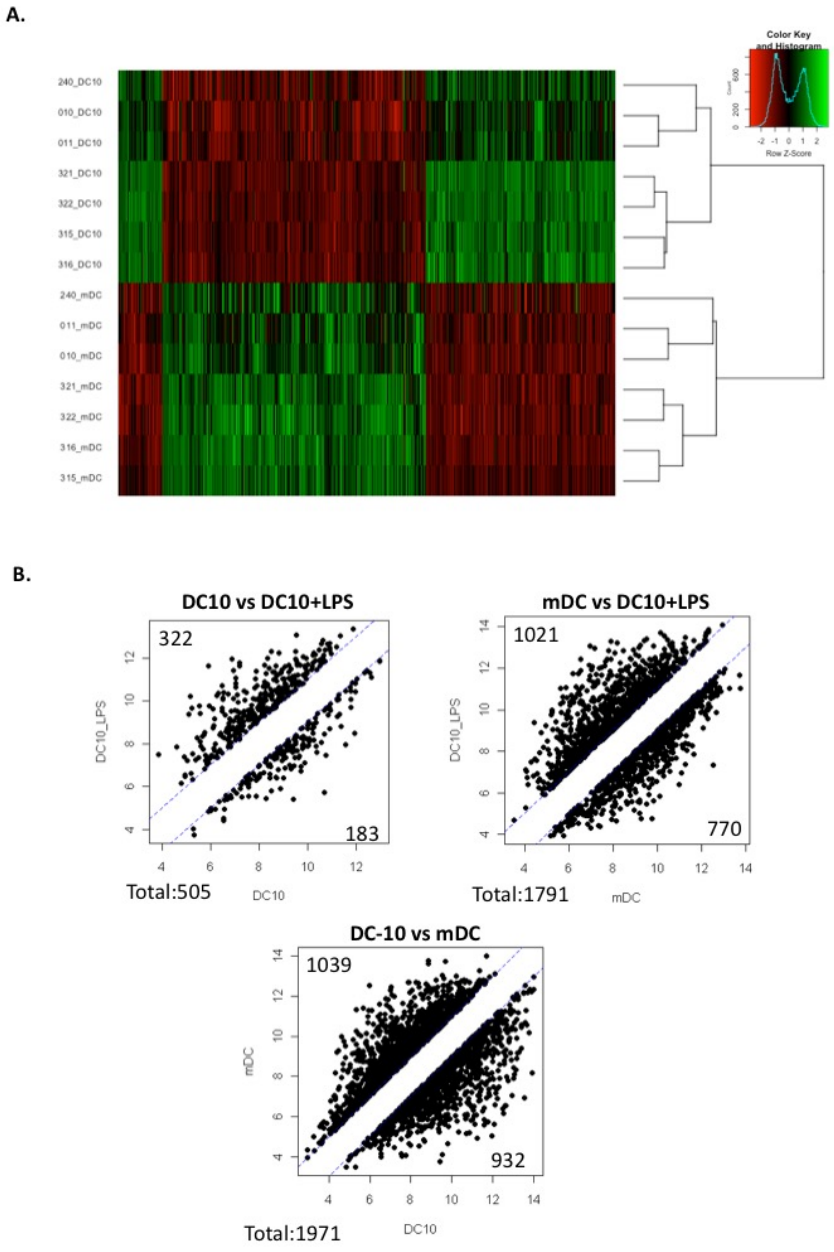
Ex vivo sorted $CD14^+CD16^+CD141^+CD163^+$ DC-10 demonstrate

functional activities similar to *in vitro* differentiated DC-10. They indeed presented the same unique cytokine profile, with high IL-10/IL-12 ratio, and both IL-6 and TNF-α secretion independently from LPS stimulation. Similar to *in vitro* differentiated DC-10, *ex vivo* sorted DC-10 prime naïve $CD4^+$ T cells, inducing a low activation and proliferation. Moreover, DC-10-primed $CD4^+$ T cells when re-stimulated with the same allogeneic antigen used in priming (allo-Ag) secrete IL-10. Thus, *ex vivo* sorted DC-10 are able to induce an allo-Ag specific IL-10 producing $CD4^+$ T cell population, resemble their *in vitro* differentiated counterpart. Further analyses will investigate whether $CD14^+CD16^+CD141^+CD163^+$ DC-10 promote allo-specific Tr1 cells.

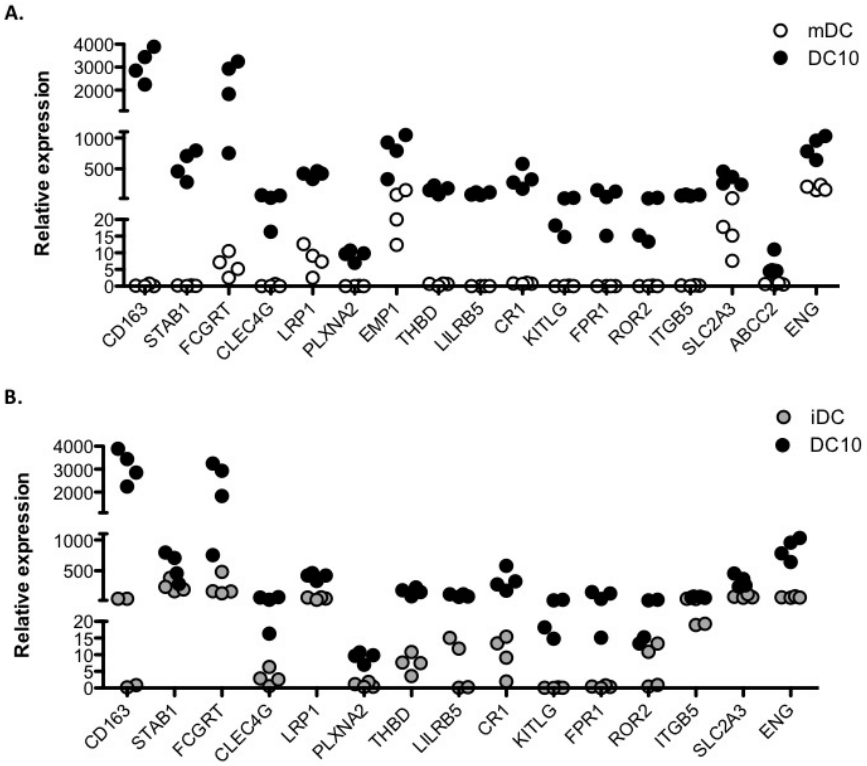
The identified combination of markers will allow *in vivo* tracking of DC-10 in physiological conditions and the study of their contribution to different organs homeostasis. DC-10 express CCR9[9], which is a gut homing receptor, and the identification of $CD14^+CD163^{high}CD160^{high}$: tolerogenic cells in the intestinal lamina propria[24] suggests the presence of DC-10 in this organ and probably an active role in the maintenance of gut homeostasis. Moreover, we are now able to evaluate the potential alteration in DC-10 frequency and functions in different clinical setting and how this could impact disease prognosis.

In summary, we have discovered two markers, CD141 and CD163, whose expression combined with CD14 and CD16 allow the identification of tolerogenic DC-10 in humans. These markers make it possible to study the *in vivo* localization of DC-10 in different physiological and pathological setting in humans, and the role of DC-10 in subjects with T cell mediated diseases.

FIGURES



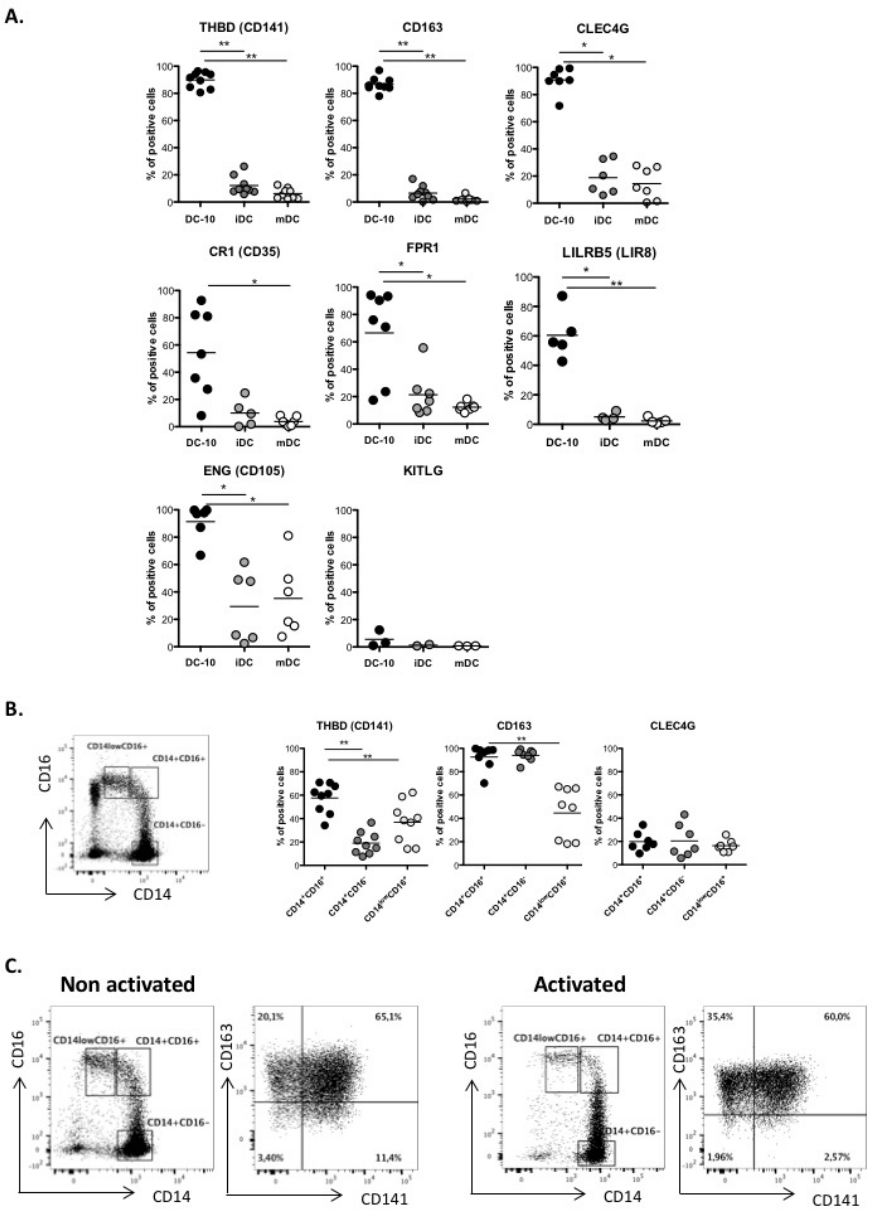
Comi M. et al., Figure 1



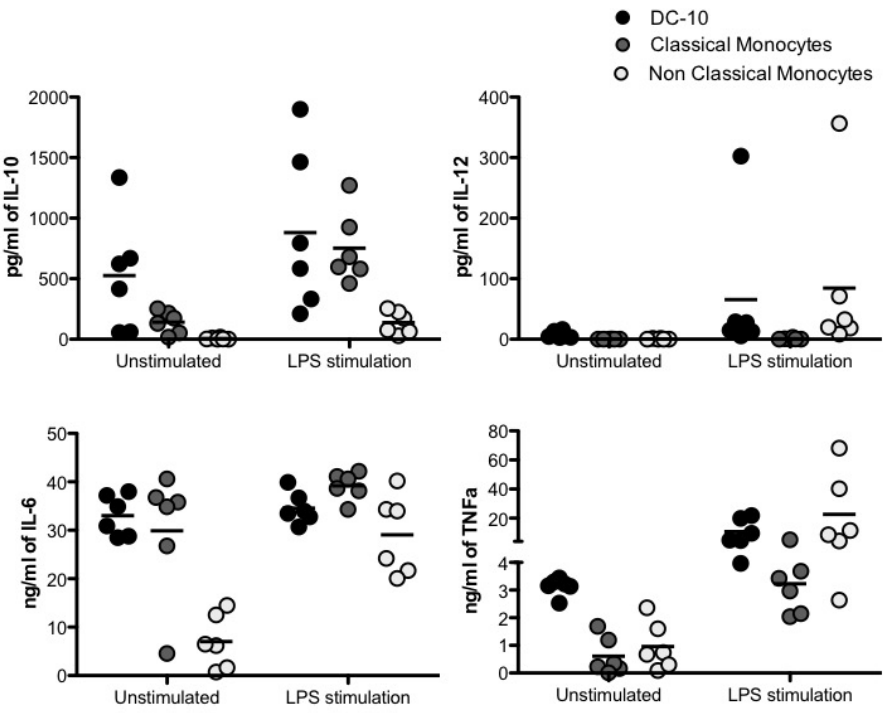
Comi M. et al., Figure 2

Gene	Fold Change to iDC
CD163	**240**
KITLG	**135**
FPR1	**126**
LILRB5	**62**
CR1	**40**
THBD	**22**
CLEC4G	**17**
ENG	**12**
LRP1	8
FCGRT	7
ROR2	7
SLC2A3	4
ITGB5	2
EMP1	2
STAB1	2

Comi M. et al., Table 1



Comi M. et al., Figure 3



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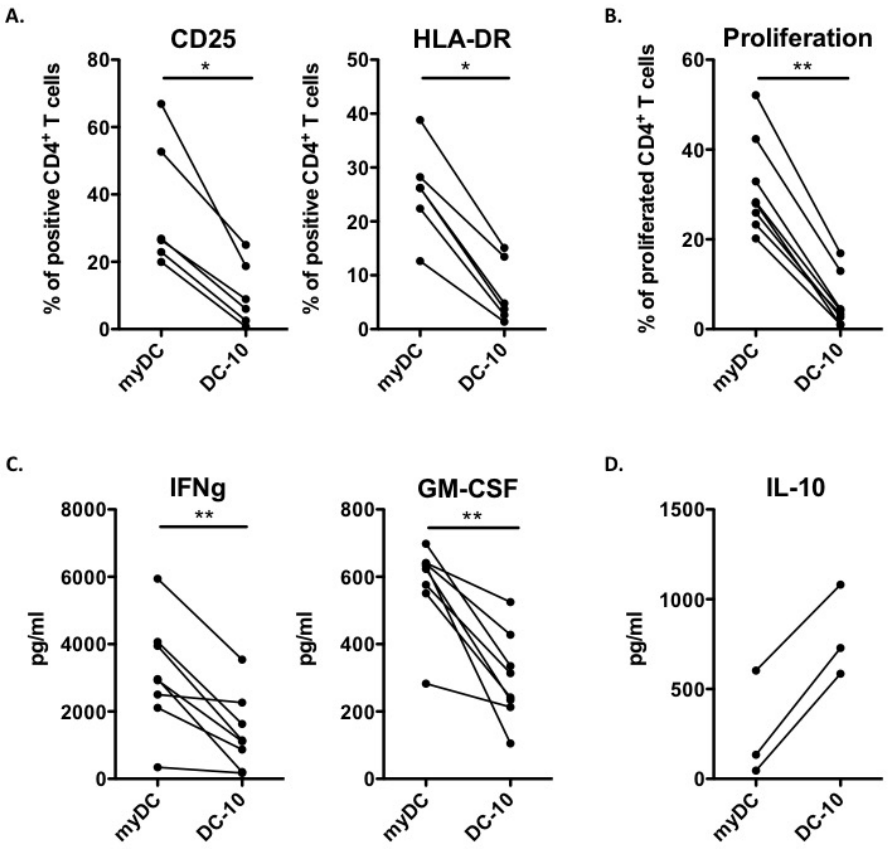

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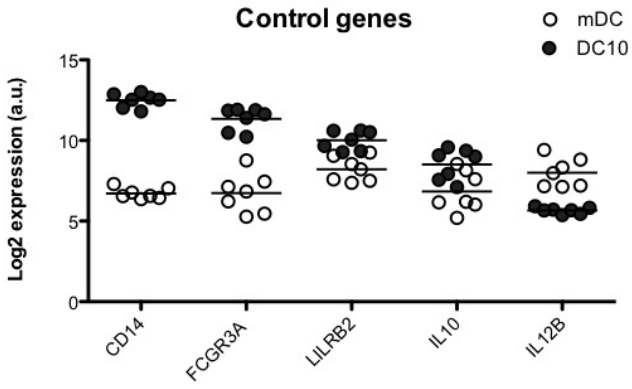
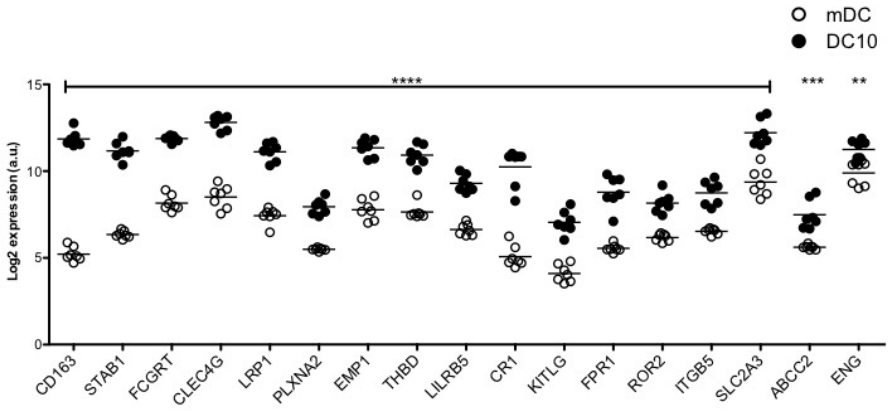
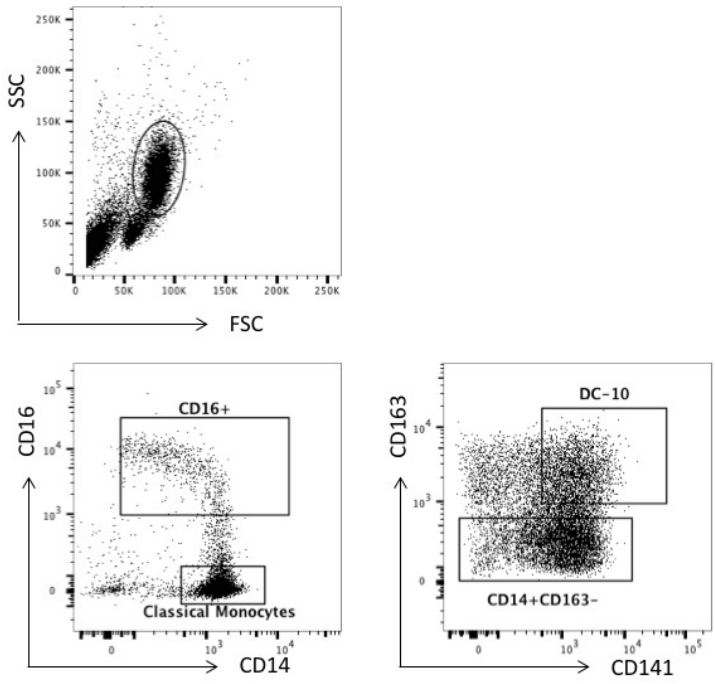


Comi M. et al., Figure S1



Comi M. et al., Figure S2



Comi M. et al., Figure S3

FIGURE LEGENDS

Figure 1. DC-10 specific transcriptome profile. A. Two-dimensional heatmaps of all significant (false discovery rate <0.05) differentially expressed genes. Genes in red are expressed at higher levels compared to the mean signal intensities in all experiments, genes in green are down-regulated, and genes in black have signal intensities close to the mean expression level. Genes are in columns and the samples are in rows. Data in rows have been clustered. **B.** Scatterplot of differential expression between mDC and DC-10. Each dot represents the averaged Log_2 expression of a single gene in mDC and DC-10. Dashed diagonal lines indicate the thresholds for Fold Change (FC>1 or FC<-1).

Figure 2. Validation and selection of putative DC-10 markers in RT-PCR. DC-10, mDC and iDC were differentiated *in vitro* from peripheral blood monocytes of 4 healthy donors. Relative mRNA amounts from DC-10, mDC (A) and iDC (B) were normalized to housekeeping gene RPL0 (deltaCt), and the relative expression values were calculated as 2^{-}

$^{(deltaCt)}*10^3$. Numbers represent arbitrary units. Each dot represents a single donor.

Table 1. Selection of top ranking genes differentially expressed in DC-10 compared to iDC. Averaged fold change expression of genes from Figure 2B in DC-10 compared to iDC are indicated in the table.

Figure 3. Co-expression of CD14, CD16, CD141 and CD163 identifies DC-10. A. DC-10, mDC and iDC were differentiated *in vitro* from peripheral blood monocytes of healthy donors. Expression of indicated markers was measured by flow cytometry. Each dot represents a single donor and lines indicate mean. *P≤0.05, **P≤0.01. **B.** Expression of CD141, CD163 and CLEC4G was evaluated on three different population of PBMC: $CD14^+CD16^+$ population that contains DC-10, $CD14^+CD16^-$ classical monocytes and $CD14l^{ow}CD16^+$ non-classical monocytes. Each dot represents a single donor and lines indicate mean. **P≤0.01. **C.** Expression of CD141 and CD163 (right) measured on $CD14^+CD16^+$ populations in non-activated and activated PBMC (left) are shown. The percentages of cells in each quadrant are indicated.

Figure 4. *Ex vivo* DC-10 produce IL-10 in absence of IL-12. DC-10, classical and non-classical monocytes were sorted from HD peripheral blood and were left un-stimulated or stimulated with LPS for 48 hours. Concentration levels of IL-10, IL-12, TNF-a, and IL-6 in culture supernatants of the indicated cells were evaluated by 4-plex BioPlex. Each dot represents a single donor and lines indicate mean

Figure 5. *Ex vivo* DC-10 induce low activation and proliferation of allogeneic naïve CD4$^+$ T cells. Naïve CD4$^+$ T cells were cultured with allogeneic DC-10 or myDC (ratio 10:1) for 5 days. **A.** Activation status of CD4$^+$ T cells were evaluated by CD25 and HLA-DR staining. Numbers indicate percentages of positive cells gated on CD4$^+$. Each dot represents a single donor *P < 0.05 **B.** Proliferative responses were evaluated by dye dilution at FACS. Each dot represents a single donor. **P < 0.01. **C.** IFN-γ and GM-CSF in culture supernatants were measured by ELISA. Each dot represents a single donor. **P < 0.01. **D.** Naive CD4$^+$ T cells cultured for 7 days with DC-10 or myDC were re-stimulated for 3 days with CD3$^-$ cells, autologous to sorted DC. IL-10 in culture

supernatants was measured by ELISA. Each dot represents a single donor.

Figure 1s. Validation of samples for transcriptome profiling and of DNA microarray results. DC-10 and mDC were differentiated *in vitro* from peripheral blood monocytes of 7 healthy donors. Log_2 expression of CD14, FCGR3A, LILRB2, IL-10 and IL12B determined by the DNA microarray is shown. Each dot represents a single donor and lines indicate mean.

Figure 2s. Selection of putative DC-10 markers by DNA microarray results. Log2 expression of DEGs encoding for surface molecules determined by the DNA microarray is shown. Each dot represents a single donor and lines indicate mean. **P≤0.01, ***P≤0.001 and ****P≤0.0001.

Figure 3s. Gating strategy for *ex vivo* DC-10 isolation.

DC-10 were identified in the peripheral blood according to FSC^{high} SSC^{high} physical parameters and CD14, CD16, CD141 and CD163 co-expression. Dot plots from one representative donor are shown.

REFERENCES

1. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **392,** 245–52 (1998).
2. Waisman, A., Lukas, D., Clausen, B. E. & Yogev, N. Dendritic cells as gatekeepers of tolerance. *Semin. Immunopathol.* 1–11 (2016). doi:10.1007/s00281-016-0583-z
3. Ziegler-Heitbrock, L. *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* **116,** e74-80 (2010).
4. Mittag, D. *et al.* Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status. *J. Immunol.* **186,** 6207–17 (2011).
5. Jongbloed, S. L. *et al.* Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* **207,** 1247–60 (2010).
6. Siegal, F. P. *et al.* The Nature of the Principal Type 1 Interferon – Producing Cells in Human Blood. *Science (80-.).* **284,** 1835–1838 (1999).
7. Moseman, E. A. *et al.* Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the

generation of CD4+CD25+ regulatory T cells. *J. Immunol.* **173,** 4433–4442 (2004).

8. Ito, T. *et al.* Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J. Exp. Med.* **204,** 105–15 (2007).

9. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–944 (2010).

10. Amodio, G. *et al.* HLA-G expressing DC-10 and CD4(+) T cells accumulate in human decidua during pregnancy. *Hum. Immunol.* **74,** 406–11 (2013).

11. Locafaro, G. *et al.* HLA-G expression on blasts and tolerogenic cells in patients affected by acute myeloid leukemia. *J. Immunol. Res.* **2014,** 636292 (2014).

12. Bacchetta, R. *et al.* Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–43 (2010).

13. Amodio, G. & Gregori, S. Human tolerogenic DC-10: perspectives for clinical applications. *Transplant. Res.* **1,** 14 (2012).

14. Petrelli, A. *et al.* Generation of Donor-specific T Regulatory Type 1 Cells From Patients on Dialysis for Cell Therapy

After Kidney Transplantation. *Transplantation* **99,** 1582–9 (2015).

15. Amodio, G. *et al*. Hla-g expression levels influence the tolerogenic activity of human DC-10. *Haematologica* **100,** 548–557 (2015).

16. Repnik, U., Knezevic, M. & Jeras, M. Simple and cost-effective isolation of monocytes from buffy coats. *J. Immunol. Methods* **278,** 283–92 (2003).

17. Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A. H. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* **192,** 1213–22 (2000).

18. Macdonald, K. P. a *et al*. Characterization of human blood dendritic cell subsets. *Blood* **100,** 4512–4520 (2002).

19. Gupta, M. R., Kolli, D. & Garofalo, R. P. Differential response of BDCA-1 + and BDCA-3 + myeloid dendritic cells to respiratory syncytial virus infection. *Respir. Res.* **14,** 1–14 (2013).

20. Chu, C.-C. *et al*. Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *J. Exp. Med.* **209,** 935–45 (2012).

21. Williams, L., Jarai, G., Smith, A. & Finan, P. IL-10 expression profiling in human monocytes. *J. Leukoc. Biol.* **72,** 800–9 (2002).

22. Philippidis, P. *et al.* Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. *Circ. Res.* **94,** 119–26 (2004).

23. Muzio, M. *et al.* Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol.* **164,** 5998–6004 (2000).

24. Barman, S. *et al.* Identification of a human intestinal myeloid cell subset that regulates gut homeostasis. *Int. Immunol.* **28,** 533–545 (2016).

CHAPTER 3

HLA-G expression levels influence the tolerogenic activity of human DC-10

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ABSTRACT

Human Leukocyte Antigen (HLA)-G is a non-classical HLA class I molecule with known immune-modulatory functions. Our group identified a subset of human dendritic cells, named DC-10, that induce adaptive IL-10-producing T regulatory type 1 (Tr1) cells via the IL-10-dependent HLA-G/ILT4 pathway. In this study we aimed at defining the role of HLA-G in DC-10-mediated Tr1 cell differentiation. We analyzed phenotype, functions, and genetic variations in the 3' un-translated region of the HLA-G locus of in vitro differentiated DC-10 from 67 healthy donors. We showed that HLA-G expression on DC-10 was donor-dependent. Functional studies demonstrated that DC-10, independently from the HLA-G expression, secrete IL-10 and negligible levels of IL-12. Interestingly, DC-10 with high HLA-G promoted allo-specific anergic T cells that contain a significantly higher frequency of Tr1 cells, defined as IL-10-producing (P=0.0121) or $CD49b^{+}LAG\text{-}3^{+}$ (P=0.0031) T cells, compared to DC-10 with low HLA-G. We found that the HLA-G expression on DC-10 was genetically imprinted, being associated with specific variations in the 3' un-translated region of the gene, and it may be finely tuned by miRNA-mediated post-transcriptional regulation. These data highlight the important role of HLA-G to boost DC-10 tolerogenic activity and confirm that IL-10 production by DC-10 is necessary but not

sufficient to promote Tr1 cells at high frequency. These new insights on the role of HLA-G in DC-10-mediated induction of Tr1 cells provide additional information for their clinical use in future Tr1- or DC-10-based cell therapy approaches.

INTRODUCTION

HLA-G is a non-classical HLA class I molecule originally identified and characterized in trophoblasts where it confers protection to the semi-allogeneic fetus from the maternal immune system[1,2]. More recently, it has become evident that HLA-G plays a role in promoting and maintaining tolerance in autoimmunity and organ transplantation, and immune escape in cancer and infectious diseases. HLA-G is characterized by a relatively low allelic polymorphism and a highly restricted tissue distribution in comparison with classical HLA class I molecules. In healthy condition, in addition to trophoblasts, HLA-G expression is restricted to amniotic cells, adult thymic medulla, erythroblasts, cornea, pancreatic islets, and endothelial and stem cells[3]. By alternative splicing of a single mRNA transcript, seven different HLA-G isoforms, four membrane-bound (HLA-G1, -G2, -G3, and -G4) and three soluble (HLA-G5, -G6, and -G7) can be generated[4,5]. Moreover, the activity of matrix metalloproteinases (MMPs) is responsible for the proteolytic cleavage of HLA-G1 from the membrane, and the release of shed HLA-G1[6]. Thus far, the best-characterized isoforms are HLA-G1, the most stable membrane-bound isoform, HLA-G5, and shed HLA-G1.

Several polymorphisms are present both in the promoter and in the 3' un-translated region (UTR) of the HLA-G gene that can

influence its expression[7]. The best-characterized polymorphism in the 3'UTR is the insertion/deletion (Ins/Del) of 14 base pairs (bp), which has been respectively associated with decreased/increased mRNA stability[8-10]. The +3142 G/C single nucleotide polymorphism (SNP) has been also proposed to control HLA-G expression, since the presence of G may increase the affinity of mRNA for the microRNA (miR)-148a, miR-148b and, miR-152[11,12]. Additional less studied SNPs are present in the 3'UTR at positions +3001 T/C, +3003 T/C, +3010 G/C, +3027 C/A, +3035 C/T, and +3196 C/G[13], which represent potential miRNA-binding sites, and thus may regulate HLA-G expression[14], and the +3187 A/G located in proximity to an AU-rich motif, which affects mRNA stability[15]. These polymorphisms cluster at least in 15 different haplotypes identified in Brazilian populations[13,16], and a number of less frequent haplotypes found in other populations[17,18].

The functions of HLA-G are oriented towards immune inhibition and induction of tolerance. Through the direct binding to the inhibitory receptors Immunoglobulin-Like-Transcript (ILT)2 and ILT4, and Killer cell Immunoglobulin-Like Receptor (KIR)2DL4[19], HLA-G mediates short-term inhibition of natural killer (NK) cells[20], cytotoxic T-lymphocyte[21], B cells[22], and dendritic cells (DC)[23,24]; and long-term induction of regulatory

cells including HLA-G-expressing T regulatory (Treg) cells[23], $CD4^{low}$ and $CD8^{low}$ suppressor T cells[25], Tr1 cells[26], and DC-10[27].

DC-10 are a subset of human tolerogenic DC that are present *in vivo*[27-29] and can be differentiated *in vitro* by culturing monocytes in the presence of IL-10. DC-10 secrete IL-10, are $CD11c^{+}$, express CD14, CD16, HLA-G and ILT4 and, although not activated, display a mature myeloid phenotype, being $CD86^{+}$ and $HLA\text{-}DR^{+}$. The secretion of IL-10 and the expression of membrane-bound HLA-G and ILT4 are critical factors involved in DC-10-mediated induction of Tr1 cells[27]. In the present study, we investigated the role of HLA-G in DC-10-mediated Tr1 cell induction and whether polymorphisms present at the 3'UTR of the gene influence the expression of membrane-bound HLA-G on DC-10.-12, DC-10 prime allogeneic naïve $CD4^{+}$ T cells, and induce an allo-specific IL-10-producing T cell population.

MATERIALS AND METHODS

Peripheral blood was obtained on informed consent in accordance with the Declaration of Helsinki under protocols approved by the ethical committee of the San Raffaele Telethon Institute for Gene Therapy.

Cells preparation

Human peripheral blood was obtained from healthy donors (HDs) upon informed consent in accordance with local ethical committee approval (Protocol TIGET03) and with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation over Lymphoprep (Nycomed Amersham).

DC differentiation

$CD14^+$ monocytes were isolated from PBMC by positive selection using CD14 MicroBeads (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Cells were cultured in RPMI 1640 (Lonza, Italy) supplemented with 10% Fetal

Bovine Serum (FBS) (Lonza, Italy) or with 5% Human Serum (HS) (EuroClone, Italy), 100 U/ml penicillin/streptomycin (Lonza, Italy), 2 mM L-Glutamine (Lonza, Italy), (DC medium) at 37°C in the presence of 10 ng/ml rhIL-4 (R&D Systems, Minneapolis MN, USA) and 100 ng/ml rhGM-CSF (Genzyme, Seattle WA, USA) with 10 ng/ml of rhIL-10 (BD, Bioscience, CA, USA) for 7 days to differentiate DC-10. Cells cultured with rhIL-4 and rhGM-CSF on day 5 were matured with 1 μg/ml of LPS (Sigma, CA, USA) for additional 2 days to generate mDC. At day 7, DC were collected, phenotypically analyzed, and used to stimulate T cells.

T-cell purification

$CD4^+$ T cells were purified from PBMC by negative selection using the untouched $CD4^+$ T cell Isolation kit (Miltenyi Biotech, Germany) according to the manufacturer's instructions. $CD4^+$ T cells were then depleted of $CD45RO^+$ cells using anti-CD45RO-coupled magnetic beads and LD negative selection columns (Miltenyi Biotech, Germany). In the purified cells the proportion of $CD4^+CD45RO^-CD45RA^+$ was consistently greater than 90%.

Flow cytometric analysis

DC were initially incubated for 15' with FcR blocking reagent (Miltenyi Biotech, Germany) and stained for additional 30' with anti-CD14 (MΦP9, BD Bioscience, CA, USA), anti-CD11c (B-ly6, BD Bioscience, CA, USA), anti-CD1a (HI149, BD Bioscience, CA, USA), anti-HLA-DR (TU36, BD Bioscience, CA, USA), anti-CD86 (BU63, Invitrogen, Camarillo CA, USA), anti-ILT4 (42D1, Beckman Coulter, Marseille, France) and, anti-HLA-G (MEM-G9, Exbio, Praha, Czech Republic) mAbs. T cells were stained with anti-CD45RA (HI100, Biolegend, San Diego, CA, USA), anti-CD4 (SK3, BD Bioscience, California, USA), anti-CD49b (AK-7, Biolegend, San Diego, CA, USA), anti-LAG-3 (FAB2319, R&D System), mAbs. The staining for CD49b and LAG-3 was performed at 37°C for 15 minutes. Samples were acquired using a FACS Canto II flow cytometer (Becton Dickinson, CA, USA), and data were analyzed with FCS express (De Novo Software, CA, USA). Quadrant markers were set accordingly to unstained controls.

Intracellular IL-10, IL-4, IFN-γ and IL-2 were detected by flow cytometry. Briefly, T cells (1 x 10^6/ml) were stimulated with leukocyte activation kit according to manufacturers' instructions (BD Bioscience, California, USA) in complete medium. 6 hours after activation, T cells were collected, washed in PBS, and incubated with anti-hCD4 (SK3, BD Bioscience, California,

USA). Subsequently, T cells were permeabilized with Cytofix/Cytoperm™ kit according to manufacturers' instructions (BD Bioscience, California, USA) and incubated with anti-hIL-2 (MQ1-17H12, BD Pharmingen), anti-hIL-4 (MP4-25D2, BD Bioscience, California, USA), anti-hIL-10 (JES3-9D7, BD Bioscience, California, USA), and anti-hIFN-γ (B27, BD Bioscience, California, USA) mAbs. Samples were acquired using a FACS Canto II flow cytometer (Becton Dickinson, CA, USA), and data were analyzed with FCS express (De Novo Software, CA, USA). Quadrant markers were set accordingly to unstained controls.

T-cell differentiation

$1x10^5$ DC were cultured with $1x10^6$ allogeneic $CD4^+CD45RO^-$ T cells in 1ml of X-vivo 15 medium (Lonza, Italy), supplemented with 5% pooled AB human serum (Lonza, Italy), and 100 U/ml penicillin/streptomycin (Lonza, Italy). After 7 days, rhIL-2 (20U/ml) (Chiron) was added, and cells were expanded for additional 7 days. Fourteen days after initiation of the culture, T cells were collected, washed, and analyzed for their functions. T cells stimulated with DC-10 are referred as T(DC-10), and T cells stimulated with mDC as T(mDC). Cultures with DC-10 typically resulted in 8-10-fold reduction in T-cell expansion

compared to cultures stimulated with mDC.

Proliferation and suppression of T cells

$CD4^+CD45RO^-$ T cells were stimulated with allogeneic DC (10:1, T:DC ratio) in a final volume of 200 µl of X-vivo 15 medium (Lonza, Italy), supplemented with 5% pooled AB human serum (Lonza, Italy), and 100 U/ml penicillin/streptomycin (Lonza, Italy) for 4 days and then pulsed for 16h with 1 µCi/well ^{3}H-thymidine.

To analyze the proliferative capacity of T(DC-10) and T(mDC) cell lines in response to allogeneic stimulation, T cells were stimulated with allogeneic mDC (10:1, T:DC ratio) in a final volume of 200 µl of medium for two day and then pulsed for 16h with 1 µCi/well ^{3}H-thymidine.

To evaluate the suppressive activity of T(DC-10), or T(mDC) cells, autologous $CD4^+$ T cells (Responders) were thawed and stained with Dye eFluor® 670 (eBioscience, California, USA) and activated with allogeneic mDC (10:1, T:DC ratio). Suppressor cells were added at a ratio of 1:1. The percentage of divided responder T cells was calculated by gating on $CD4^+$ cells, as described elsewhere[1].

Cytokine determination: ELISA

To measure IL-10, IL-12, IL-6, and TNF-α, DC were left un-stimulated or activated with 50 ng/ml of rhIFN-γ (R&D Systems, Minneapolis MN, USA) and 200 ng/ml of LPS (Sigma, CA, USA) for 2 days. Cytokine levels were determined in culture supernatants by ELISA according to the manufacturer's instructions (BD Bioscience, California, USA). The limits of detection were as follows: IL-6: 15 pg/ml, IL-10: 5 pg/ml, IL-12: 30 pg/ml, and TNF✔: 5 pg/ml. To measure IFN-γ production by T cells, culture supernatants were harvested after 48, 72, and 96 hours of culture and levels of IFN-γ were determined by ELISA according to the manufacturer's instructions (BD Biosciences). The limit of detection was 30 pg/ml.

Detection of soluble HLA-Gs

Levels of shed HLA-G1 and soluble HLA-G5 were determined by enzyme-linked immunosorbent assay (ELISA), as previously described[2,3]. To detect sHLA-G (shed HLA-G1 and HLA-G5) plates (Nunc-Immuno Plate PolySorp, Thermoscientific, Denmark) were coated with the mAb G233 (Exbio, Czech Republic), whereas to detect HLA-G5 plates were coated with the mAb 5A6G7 (Exbio, Czech Republic). sHLA-G or HLA-G5 were detected with biotinylated β-microglobulin and W6/32

mAbs (Exbio, Czech Republic), respectively. Supernatants from HLA-G transfected LCL721.221 cells[4], and HeLa HLA-G5-transfected cells (kindly provided by Dr. Rizzo, Università di Ferrara) purified by affinity chromatography by using the W6/32 mAb were used for the generation of standard calibration curves for shed HLA-G and HLA-G5, respectively. The limit of sensitivity was 1 ng/ml.

Amplification and sequencing of 3'UTR of the HLA-G gene

Genomic DNA was extracted from PBMCs using a commercial kit (QIAamp, QIAGEN, Italy) according to the manufacturer' s instructions. Briefly, 100 ng of genomic DNA were amplified in a 25 µl reaction containing 1X polymerase chain reaction (PCR) buffer (Roche, USA), 0.2 mM dNTP mix (Roche, USA), 1.5 mM $MgCl_2$ (Roche, USA), 0.8 U *Taq* Polymerase (Roche, USA), and 1 µM of each primer (For: 5' TCACCCCTCACTGTGACTGA 3'; Rev: 5' TTCTCATGTCTTCCATTTATTTTGTC 3'). The initial denaturation step was carried out at 95° C for 3 min, followed by 30 cycles at 93° C for 60 s, 58° C for 60 s, 72° C for 60 s, and by a final extension step at 72° C for 10 min. The amplification product was evaluated using a 2.5% agarose gel, purified using a commercial kit (Wizard SV Gel and PCR Clean-Up System,

Promega, WI, USA) according to the manufacturer' s instructions, and subjected to direct sequencing on both strands. All polymorphic sites observed at the 3'UTR were individually annotated and named according to previous reports[5].

miRNA extraction and quantification

Cells were thawed as dry pellet, small RNA were extracted using mirVana Isolation Kit following manufacturer's instructions (Ambion, Austin, TX). Real-time RT-PCR was used to quantify the amounts of miR-152-3p, miR-2110 and, miR-93* (Applied Biosystems, Foster City, CA) transcripts in DC-10 compared to those of $CD14^+$ precursor cells. Briefly, PCR was carried out for 40 amplification rounds in the presence of Taqman® microRNA Assay system (Applied Biosystems, Foster City, CA), using miR-let-7a (Applied Biosystems, Foster City, CA) as endogenous control. Reactions were carried out in duplicate or triplicate in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) and SDS 2.2.1 software was used to analyze the data. The same threshold was applied to calculate the Ct values for each PCR reaction. Quantification relative to $CD14^+$ precursor cells was carried out using the comparative CT method: $\Delta CT = CT_{miR\text{-}X} - CT_{miR\text{-}let\text{-}7a}$; $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{CD14+}$; relative miR-X expression = $2^{-\Delta\Delta CT}$.

Real-time quantitative PCR analyses

Total RNA was extracted with the RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized with the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time analysis were performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) and SDS 2.2.1 software to analyze the data. Real-time RT-PCR was used to quantify the amounts of HLA-G transcripts compared with those of JEG-3 as previously described [6]. Briefly, duplex PCR was carried out for 40 amplification rounds in the presence of TaqMan Universal PCR Master Mix, using the pre-developed TaqMan assay reagent GAPDH as endogenous control [probe with VIC reporter and 6-carboxytetramethylrhodamine (TAMRA) quencher (Applied Biosystems, Foster City, CA)], HLA-G-specific probe located in exon 5 [200 nM; (Applied Biosystems, Foster City, CA): 5'-CACTGGAGCTGCGGTCGCTGCT; 6-carboxyfluorescein (FAM) reporter and TAMRA quencher] and HLA-G-specific primers [300 nM (Applied Biosystems, Foster City, CA): forward 5'-CTGGTTGTCCTTGCAGCTGTAG; reverse 5'-CCTTTTCAATCTGAGCTCTTCTTTCT]. Quantification relative to JEG-3 was carried out in duplicate, using the

comparative CT method: $\Delta CT = CT_{HLA\text{-}G} - CT_{GAPDH}$; $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{JEG\text{-}3}$; relative HLA-G expression = $2^{-\Delta\Delta CT}$.

Statistical analysis

Sample mean results were compared using the non-parametric Mann-Whitney U test for continuous variable. HLA-G 3'UTR allele and genotype frequencies were obtained by direct count. Comparison of allele and genotype frequencies between populations was performed using the chi-squared test. Correlation between membrane-bound HLA-G and ILT4 expression was performed using the Spearman correlation test. Comparison between DC-10^{FBS} and DC-10^{HS} was performed by using paired t-Test. All results are presented as mean values±SEM. Differences were regarded as significant at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. The results were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA).

RESULTS

In vitro differentiated DC-10 express variable levels of membrane-bound HLA-G

Independently from the donor, DC-10 differentiated *in vitro* as described in Material and Methods, were $CD11c^{+}CD14^{+}CD1a^{-}CD86^{+}HLA\text{-}DR^{+}$ (Figure 1A and 1B). High variability in the expression of membrane-bound HLA-G (ranging from 3.5% to 97.7%) and of ILT4 (ranging from 0.5% to 70.7%; Figure 1A and 1B) was observed. Notably, ILT4 expression correlated with that of HLA-G (R^2=0.46, P<0.0001). mDC differentiated from the same donors of DC-10 were $CD11c^{+}CD1a^{+}CD14^{-}CD86^{bright}$, and did not express HLA-G (<3%) (Figure 1C). Based on the expression of HLA-G, DC-10 were classified as $HLA\text{-}G^{high}$ and $HLA\text{-}G^{low}$ when the percentage of HLA-G-expressing cells was higher or lower than 38%, respectively. This threshold value was determined as <95% of confidence interval of the mean of HLA-G-expressing cells among all DC-10 tested.

$HLA\text{-}G^{high}$ and $HLA\text{-}G^{low}$ DC-10 spontaneously secreted IL-10 at comparable levels, and negligible levels of IL-12 and TNF-α (Figure 1D). Upon LPS and IFN-γ stimulation, the release of IL-10, but not of IL-12, by DC-10 increased, and no differences were observed between $HLA\text{-}G^{high}$ and $HLA\text{-}G^{low}$ DC-10. Upon

activation HLA-G^{high} and HLA-G^{low} DC-10 secreted comparable amounts of TNF-α (Figure 1D). As expected, mDC differentiated *in vitro* from the same donors from whom DC-10 were generated, secreted high levels of IL-12, and limited amounts of TNF-α, and of IL-10 at steady state and upon activation (Figure 1D).

To investigate whether the expression of HLA-G by DC-10 was influenced by the culture conditions used for their differentiation (i.e. the presence of FBS), we compared the phenotype and cytokine production profile of DC-10 generated in media supplemented with FBS (DC-10^{FBS}) or HS (DC-10^{HS}). DC-10, independently from the media used, were CD14^{+}CD1a^{-} CD86^{+}HLA-DR^{+} and expressed both HLA-G and ILT4 at variable levels (Figure 2A). DC-10^{FBS} and DC-10^{HS} produced high levels of IL-10 and low amounts of IL-12 spontaneously and upon LPS and IFN-γ stimulation, and TNF-α upon stimulation (Figure 2B). Thus, the phenotype of DC-10 is not affected by the culture conditions used for their generation.

Overall, DC-10 generated from different donors are mature myeloid CD14^{+} cells, with a high IL-10/IL-12 ratio, and express variable levels of membrane-bound HLA-G and ILT4.

DC-10 with high membrane-bound HLA-G promote T-cell anergy

Naïve $CD4^+$ T cells stimulated with allogeneic HLA-G^{high} and HLA-G^{low} DC-10, at 10:1 ratio, showed a significantly lower proliferative response compared to T cells primed with mDC generated from the same donor. A reduction in proliferation of 91±7.4% (mean±SEM, n=5) and of 91.3±2.4% (mean±SEM, n=7) induced by HLA-G^{high} and HLA-G^{low} DC-10, respectively, was observed (Figure 3A and Supplementary Figure 1A). Accordingly, the levels of IFN-γ secreted by T cells stimulated with HLA-G^{high} or HLA-G^{low} DC-10 were lower than those produced by T cells primed with mDC (Figure 3A). Similar results were obtained using DC-10^{HS}: 82.5±5.2% (mean±SEM, n=8) reduction in proliferation of T cells stimulated with DC-10^{HS} compared to that obtained with T cells primed with mDC, and low IFN-γ production by T cells (Figure 3B), confirming that culture conditions have not effect on the functional activity of DC-10.

To investigate the function of T cells primed with HLA-G^{high} and HLA-G^{low} DC-10, secondary responses were evaluated. After one round of stimulation, T cells primed with HLA-G^{high} DC-10 [T(DC-10^{high}) cells] were hypo-responsive to re-activation with mDC differentiated from the same donor used in primary

stimulation, showing a reduction in proliferation of 61.9±18% (mean±SEM, n=5) compared to T cells primed with mDC (T(mDC) cells) (Figure 3C and Supplementary Figure 1B). Conversely, T cells obtained with HLA-G^{low} DC-10 [T(DC-10^{low}) cells] showed high proliferative responses when re-activated with mDC with a limited reduction in proliferation (40.3±13.8%, mean±SEM, n=7; Figure 3C and Supplementary Figure 1B). IFN-γ production by T(DC-10^{high}) cells, and at lower extend, by T(DC-10^{low}) cells, re-challenged with mDC, was also reduced compared to that secreted by T(mDC) cells (Figure 3C). Thus, high expression of membrane-bound HLA-G on DC-10 is required to promote T-cell anergy in allogeneic naïve CD4$^+$ T cells.

DC-10 with high membrane-bound HLA-G induce Tr1 cell differentiation

T(DC-10^{high}) cells contained a significantly higher frequency of IL-10-producing cells (on average 10%) compared to both T(DC-10^{low}) cells (on average 3.4%, *P*=0.0121) and T(mDC) cells (on average 3.5%, *P*=0.0127) (Figure 4A). Moreover, in T(DC-10^{high}) cell cultures IFN-γ producing cells were on average 58.1%, and the frequency of IL-4-producing cells was very low (2.5±1.2%, mean±SEM, n=3; Figure 4A). Conversely, T(DC-

10^{low}) cells contained variable percentage of IFN-γ T cells (ranging from 0.3% to 60.8%), and low frequency of IL-4^+ T cells (1.9±0.93%, mean±SEM, n=8) (Figure 4A). The percentages of IFN-γ and IL-4^+ in T(mDC) cells generated in parallel were comparable to those observed in T(DC-10^{high}) and T(DC-10^{low}) cells (Figure 4A).

Tr1 cells can be identified by the co-expression of CD49b and LAG-3[30]. Thus, we evaluated the frequency of $CD49b^+$LAG-$3^+CD45RA^-$ cells in T(DC-10^{high}) and T(DC-10^{low}) cells. Results indicated that T(DC-10^{high}) cells contained a higher frequency of $CD49b^+$LAG-$3^+CD45RA^-$ cells (on average 11.3%) compared to both T(DC-10^{low}) (on average 4.3%) and T(mDC) cells (on average 2.2%, *P*=0.0031) (Figure 4B). In line with the presence of high frequency of Tr1 cells, T(DC-10^{high}) cells suppressed the proliferation of autologous $CD4^+$ T cells activated with mDC (40.3±11.1%, mean±SEM, n=3), while T(DC-10^{low}) cells did not (Figure 4C).These findings indicate that,high levels of membrane-bound HLA-G on DC-10 improved their ability to prime T cells to become IL-10-producing Tr1 cells *in vitro*.

HLA-G^{high} and HLA-G^{low} DC-10 secrete comparable levels of shed HLA-G1

To determine whether the low expression of membrane-bound HLA-G observed on HLA-G^{low} DC-10 was ascribed to their ability to shed HLA-G1 from the membrane, we evaluated in culture supernatants the amounts of shed HLA-G1. We detected shed HLA-G1 in 4 out of 16 unstimulated DC-10 (1.9±0.4 ng/ml, mean±SEM) and in 9 out of 16 stimulated DC-10 (3±0.4 ng/ml, mean±SEM; Figure 5A). Conversely, soluble HLA-G5 was never detected in DC-10 culture supernatants. Interestingly, mDC generated in parallel secreted higher levels of shed HLA-G1 than DC-10 both in steady state and upon activation (P=0.04) (Figure 5A). No differences were observed in the ability of HLA-G^{high} and HLA-G^{low} DC-10 to release shed HLA-G1 (Figure 5B). Thus, *in vitro* differentiated DC-10 secrete low levels of shed HLA-G1, but not HLA-G5. Moreover, the low expression of membrane-bound HLA-G observed on HLA-G^{low} DC-10 is not associated with an increased release of shed HLA-G1.

Frequencies of 3'UTR HLA-G genotypes in donors with HLA-G^{high} or HLA-G^{low} DC-10

Polymorphisms at 3'UTR of the HLA-G locus influence its expression[31], thus we investigated whether variability in this region can be associated with membrane-bound HLA-G

expression on DC-10. Donors from whom we differentiated DC-10 were typed at the 3'UTR and the presence of eight different polymorphic sites was inferred: the 14bp Ins/Del (rs1704), +3003 C/T (rs1707), +3010 C/G (rs1710), +3027 A/C (rs17179101), +3035 C/T (rs17179108), +3142 C/G (rs1063320), +3187 A/G (rs9380142) and +3196 C/G (rs1610696). We observed that the 14bp Ins allele was significantly highly represented both in single dose (66%) and in double copy (45%) in donors with HLA-G^{low} DC-10 compared to those with HLA-G^{high} DC-10 (36%, P= 0.0009 and 14%, P= 0.0065, respectively; Figure 6A). Since 14bp Ins is in strong linkage disequilibrium with G in position +3142[32], we classified donors according to the presence of the 14bp Ins/Del and +3142 C/G polymorphisms as following: InsG/InsG, DelC/DelC, DelC/InsG, and DelG/X. The relative frequencies of these alleles and genotypes are shown in Figure 6B. As expected, we confirmed the high frequency of the InsG allele in donors with HLA-G^{low} DC-10 and we found that the DelC allele was significantly less represented in donors with HLA-G^{low}compared to HLA-G^{high} DC-10 (P= 0.0213; Figure 6B).

We next defined the frequencies of HLA-G 3' UTRs according to Castelli et al. classification[13], and we found that UTR-1 and UTR-2 were overall the most frequent haplotypes in our cohort of donors, being UTR-1 observed in 40% of donors with HLA-

G^{high} DC-10, and UTR-2 in 39% of donors with HLA-G^{low} DC-10 (Table 1). Accordingly, UTR-2/UTR-2 was significantly higher represented in donors with HLA-G^{low} compared to HLA-G^{high} DC-10 (P= 0.036; Table 1). Moreover, 19% of donors with HLA-G^{high} DC-10 were UTR-3/DelC, whereas none of the donors with HLA-G^{low} DC-10 had this genotype (P= 0.013; Table 1).

The presence of the 14bp Ins has been associated with low mRNA stability and reduced soluble HLA-G protein production[33]. We therefore investigated whether the levels of mRNA for HLA-G were variable in DC-10 from different 3'UTR HLA-G typed donors. Overall the amount of HLA-G transcripts was very low, and in 7 out of 13 samples resulted below the detection limit. Nevertheless, the highest amounts of HLA-G transcript were from donor with 14bp Ins/Del and 14bp Del/Del genotypes (Supplementary Figure 2).

These findings demonstrate that the expression levels of membrane-bound HLA-G on DC-10 can be influenced by specific polymorphisms at the 3'UTR of the HLA-G locus, and that the UTR-2/UTR-2 genotype is more frequent among donors from whom DC-10 with low HLA-G were generated, while UTR-3/DelC genotype in donors with high HLA-G expression on DC-10.

miR-152 is highly expressed in *in vitro* differentiated DC-10

We hypothesized that miR-152, miR-148a, and miR-148b, which target G at position +3142, may be responsible for the low membrane-bound HLA-G expression observed in DC-10 from donors carrying the UTR-2/UTR-2 genotype. We evaluated the expression of miR-152, miR-2110 and, miR-93a predicted to have high binding affinity for HLA-G mRNA [14] in *in vitro* differentiated DC-10 compared to $CD14^+$ precursors. Results showed that miR-152 was expressed on average 2.1 fold more (n=5) in DC-10 than in $CD14^+$ cells (Figure 7). Conversely, miR-2110 and miR-93a were respectively equally or less expressed in DC-10 than in $CD14^+$ cells (Figure 7). Thus, the expression of miR-152 can be involved in the post-transcriptional regulation of HLA-G in DC-10, especially in those donors with 3'UTR haplotypes with G in position +3142.

DISCUSSION

In the present study, we defined the important role of HLA-G in DC-10-mediated induction of Tr1 cells. We showed that the expression of membrane-bound HLA-G on DC-10 is donor-dependent and that, in the presence of similar levels of IL-10, a high expression of HLA-G on DC-10 is required to induce at high frequency Tr1 cells *in vitro*. We also established that 3'UTR genetic variations influence the HLA-G expression on DC-10 that might be finely tuned by miRNA-mediated post-transcriptional regulation.

We confirmed that, similar to other populations of tolerogenic DC previously described, G-CSF-induced DC[34,35] and dermal $CD141^+$ cells[36], DC-10 differentiated *in vitro* from monocytes using our standardized protocol[27] are $CD14^+$. In addition, DC-10 expressed variable levels of HLA-G depending on the donor, and according to this, DC-10 can be classified in $HLA\text{-}G^{high}$ or $HLA\text{-}G^{low}$. Notably, we found that the expression of HLA-G on DC-10 correlated with that of ILT4, but not with the expression of ILT2 or of ILT3, which were highly expressed on DC-10 (data not shown). These findings are in line with a previous report showing that in transfected antigen-presenting cell (APC) lines the ectopic expression of HLA-G promoted the up-regulation of ILT2, ILT3, and ILT4[37].

By proteolytic shedding, the HLA-G1 trans-membrane isoform can be released as shed HLA-G1, which retains all the functions of the membrane counterpart[6,38]. We postulated that low levels of HLA-G on DC-10 were associated with an increased production of shed HLA-G1. However, comparable low amounts of shed HLA-G1 were generated from both HLA-G^{low} and HLA-G^{high} DC-10. The production of shed HLA-G1 is regulated by the activity of MMPs[38], and specifically by MMP-2[39]. Data from the gene expression profile of *in vitro* differentiated DC-10 indicated that MMPs are present in DC-10 (Comi et al., unpublished data). Thus, we can hypothesize that in HLA-G^{low} DC-10 the great majority of HLA-G1 molecules is rapidly cleaved and released as shed HLA-G1, whereas the higher amounts of HLA-G1 synthesized in HLA-G^{high} DC-10 saturate the activity of MMPs and, as a result, more HLA-G1 is expressed as trans-membrane isoform.

It has been previously reported that constitutive high HLA-G expression confers to APCs the ability to inhibit primary T-cell responses[23]. Conversely, we herein showed that DC-10 displayed low stimulatory activity regardless to HLA-G expression levels. This result is not surprising, since DC-10, independently from HLA-G expression, secrete high levels of IL-10, which is known to directly inhibit T-cell responses *in vitro*[40-43]. We previously demonstrated that IL-10, either exogenously added or derived by

DC-10, inhibited the proliferation of allogeneic naïve T cells *in vitro*[44,45].

DC-10 prime T cells to become Tr1 cells *via* the IL-10-induced HLA-G/ILT4 pathway[27]. Although IL-10, ILT4, and HLA-G are important for DC-10 tolerogenic activity, their relative contributions in inducing Tr1 cells were not investigated. The identification of DC-10 that spontaneously expressed high or low HLA-G, allowed us to finally demonstrate that the high levels of HLA-G on DC-10 are required to efficiently induce anergic T cells, and consequently Tr1 cells. We indeed proved that HLA-G^{high} DC-10 promoted Tr1 cells, identified as IL-10-producing or as $CD49b^{+}LAG\text{-}3^{+}CD45RA^{-}$[30] T cells, at higher frequency than HLA-G^{low} DC-10. In addition, we confirmed the key role of IL-10 in DC-10-induced T-cell anergy (and Tr1 cells)[27,43-45], since, in a limited number of donors, we found that T cells primed with HLA-G^{low} DC-10 became hypo-responsive to secondary stimulation. In this study we showed that although IL-10-derived by DC-10 is necessary for promoting T-cell anergy, it is not sufficient to promote the differentiation of Tr1 cells at high frequency. The low HLA-G (and ILT4) expression on DC-10 hampers the amplification of the DC-10-mediated tolerogenic loop, consisting in inhibiting T cell activation *via* ILT2 and promoting HLA-G and ILT4 expression on neighboring DC-10, and the consequent induction of IL-10,

which sustains Tr1 cell generation. Being HLA-G expression on DC-10 genetically determined, future studies aimed at up-regulating HLA-G in donors prone to generate HLA-G^{low} DC-10 will definitively prove our hypothesis.

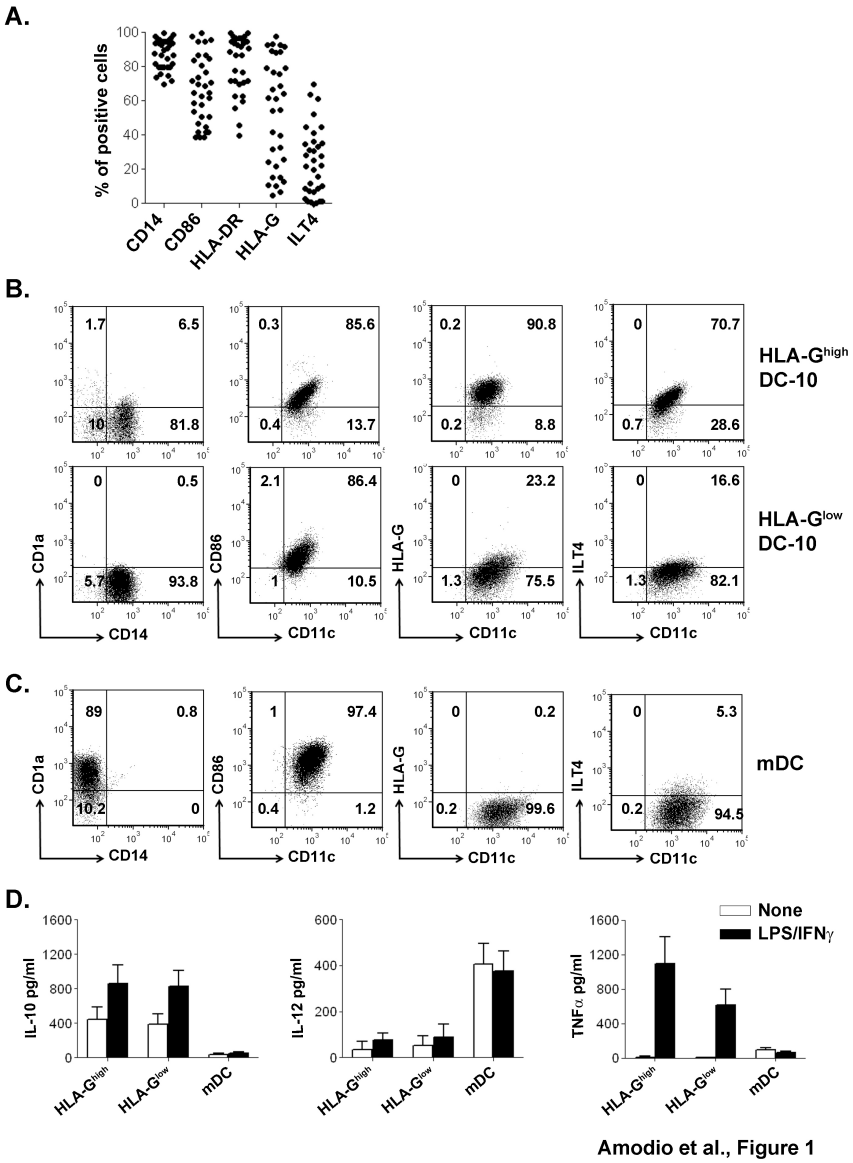
Studies investigating 3'UTR HLA-G polymorphisms and HLA-G expression primarily focused on the association between the 14bp Ins/Del polymorphism and soluble HLA-G isoforms[33,46]. Only recently these analyses have been enlarged to other polymorphisms in the 3'UTR region of HLA-G and to membrane-bound HLA-G[47,48]. In the present study, we screened the association of multiple variations in the 3'UTR HLA-G, considering alleles, genotypes, haplotypes, and diplotypes with membrane-bound HLA-G expression on DC-10. We reported that the majority of donors with HLA-G^{low} DC-10 carried haplotypes, such as UTR-2, UTR-5, UTR-7, containing 14bp Ins and G in position +3142, both variations previously associated with low mRNA stability[10,14]. These results are in line with a recent work showing that UTR-2 and UTR-5 associated with low expression of soluble HLA-G [48]. The presence of G at position +3142 increases the affinity of miR-152, miR-148a, and miR-148b for mRNA, promoting the repression of HLA-G expression[14]. Moreover, the over-expression of miR-148a or miR-152 in JEG-3 or LCL721.221 cell lines promotes post-transcriptional down-regulation of membrane-bound HLA-G[49,50].

Interestingly, analysis of the miRNome profile of DC-10, in comparison to that of mature DC, identified 10 differentially expressed miRNAs, and among those miR-148a (Comi et al. unpublished data). Moreover, miR-152 was highly expressed in DC-10 as compared to $CD14^{+}$ precursors. Thus, it can be postulated that post-transcriptional regulation of mRNA encoding for HLA-G may occur in DC-10 generated from donors carrying double dose of 3'UTRs haplotypes with +3142 G. The higher frequency of UTR-2, UTR-5, and UTR-7 in donors with HLA-G^{low} DC-10 supports this hypothesis. Although several questions regarding the mechanisms associated with polymorphic sites at 3'UTR of HLA-G need to be addressed, the present study evidences a link between the presence of 14bp Ins and the low expression of HLA-G, and indicates that this effect can be finely tuned by additional variations present at the 3'UTR, such as the +3142 C/G possibly *via* miRNA-mediated post-transcriptional regulation.

In conclusion, this study provides new insights on the role of HLA-G in DC-10-mediated Tr1 cell induction: i) high expression of HLA-G is required for efficient induction of Tr1 cells *via* DC-10; ii) the contribution of soluble HLA-G isoforms in DC-10-mediated induction of Tr1 cells is negligible; iii) 3'UTR HLA-G haplotypes and genotypes can be associated with the expression of membrane-bound HLA-G on DC-10 and,

consequently, with their ability to prime naïve T cells to become Tr1 cells. To our knowledge, this is the first comprehensive study in which 3'UTR polymorphisms have been associated with membrane-bound HLA-G expression and with tolerogenic properties of a specific cell subset. DC-10 are currently used to generate allo-specific Tr1 cells for adoptive Treg-based cell therapy, but they represent an interesting therapeutic tool to induce or re-establish immunological tolerance in different clinical settings including allogeneic transplantation or autoimmune diseases. Results on the influence of 3'UTR genotypes on HLA-G expression on DC-10 and their tolerogenic activity provide new important tools for donor selection in several clinical settings such as hematopoietic stem cell and solid organ transplantation, and will also be relevant for understanding the immunological mechanisms underlying autoimmune diseases and cancer.

FIGURES



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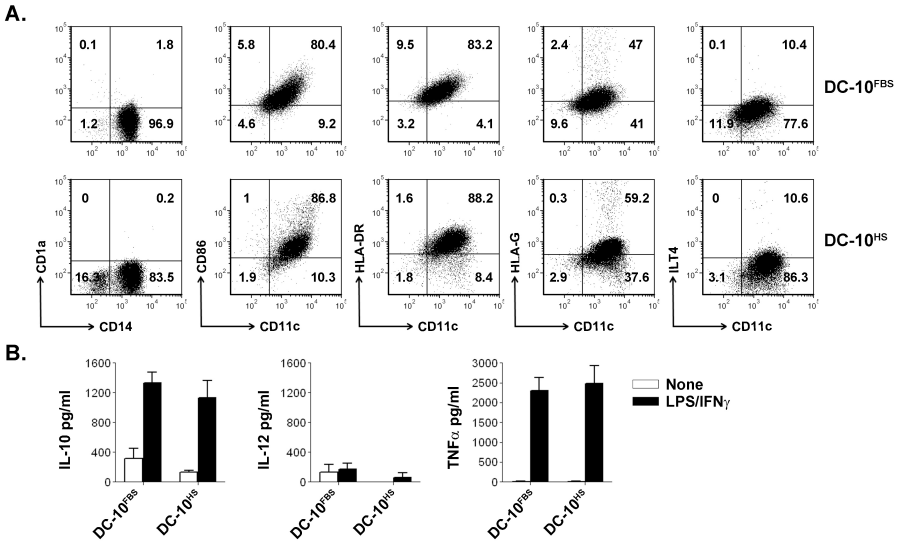

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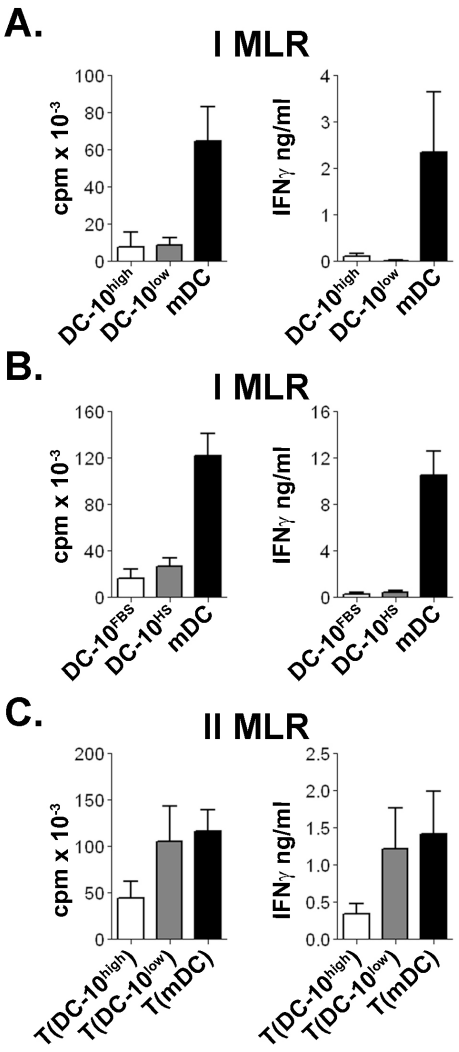

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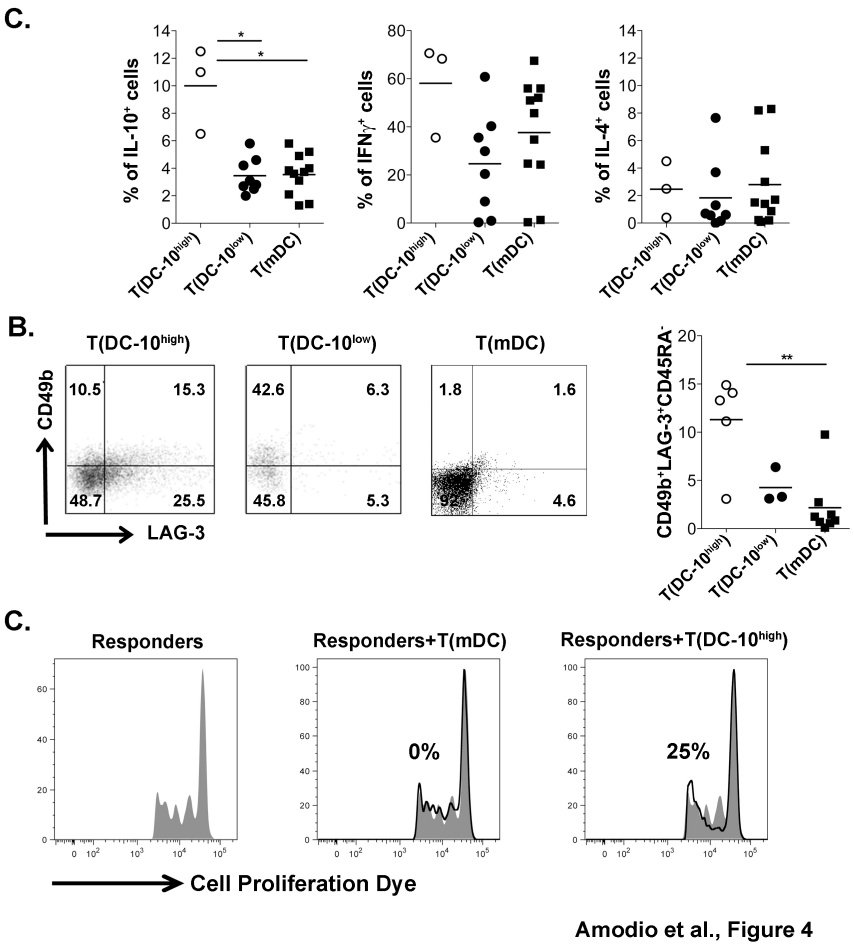

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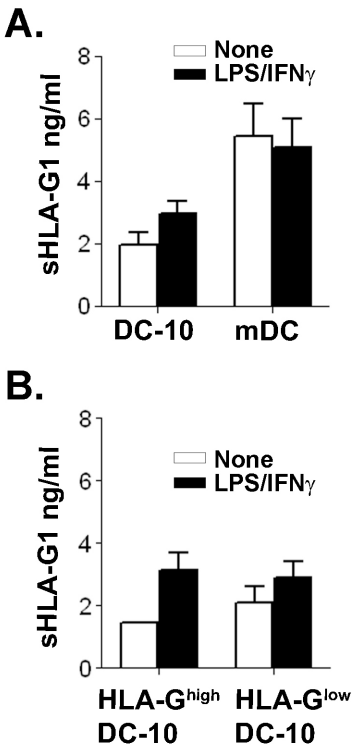

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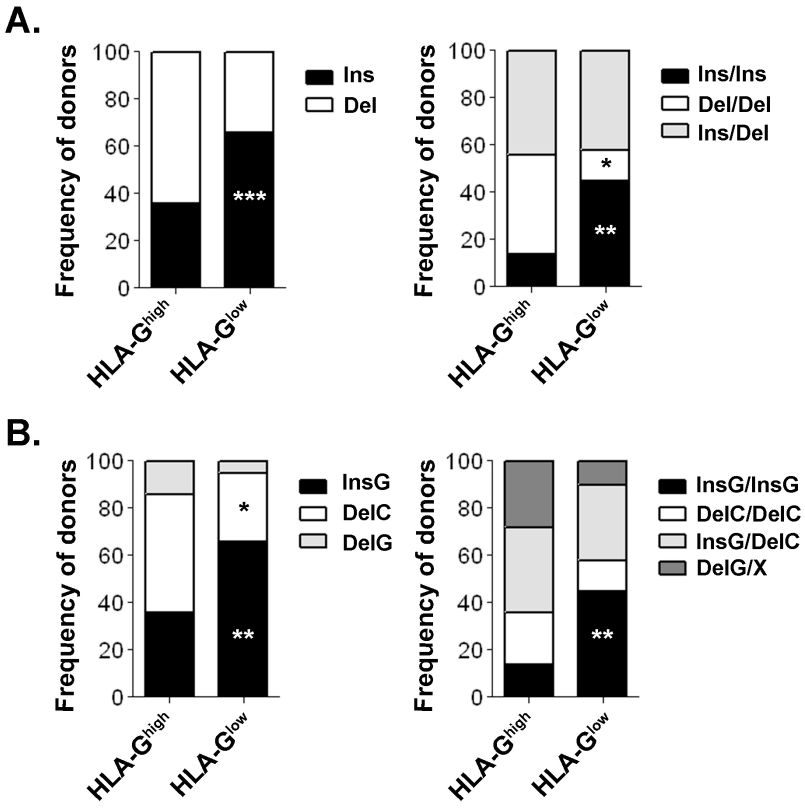

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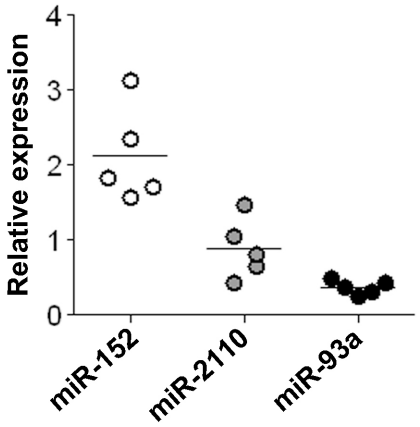

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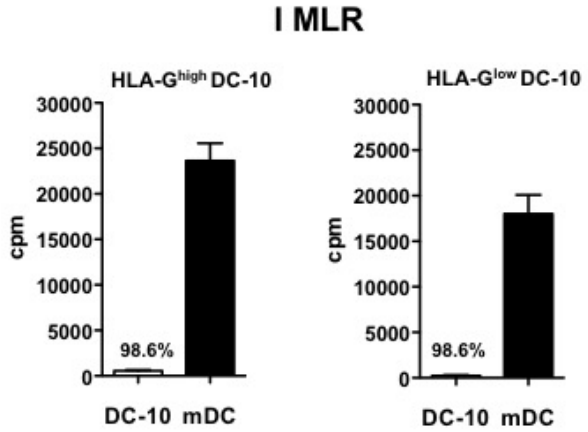
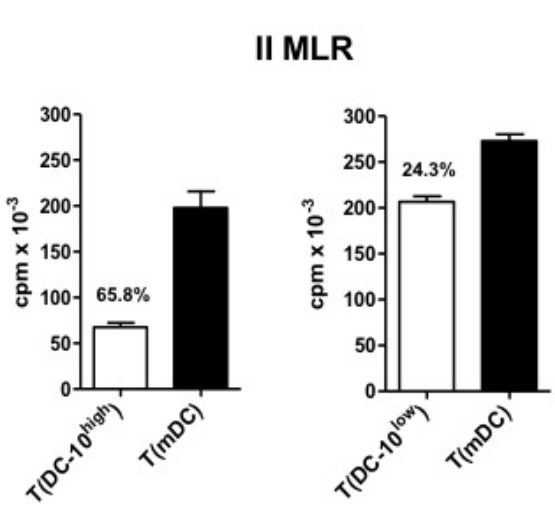
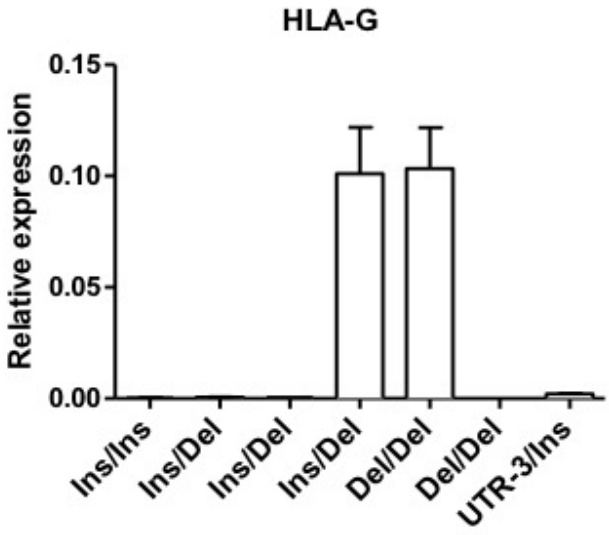
A.



B.



Amodio et al., Supplementary Figure 1



Amodio et al., Supplementary Figure 2

FIGURE LEGENDS

Figure 1. *In vitro* differentiated DC-10 express variable levels of membrane-bound HLA-G. Expression levels of CD14, CD1a, CD11c, CD86, HLA-DR, HLA-G, and ILT4 on *in vitro* differentiated DC-10 and mDC were evaluated by FACS analysis. **(A)** Percentages of positive cells for the indicated markers in n= 33 independent donors are shown**. (B)** One representative donor out of 30 HLA-G^{high} DC-10 (upper panels), and one out of 24 HLA-G^{low} DC-10 (lower panels) are presented. Numbers represent percentages of positive cells in each quadrant. **(C)** One representative donor out of 54 mDC is presented. Numbers represent percentages of positive cells in each quadrant. **(D)** DC-10 were left un-stimulated or stimulated with LPS and IFN-γ for additional 2 days. Concentration levels of IL-10, IL-12, and TNF-α in culture supernatants of the indicated cells are shown. Mean±SEM of n=11 (HLA-G^{high} DC-10), n=15 (HLA-G^{low} DC-10), and n=26 (mDC) independent experiments.

Figure 2. DC-10 differentiated with FBS or HS have comparable phenotype and cytokine profile. (A)

Expression levels of CD14, CD1a, CD11c, CD86, HLA-DR, HLA-G, and ILT4 on DC-10 differentiated from $CD14^+$ cells isolated from the same donor with FBS ($DC\text{-}10^{FBS}$) or HS ($DC\text{-}10^{HS}$) were evaluated by FACS analysis. One representative donor out of 9 $DC\text{-}10^{FBS}$ (upper panels), and $DC\text{-}10^{HS}$ (lower panels) are presented. Numbers represent percentages of positive cells in each quadrant. **(B)** DC-10 were left un-stimulated or stimulated with LPS and IFN-γ for additional 2 days. Concentration levels of IL-10, IL-12, and TNF-α in culture supernatants of the indicated cells are shown. Mean±SEM of n= 6 independent experiments.

Figure 3. DC-10 with high levels of membrane-bound HLA-G promote T-cell anergy. **(A)** Naive $CD4^+$ T cells were cultured with allogeneic *in vitro* differentiated $HLA\text{-}G^{high}$ DC-10, $HLA\text{-}G^{low}$ DC-10, and mDC (ratio 10:1). Proliferative responses were evaluated 4 days after culture by [^{3}H]-thymidine incorporation for additional 16 hours (left panel). Mean±SEM of n=5 $DC\text{-}10^{high}$, n=7 $DC\text{-}10^{low}$, and n=12 mDC independent experiments. IFN-γ production was evaluated 4 days after culture (right panel). Mean±SEM of n=4 $DC\text{-}10^{high}$, n=6 $DC\text{-}10^{low}$, and n=10 mDC independent experiments. **(B)** Naive $CD4^+$ T cells were cultured with allogeneic *in vitro* differentiated $DC\text{-}10^{FBS}$, and $DC\text{-}10^{HS}$ from $CD14^+$ cells isolated from the same

donor (ratio 10:1). Proliferative responses were evaluated 4 days after culture by [^{3}H]-thymidine incorporation for additional 16 hours (left panel); IFN-γ production was evaluated 4 days after culture (right panel). Mean±SEM of n=8 independent experiments. **(C)** Naive CD4$^+$ T cells were stimulated with allogeneic HLA-G^{high} DC-10 [T(DC-10^{high})], HLA-G^{low} DC-10 [T(DC-10^{low})], or mDC [T(mDC)] for 14 days. After culture, T cells were tested for their ability to proliferate in response to mDC from the same allogeneic donor used in priming. Proliferative responses were evaluated 2 days after culture by [^{3}H]-thymidine incorporation for additional 16 hours (left panel). Mean±SEM of n=5 [T(DC-10^{high})], n=7 [T(DC-10^{low})], and n=12 [T(mDC)] independent experiments. IFN-γ production was evaluated 2 days after culture (right panel). Mean±SEM of n=5 [T(DC-10^{high})], n=6 [T(DC-10^{low})], and n=11 [T(mDC)] independent experiments.

Figure 4. DC-10 with high membrane-bound HLA-G induce Tr1 cell differentiation. Naive CD4$^+$ T cells were stimulated with allogeneic HLA-G^{high} DC-10 [T(DC-10^{high})], HLA-G^{low} DC-10 [T(DC-10^{low})], or mDC [T(mDC)] for 14 days. **(A)** After culture, T cells were activated with PMA/IONO (leukocyte activation kit) for 6 hours (as described in Methods), and cytokine production was determined by intracytoplasmic

staining. Percentages of IL-10-, IFN-γ, and IL-4-producing cells in T(DC-10^{high}) (n= 3), T(DC-10^{low}) (n= 8), and T(mDC) (n= 11) cells are presented. Each dot represents a single donor and lines indicate mean, *P < 0.05. **(B)** After culture, the frequencies of $CD4^+$ T cells co-expressing CD49b and LAG-3 in T(DC-10^{high}), T(DC-10^{low}), and T(mDC) cells were analyzed by FACS. One representative plot for each cell lines (left panels) and percentages of $CD49b^+LAG\text{-}3^+CD45RA^-$ in 5, 3, and 8 independent experiments for T(DC-10^{high}), T(DC-10^{low}), and T(mDC) cells, respectively, are shown (right panel). Numbers represent percentages of positive cells in each quadrant. Each dot represents a single donor and lines indicate mean, ** P <0.01. **(C)** T(DC-10^{high}) and T(mDC) cells were tested for their ability to suppress responses of autologous $CD4^+$ T cells activated with mDC (Responders). Percentages of suppression mediated by the indicated T cell populations are indicated. One representative donor out of three tested is showed.

Figure 5. HLA-G^{high} and HLA-G^{low} DC-10 secrete comparable levels of shed HLA-G1. DC-10 and mDC were left un-stimulated or stimulated with LPS and IFN-γ for additional 2 days. **(A)** Concentration levels of shed HLA-G1 in culture supernatants of DC-10 and mDC are shown. mean±SEM; n=4 (DC-10) and n=9 (mDC) unstimulated and n=8 (DC-10) and

n=12 (mDC) stimulated with LPS and IFN-γ **(B)** Concentration levels of shed HLA-G1 in culture supernatants of HLA-G^{high} and HLA-G^{low} DC-10 are shown. Mean±SEM; n=1 (HLA-G^{high} DC-10) and n=3 (HLA-G^{low} DC-10) unstimulated and n=3 (HLA-G^{high} DC-10) and n=6 (HLA-G^{low} DC-10) stimulated with LPS and IFN-γ.

Figure 6. Frequencies of 3'UTR genotypes in donors with HLA-G^{high} and HLA-G^{low} DC-10. **(A)** Allele and genotype frequencies for 14 bp Ins/Del polymorphism, and for **(B)** 14 bp Ins/Del and +3142 G/C polymorphisms in 67 healthy donors (n= 36, HLA-G^{high} DC-10 and n= 31 HLA-G^{low} DC-10) are shown. * P <0.05, ** P <0.01, *** P <0.001.

Figure 7. miR-152 is highly expressed by *in vitro* differentiated DC-10. miRNAs were extracted from *in vitro* differentiated DC-10 and the expression of the indicated miRNAs was evaluated by RT-PCR. Following normalization to miR-let7a, relative mRNA amounts from DC-10 cells were adjusted to corresponding expression levels of a calibrator (corresponding $CD14^+$ precursors). Numbers represent arbitrary

units. Each dot represents a single cell preparation and lines indicate mean.

Figure S1. DC-10 with high levels of membrane-bound HLA-G promote T-cell anergy. **(A)** Naive $CD4^+$ T cells were cultured with allogeneic *in vitro* differentiated HLA-G^{high} DC-10, HLA-G^{low} DC-10, and mDC (ratio 10:1). Proliferative responses were evaluated 4 days after culture by [^{3}H]-thymidine incorporation for additional 16 hours. One representative donor out of 6 for HLA-G^{high} DC-10 and one out of 8 for HLA-G^{low} DC-10 independent donors tested are presented. Numbers represent the percentages of reduction in proliferation of naïve $CD4^+$ T cells stimulated with DC-10 compared to those activated with mDC. **(B)** Naive $CD4^+$ T cells were stimulated with allogeneic HLA-G^{high} DC-10 [T(DC-10^{high})], HLA-G^{low} DC-10 [T(DC-10^{low})], or mDC [T(mDC)] for 14 days. After culture, T cells were tested for their ability to proliferate in response to mDC from the same allogeneic donor used in priming. Proliferative responses were evaluated 2 days after culture by [^{3}H]-thymidine incorporation for additional 16 hours. One representative donor out of 6 HLA-G^{high} DC-10 and one out of 8 HLA-G^{low} DC-10 independent donors tested are presented. Numbers represent the percentages of anergy of T(DC-10)

compared to T(mDC) calculated as follows: 100-[(T(mDC cpm)-T(DC-10 cpm)/T(mDC cpm)*100].

Figure S2. Quantification of HLA-G mRNA transcripts in DC-10. DC-10 were differentiated in the presence of IL-4, GM-CSF and IL-10 for 7 days. On day 7, total mRNA was extracted from DC-10 and expression of HLA-G gene was evaluated by RT-PCR. Following normalization to GAPDH, relative mRNA amounts from DC-10 cells were adjusted to corresponding expression levels of a calibrator (JEG-3 cell line). Numbers represent arbitrary units. DC-10 were obtained from the indicated 3'UTR genotyped donors.

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REFERENCES

1. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. *Science*. 1990;248(4952):220-223.

2. Rouas-Freiss N, Goncalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc Natl Acad Sci U S A*. 1997;94(21):11520-11525.

3. Curigliano G, Criscitiello C, Gelao L, Goldhirsch A. Molecular pathways: human leukocyte antigen G (HLA-G). *Clin Cancer Res*. 2013;19(20):5564-5571.

4. Fujii T, Ishitani A, Geraghty DE. A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. *J Immunol*. 1994;153(12):5516-5524.

5. Ishitani A, Geraghty DE. Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. *Proc Natl Acad Sci U S A*. 1992;89(9):3947-3951.

6. Park GM, Lee S, Park B, et al. Soluble HLA-G generated by proteolytic shedding inhibits NK-mediated cell lysis. *Biochem Biophys Res Commun*. 2004;313(3):606-611.

7. Carosella ED. The tolerogenic molecule HLA-G. *Immunol Lett*. 2011.

8. Rousseau P, Le Discorde M, Mouillot G, Marcou C, Carosella ED, Moreau P. The 14 bp deletion-insertion polymorphism in the 3' UT region of the HLA-G gene influences HLA-G mRNA stability. *Hum Immunol*. 2003;64(11):1005-1010.

9. Rebmann V, van der Ven K, Passler M, Pfeiffer K, Krebs D, Grosse-Wilde H. Association of soluble HLA-G plasma levels with HLA-G alleles. *Tissue Antigens*. 2001;57(1):15-21.

10. Hviid TV, Hylenius S, Rorbye C, Nielsen LG. HLA-G allelic variants are associated with differences in the HLA-G mRNA isoform profile and HLA-G mRNA levels. *Immunogenetics*. 2003;55(2):63-79.

11. Tan Z, Randall G, Fan J, et al. Allele-specific targeting of microRNAs to HLA-G and risk of asthma. *Am J Hum Genet*. 2007;81(4):829-834.

12. Veit TD, Chies JA. Tolerance versus immune response -- microRNAs as important elements in the regulation of the HLA-G gene expression. *Transpl Immunol*. 2009;20(4):229-231.

13. Castelli EC, Mendes-Junior CT, Deghaide NH, et al. The

genetic structure of 3'untranslated region of the HLA-G gene: polymorphisms and haplotypes. *Genes Immun*. 2010;11(2):134-141.

14. Castelli EC, Moreau P, Oya e Chiromatzo A, et al. In silico analysis of microRNAS targeting the HLA-G 3' untranslated region alleles and haplotypes. *Hum Immunol*. 2009;70(12):1020-1025.

15. Yie SM, Li LH, Xiao R, Librach CL. A single base-pair mutation in the 3'-untranslated region of HLA-G mRNA is associated with pre-eclampsia. *Mol Hum Reprod*. 2008;14(11):649-653.

16. Lucena-Silva N, Monteiro AR, de Albuquerque RS, et al. Haplotype frequencies based on eight polymorphic sites at the 3' untranslated region of the HLA-G gene in individuals from two different geographical regions of Brazil. *Tissue Antigens*. 2012;79(4):272-278.

17. Larsen MH, Hylenius S, Andersen AM, Hviid TV. The 3'-untranslated region of the HLA-G gene in relation to pre-eclampsia: revisited. *Tissue Antigens*. 2010;75(3):253-261.

18. Alvarez M, Piedade J, Balseiro S, Ribas G, Regateiro F. HLA-G 3'-UTR SNP and 14-bp deletion polymorphisms in Portuguese and Guinea-Bissau populations. *Int J Immunogenet*. 2009;36(6):361-366.

19. Shiroishi M, Kuroki K, Rasubala L, et al. Structural basis for recognition of the nonclassical MHC molecule HLA-G by the leukocyte Ig-like receptor B2 (LILRB2/LIR2/ILT4/CD85d). *Proc Natl Acad Sci U S A*. 2006;103(44):16412-16417.

20. Riteau B, Menier C, Khalil-Daher I, et al. HLA-G1 co-expression boosts the HLA class I-mediated NK lysis inhibition. *Int Immunol*. 2001;13(2):193-201.

21. Le Gal FA, Riteau B, Sedlik C, et al. HLA-G-mediated inhibition of antigen-specific cytotoxic T lymphocytes. *Int Immunol*. 1999;11(8):1351-1356.

22. Naji A, Menier C, Morandi F, et al. Binding of HLA-G to ITIM-bearing Ig-like transcript 2 receptor suppresses B cell responses. *J Immunol*. 2014;192(4):1536-1546.

23. LeMaoult J, Krawice-Radanne I, Dausset J, Carosella ED. HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4+ T cells. *Proc Natl Acad Sci U S A*. 2004;101(18):7064-7069.

24. Horuzsko A, Lenfant F, Munn DH, Mellor AL. Maturation of antigen-presenting cells is compromised in HLA-G transgenic mice. *Int Immunol*. 2001;13(3):385-394.

25. Naji A, Le Rond S, Durrbach A, et al. CD3+CD4low and CD3+CD8low are induced by HLA-G: novel human

peripheral blood suppressor T-cell subsets involved in transplant acceptance. *Blood*. 2007;110(12):3936-3948.

26. Gregori S, Magnani CF, Roncarolo MG. Role of human leukocyte antigen-G in the induction of adaptive type 1 regulatory T cells. *Hum Immunol*. 2009;70(12):966-969.

27. Gregori S, Tomasoni D, Pacciani V, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood*. 2010;116(6):935-944.

28. Amodio G, Mugione A, Sanchez AM, et al. HLA-G expressing DC-10 and CD4(+) T cells accumulate in human decidua during pregnancy. *Hum Immunol*. 2013;74(4):406-411.

29. Locafaro G, Amodio G, Tomasoni D, Tresoldi C, Ciceri F, Gregori S. HLA-G expression on blasts and tolerogenic cells in patients affected by Acute Myeloid Leukemia. *J Immunol Res*. 2014;2014.

30. Gagliani N, Magnani CF, Huber S, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med*. 2013;19(6):739-746.

31. Donadi EA, Castelli EC, Arnaiz-Villena A, Roger M, Rey D, Moreau P. Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association.

Cell Mol Life Sci. 2011;68(3):369-395.

32. Castelli EC, Mendes-Junior CT, Veiga-Castelli LC, Roger M, Moreau P, Donadi EA. A comprehensive study of polymorphic sites along the HLA-G gene: implication for gene regulation and evolution. *Mol Biol Evol*. 2011;28(11):3069-3086.

33. Hviid TV, Rizzo R, Christiansen OB, Melchiorri L, Lindhard A, Baricordi OR. HLA-G and IL-10 in serum in relation to HLA-G genotype and polymorphisms. *Immunogenetics*. 2004;56(3):135-141.

34. Rutella S, Bonanno G, Pierelli L, et al. Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-alpha. *Eur J Immunol*. 2004;34(5):1291-1302.

35. Rossetti M, Gregori S, Roncarolo MG. Granulocyte-colony stimulating factor drives the in vitro differentiation of human dendritic cells that induce anergy in naive T cells. *Eur J Immunol*. 2010;40(11):3097-3106.

36. Chu CC, Ali N, Karagiannis P, et al. Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *J Exp Med*. 2012;209(5):935-945.

37. LeMaoult J, Zafaranloo K, Le Danff C, Carosella ED. HLA-

G up-regulates ILT2, ILT3, ILT4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells. *FASEB J*. 2005;19(6):662-664.

38. Dong Y, Lieskovska J, Kedrin D, Porcelli S, Mandelboim O, Bushkin Y. Soluble nonclassical HLA generated by the metalloproteinase pathway. *Hum Immunol*. 2003;64(8):802-810.

39. Rizzo R, Trentini A, Bortolotti D, et al. Matrix metalloproteinase-2 (MMP-2) generates soluble HLA-G1 by cell surface proteolytic shedding. *Mol Cell Biochem*. 2013;381(1-2):243-255.

40. Taga K, Mostowski H, Tosato G. Human interleukin-10 can directly inhibit T-cell growth. *Blood*. 1993;81(11):2964-2971.

41. de Waal Malefyt R, Yssel H, de Vries JE. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. *J Immunol*. 1993;150(11):4754-4765.

42. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *Journal Exp Med*. 1996;184(1):19-29.

43. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset

inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. 1997;389(6652):737-742.

44. Bacchetta R, Gregori S, Serafini G, et al. Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica*. 2010;95(12):2134-2143.

45. Gregori S, Roncarolo MG, Bacchetta R. Methods for in vitro generation of human type 1 regulatory T cells. *Methods Mol Biol*. 2011;677:31-46.

46. Rizzo R, Hviid TV, Stignani M, et al. The HLA-G genotype is associated with IL-10 levels in activated PBMCs. *Immunogenetics*. 2005;57(3-4):172-181.

47. Rizzo R, Audrito V, Vacca P, et al. HLA-G is a component of the CLL escape repertoire to generate immune suppression: impact of HLA-G 14 bp (rs66554220) polymorphism. *Haematologica*. 2013.

48. Martelli-Palomino G, Pancotto JA, Muniz YC, et al. Polymorphic sites at the 3' untranslated region of the HLA-G gene are associated with differential hla-g soluble levels in the Brazilian and French population. *PloS one*. 2013;8(10):e71742.

49. Zhu XM, Han T, Wang XH, et al. Overexpression of miR-152 leads to reduced expression of human leukocyte antigen-

G and increased natural killer cell mediated cytolysis in JEG-3 cells. *Am J Obstet Gynecol*. 2010;202(6):592 e591-597.

50. Manaster I, Goldman-Wohl D, Greenfield C, et al. MiRNA-mediated control of HLA-G expression and function. *PloS one*. 2012;7(3):e33395.

CHAPTER 4

Human tolerogenic DC-10 modulate allo-specific T cell responses

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ABSTRACT

DC-10 are a subset of human tolerogenic DC characterized by the ability to produce IL-10 and by the expression of HLA-G and ILT4. DC-10 are inducible *in vitro* by culturing peripheral blood monocytes with IL-10, and are present *in vivo* in blood, secondary lymphoid organs, and in decidua of pregnant women. DC-10 modulate $CD4^+$ T cell responses and promote the differentiation of naïve $CD4^+$ T cells in T regulatory type 1 (Tr1) cells *in vitro*. Thus far, little is known about the role of DC-10 on $CD8^+$ T cell responses. In this work, we investigated the *in vivo* and *in vitro* DC-10-mediated modulation of $CD8^+$ T cells. In humanized NSG mice DC-10 primed both $CD8^+$ and $CD4^+$ T cells, anergizing them against allogeneic antigen (allo-Ag). DC-10 activated allogeneic $CD8^+$ T cells *in vitro* at lower levels compared to mature DC (mDC), induced anergic allo-specific $CD8^+$ T cells, and suppressed allogeneic $CD8^+$ T cells activated with mDC in a contact-independent manner. These findings indicate that DC-10 modulate $CD8^+$ T cell responses *in vitro* and and *in vivo*. The ability of DC-10 to limit allo-reactive $CD8^+$ T cell responses allows the long-term allo-specific tolerance *via* the generation of Tr1 cells and support the potential use of DC-10 as DC-based therapy to prevent graft rejection.

INTRODUCTION

Dendritic cells (DC) are highly specialized antigen presenting cells, important initiators of primary T cell responses. DC activate lymphocytes, but they also tolerize T cells to self- and not harmuful antigens (Ag), minimizing autoimmune reactions[1]. The immunosuppressive and immunomodulatory abilities of DC make them interesting tool for cell therapy to promote/restore tolerance in T cell mediated diseases[2]. Tolerogenic DC can lead T regulatory (Treg) cells induction from naïve T cells, and anergy or deletion of effector T cells. Several phase I clinical trials are currently ongoing, and first results demonstrated that tolerogenic DC are well tolerated[3].

Among different tolerogenic DC subsets, we focused our study on DC-10 that can be differentiated *in vitro* by culturing peripheral blood monocytes in the presence IL-10[4]. DC-10 express CD11c, CD14, CD16, CD141 and CD163 and, although not activated, display a mature myeloid phenotype, being $CD80^+$, $CD86^+$ and $HLA\text{-}DR^+$. Secretion of high levels of IL-10 and expression of membrane-bound HLA-G and ILT4 are critical factors involved in DC-10-mediated induction of T Regulatory Type 1 (Tr1) cells[4,5]. Based on these findings, DC-10 are currently used in the clinic to generate allo-specific IL-10-producing Tr1 cells from cell-based therapies[6–8].

The aim of the present work was to investigate the potential use of DC-10 as DC-based cell therapy approach to induce tolerance. We demonstrated that DC-10 prime $CD8^+$ and $CD4^+$ T cells *in vivo* and *in vitro*, inducing hypo-responsive allo-specific T cells. *In vitro* studies showed that DC-10 induce low allogeneic $CD8^+$ T cell activation and proliferation and DC-10-primed $CD8^+$ T cells are hypo-proliferative upon restimulation. Finally, DC-10 suppress allogeneic $CD8^+$ T responses induced by activation with mature DC in a contact-independent manner.

MATERIALS AND METHODS

Cell preparation

Human peripheral blood was obtained from healthy donors in accordance with local committee approval, and informed consent was provided for the use of blood samples according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated *via* density gradient centrifugation over Lymphoprep (Axis-Shield PoC AS, Oslo, Norway).

DC differentiation

$CD14^+$ monocytes were isolated from PBMC by positive selection using CD14 MicroBeads (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Cells were cultured in RPMI 1640 (Lonza, Switzerland) supplemented with 10% FCS (Euroclone, Italy), 100 U/ml penicillin/streptomycin (Lonza, Switzerland), 2 mM L-glutamine (Lonza, Switzerland), supplemented with rhGM-CSF (Miltenyi Biotech, Germany) at 100 ng/ml, rhIL-4 (Miltenyi Biotech, Germany) at 10 ng/ml, and rhIL-10 (CellGenix, Germany) at 10 ng/ml for 7 days to obtained DC-10. On day 3, half of differentiation medium was added to

wells. On day 5, DC differentiated in the absence of rhIL-10 were matured by addition of 1 μg/ml of LPS (Sigma Aldrich, CA, USA) for additional 2 days to obtain mature DC (mDC) or left un-stimulated to generate immature DC (iDC). Resulting DC were recovered on day 7 and their phenotype was evaluated by flow cytometry.

In vivo experiments

6- to 8-week-old female NSG mice were obtained from Charles-River Italia (Calco, Italy). The experimental protocol was approved by the internal committee for animal studies of our institution (Institutional Animal Care and Use Committee [IACUC #632]). On day 0 mice were injected with PBMC (10^7) alone or in combination with allogeneic mDC or DC-10 (10^6). Cells were re-suspended in 250 μl of PBS and infused intraperitoneally. Mice were euthanized at day 14 to analyse human cells in the spleen. $2x10^5$ total splenocytes were then plated with 10^4 mDC, from the same donor of injected DC (allo-mDC) or from an un-related donor (third party mDC). After 3 days ^{3}H-timidine was added in the last 16 hours of co-culture to quantify proliferation. In some experiments, total splenocytes were labelled with Cell Proliferation Dye eFluor® 670

(eBioscience, CA, USA) and proliferation was evaluated by proliferation dye diluition.

T cell purification and culture

$CD8^+$ T cells were purified from PBMC by negative selection using the $CD8^+$ T cell Isolation kit (Miltenyi Biotech, Germany) according to the manufacturer's instructions. For naïve $CD8^+$ T cell isolation, the recovered $CD8^+$ T cells were depleted of $CD45RO^+$ cells using anti-CD45RO-coupled magnetic beads (Miltenyi Biotech, Germany).

10^5 allogeneic $CD8^+$ T cells were cultured with 10^4 DC (DC-10, mDC or iDC) in 200 µl of X-VIVO 15 medium (Lonza, Switzerland), supplemented with 5% human serum (Sigma Aldrich, CA, USA), and 100 U/ml penicillin/streptomycin (Lonza, Switzerland). In same experiment, autologous DC-10 were added at 1:1 ratio to cultures (10^4). After 5 days, T cells were collected, washed, and stained for activation markers. Alternatively, ^{3}H-timidine was added in the last 16 hours of co-culture to quantify proliferation.

10^6 naïve $CD8^+$ T cells were cultured with 10^5 allogeneic mDC or DC-10 with addition of 5 ng/ml of IL-7 (PeproTech, NJ, USA) and IL-15 (R&D System, MN, USA). After 7 days, cells

were collected and 10^5 primed $CD8^+$ T cells were re-stimulated with 10^4 mDC from the same donor of injected DC (allo-mDC) for 3 days or from an un-related donor (third party mDC) for 5 days. ^{3}H-timidine was added in the last 16 hours of co-culture to quantify proliferation.

In transwell experiments, 10^6 $CD8^+$ T cells were stimulated with 10^5 allogeneic mDC in the lower chamber of transwell, while in the upper chamber $5*10^5$ $CD8^+$ T cells were co-cultured with 10^5 allogeneic DC-10, syngenic to mDC. As control, 10^6 $CD8^+$ T cells stimulated with 10^5 allogeneic mDC alone or in combination with 10^5 allogeneic DC-10 were used. After 5 days, T cells were collected, washed, and stained for activation markers and proliferation.

Flow cytometry

To evaluate DC phenotype, cells at day 7 of differentiation protocol were stained with antibodies against CD1a, CD14, CD16, HLA-DR, CD11c (Becton Dickinson, NJ, USA).

To quantify human chimerism in spleens of NSG mice, splenocytes were co-stained with anti-human CD45 (Biolegend), anti-human CD3, anti-human CD8, anti-CD4 and anti-murine CD45 (Becton Dickinson, CA, USA) antibodies. Total

splenocytes were labelled with Cell Proliferation Dye eFluor® 670 (eBioscience, CA, USA) and proliferation was evaluated by proliferation dye diluition.

To study activation of $CD8^+$ T cells, at the end of co-cultures cells were washed and stained with anti-CD3 (Biolegend), anti-CD8, anti-CD25, anti-CD71 and anti-CD137 antibodies (Becton Dickinson, CA, USA). To measure $CD8^+$ T cell proliferation in transwell experiments, intracellular staining was performed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience CA, USA) and anti-Ki67 antibody (Becton Dickinson, CA, USA).

FcR Blocking Reagent (Miltenyi Biotec, German) is used to avoid not specific staining. Samples were acquired using a FACS Canto II Flow Cytometer (Becton Dickinson, CA, USA), and data were analysed with FlowJo software (FlowJo, LLC Oregon, USA).

ELISA

To measure IFN-γ released by activated $CD8^+$ T cells, co-culture supernatants were tested in classical sandwich ELISA following manufacturer's instruction (Becton Dickinson, CA, USA). Levels of sHLA-G were determined as previously described[9]. To

detect sHLA-G plates were coated with purified G233 antibody (Exbio, Czech Republic), while biotinylated W6/32 (Exbio, Czech Republic) was used as secondary antibody. Supernatants from HLA-G transfected LCL721.221 cells[10] purified by affinity chromatography by using the W6/32 mAb were used for the generation of standard calibration curves for shed HLA-G.

Statistical analysis

Non-parametric Mann-Whitney U test for continuous variable or Wilcoxon matched pairs test (two-tailed) were used for statistical analysis. All results are presented as mean values±SEM. Differences were regarded as significant at *P<0.05, **P<0.01, ***P<0.001 and ****P<0.001. Results were analysed using GraphPad Prism 5.0 (GraphPad Software, CA, USA).

RESULTS

DC-10 prime $CD4^+$ and $CD8^+$ T cells *in vivo*, inducing an allo-specific hypo-responsiveness

We studied the ability of *in vitro* differentiated DC-10 to prime T cell responses in humanized mice. NSG mice were co-injected with PBMC and allogeneic DC-10 or mature DC (mDC). After 14 days, the human chimerism within the spleen was similar in mice injected with DC-10 and mDC, an average 49% (±3.7%, ±SEM) and 65% (±2.3%, ±SEM) in DC-10-injected and mDC-injected mice, respectively. The majority of human cells were $CD3^+$ (89.34±3.08% and 86.28±1.43%, mean±SEM, n=5, in DC-10 and mDC injected mice, respectively), whereas the CD4/CD8 ratio was different (Suppl. Fig.1). In DC-10-injected mice an equal percentage of $CD4^+$ and $CD8^+$ T cells was observed, while in mDC-injected mice we found an expansion of $CD4^+$ T cells (60.88±3.18%, mean±SEM, n=5), which were 2-fold higher compared to $CD8^+$ T cells (28.46±1.37%, mean±SEM, n=5). Upon *in vitro* re-challenge of spleen cells with mDC autologous to injected DC (allo-mDC), T cells from DC-10-injected mice proliferated significantly (p=0.008) less compared to those of mDC-injected mice (Fig.1A). Conversely, T cells from DC-10-injected and mDC-injected mice poorly proliferated upon re-stimulation with a third party mDC, demonstrating an effective

in vivo allo-specific priming. We next investigated the proliferative response of $CD4^+$ and $CD8^+$ T cells from spleen of mice injected with PBMC and allogeneic DC-10 or mDC. Results showed that $CD4^+$ T cell response to mDC autologous to injected DC (allo-mDC) was significantly ($p=0.011$) lower in DC-10-injected mice compared to that in mDC-injected mice, but significantly higher ($p=0.047$) compared to that in mice injected with PBMC alone (Fig.1B). Similarly, $CD8^+$ T cells from DC-10-injected mice showed a significant lower ($p=0.006$) proliferative response to allo-mDC, but comparable to that of $CD8^+$ T cells from mice injected with PBMC alone. $CD4^+$ and $CD8^+$ T cell proliferation to third party mDC was similar in the three groups (Fig.1B). These data indicate that DC-10 prime T cells *in vivo*, and promote allo-specific hypo-responsiveness, in both $CD4^+$ and $CD8^+$ T cells.

DC-10 induce low activation and proliferation of allogeneic $CD8^+$ T cells

To define the ability of DC-10 to modulate allogeneic $CD8^+$ T cell responses, we next performed *in vitro* experiments. $CD8^+$ T cells were stimulated with allogeneic DC-10 for 5 days and the levels of CD71, CD25, and CD137 expression was monitored. As control, $CD8^+$ T cells were stimulated with mDC and

immature DC (iDC) generated from the same donor of DC-10. Results shown in Figure 2A indicate that $CD8^+$ T cells stimulated with allogeneic DC-10 expressed significantly lower levels of the activation markers CD71, CD25, and CD137 ($p=0.004$, $p=0.004$ and $p=0.008$, respectively) compared to T cells stimulated with mDC (Fig.2A). Moreover, the percentages of $CD71^+$ $CD8^+$ and $CD25^+$ $CD8^+$ cells in DC-10 co-cultures were also significantly lower ($p=0.008$) compared to those observed in T cells stimulated with iDC. Although DC-10 promoted mild up-regulation of the abovementioned activation markers, the frequencies of positive cells were significantly higher ($p=0.004$) compared to those of un-stimulated $CD8^+$ T cells (Fig.2A). The proliferative response of allogeneic $CD8^+$ T cells stimulated with DC-10 was in line with the activation marker profile. DC-10 induced a significantly lower proliferation compared to both mDC and iDC ($p<0.0001$), but higher compared to that of un-stimulated T cells ($p<0.0001$) (Fig.2B). In 3 out of 13 donors tested, we observed in DC-10 co-cultures IFN-γ production, which was lower compared to that measured in both iDC and mDC co-cultures (Fig.2C). In conclusion, these data indicate that DC-10 activate and induce proliferation in $CD8^+$ T cells at low levels.

DC-10 anergize naïve CD8$^+$ T cells against an antigen-specific re-stimulation

We next studied the potential of DC-10 to induce anergic allo-specific CD8$^+$ T cells. We therefore primed naïve CD8$^+$ T cells with allogeneic DC-10, and test their response upon secondary stimulation. Results depicted in Figure 3A showed that DC-10-primed CD8$^+$ T cells proliferated at lower levels compared to mDC-primed T cells, when allo-mDC were used as stimulators. Conversely, the proliferative response towards third party mDC was similar in both DC-10- and mDC-primed CD8$^+$ T cells. Upon allo-mDC CD8$^+$ T cells primed with DC-10 secreted lower levels of IFN-γ compared to T cells primed with mDC. Differently from what we observed in proliferation, also third party mDC promoted lower IFN-γ secretion by DC-10-primed T cells. These results demonstrated that DC-10 prime naïve CD8$^+$ T cells and induce allo-specific anergy.

DC-10 suppress mDC-stimulated CD8$^+$ T cell response

To evaluate whether DC-10 can modulate an ongoing immune response, we assessed their suppressive ability on activated allogeneic CD8$^+$ T cells. To this end, CD8$^+$ T cells were

stimulated with allogeneic mDC in the presence or absence of DC-10 autologous to mDC. Addition of DC-10 to co-culture significantly ($p=0.002$) dampened mDC-mediated $CD8^+$ T cell responses, with an average of 39.4% (n=10) of suppression of proliferation (Fig.4A). Similarly, DC-10 significantly ($p=0.01$) inhibited the secretion of IFN-γ induced by mDC (5.14±0.41 pg/ml vs 8.55±1.15 pg/ml, mean±SEM, n=10) (Fig.4B). Thus, DC-10 inhibit allo-specific $CD8^+$ T cell responses mediated by autologous mDC.

We next investigated whether DC-10-mediated suppression of $CD8^+$ T cells activated by allogeneic mDC was contact-dependent. $CD8^+$ T cells were stimulated with allogeneic mDC in the lower chamber of transwell, while in the upper chamber $CD8^+$ T cells were co-cultured with DC-10. After 5 days, the proliferative response of allogeneic $CD8^+$ T cells in the lower chamber was evaluated by Ki67 staining. DC-10 in transwell condition suppressed the proliferation of mDC-stimulated $CD8^+$ T cells, as demonstrated by the lower percentages of $Ki67^+$ cells (4.2±1.36% and 18.12±3.95%, mean±SEM, n=6, respectively) (Fig.4C). Surprisingly, the inhibition of $CD8^+$ T cell proliferation mediated by DC-10 in transwell was even higher compared to that exerted by DC-10 in co-culture (8.58±2.38% of $Ki67^+$ cells, mean±SEM, n=6). An average of 76.33% and 52.5% of DC-10-mediated suppression in transwell and co-cultures, respectively,

was observed. Moreover, DC-10 in transwell reduced IFN-γ production by $CD8^+$ T cells stimulated with mDC at similar levels that those observed with DC-10 in co-culture (Fig.4D). In conclusion, DC-10 suppress proliferation and consequent IFN-γ production of allo-stimulated $CD8^+$ T cells in a contact-independent manner.

The three key molecules for $CD4^+$ T regulatory Type 1 (Tr1) cells induction by DC-10 are IL-10, HLA-G and its receptor ILT-4. IL-10 levels in the co-colture supernatants were very low (data not shown), indicating that IL-10 secreted by DC-10 cannot mediated $CD8^+$ T cell suppression. HLA-G is not only expressed as membrane-bound, but can also be secreted or released from cell membrane by shedding[11]. We therefore evaluated the sHLA-G levels in the supernatants. Interestingly, addition of DC-10 in transwell resulted in a 2-fold increase in sHLA-G levels compared to those found in mDC-CD8 co-culture (3988±612 and 1723±278 pg/ml, mean±SEM, n=5) (Fig.4E). Surprisingly, sHLA-G in DC-10 co-colture showed similar concentration to that in mDC-co-colture (2112±182 pg/ml, mean±SEM, n=5) (Fig.4E). These results suggested DC-10 suppressive effect on $CD8^+$ T cells could be mediated by sHLA-G.

DISCUSSION

We showed that DC-10 modulate $CD4^+$ and $CD8^+$ allogeneic T cell, both *in vivo* and *in vitro*. DC-10 injected in NSG mice prime allogeneic $CD4^+$ and $CD8^+$ and promote their allo-specific hypo-responsiveness. The low activation and proliferation of $CD8^+$ T cells is observed also *in vitro*, when DC-10 are co-cultured with allogeneic $CD8^+$ T cells. Moreover, allogeneic naïve $CD8^+$ T cells primed by DC-10 are anergic and secrete very low levels of IFN-γ when restimulated with the same allo-antigen (allo-Ag) used for their priming, while responsiveness to unrelated Ags is maintained. Finally, addition of DC-10 modulate proliferation and IFN-γ production of $CD8^+$ T cells activated with mature DC (mDC). The suppressive mechanism mediated by DC-10 is contact-independent and might be mediated by the secretion and shedding of soluble HLA-G.

DC-10 modulation of $CD4^+$ T cell responses has been already demonstrated[4]. DC-10 are currently used for generating T regulatory Type 1 (Tr1) cells for a Treg-based cell therapy. Less is known about the modulatory activity exerted by DC-10 on $CD8^+$ T cells. In this study, we showed that DC-10 activate at low levels allogeneic $CD8^+$ T cells, both *in vitro* and *in vivo*. The low level of activation is not dependent on IL-10 secreted by DC-10. Differently from $CD4^+$ T cells, IL-10 does not

suppressed $CD8^+$ T cell proliferation[12], and addition of neutralizing anti-IL-10R antibodies during activation of $CD8^+$ T cells by DC-10 or IL-10 addition during activation of $CD8^+$ by allogeneic mDC do not increase proliferation (data not shown). The low proliferative response induced by DC-10 in allogeneic $CD8^+$ T cells is not associated with the absence of signals necessary for their proper activation. Three signals are required for optimal $CD8^+$ T cell activation: antigen presentation to TCR, engagement of CD28 by costimulatory molecules, and cytokines-mediated signals, predominantly *via* IL-12. DC-10 express HLA class I molecules at levels comparable to that expressed by both immature (iDC) and mature DC, and display a mature phenotype, with CD80 and CD86 expression higher than iDC. Moreover, addition of exogenous IL-12 during activation of $CD8^+$ T cells by DC-10 partially increased T cell proliferation (data not shown), which, however, never reached the levels observed when $CD8^+$ T cells are stimulated with mDC.

Based on these data, we hypothesized that DC-10 should provide negative signals to $CD8^+$ T cells that prevent their activation and proliferation. Among molecules highly expressed by DC-10, which may provide inhibitory signals to $CD8^+$ T cells, HLA-G might be the player. However, results with neutralizing anti-HLA-G antibodies added during DC-10-mediated activation of $CD8^+$ T cells did not reverse the low proliferative response.

Notably, DC-10 expressed high levels of ILT-3, molecule known to inhibit T cell proliferation and suppress differentiation of IFN-γ $CD8^+$ cytotoxic T cells[13]. We are currently investigating whether signal mediated by ILT3 to $CD8^+$ T cells may limit their activation and consequent proliferation. The presence of an inhibitory signal provided by DC-10 to $CD8^+$ T cells is further confirmed by the induction of allo-specific anergic T cells. Anergic $CD8^+$ T cells have been described to be induced upon IL-10-treated DC stimulation[14]. However, differently from the latter $CD8^+$ T cells, the phenotype of $CD8^+$ T cells generated upon stimulation with DC-10 did not express up-regulated levels of CTLA-4 (data not shown). A population of allo-specific suppressor $CD8^+$ T cells have been described by Liu et al.[15], the $CD8^+CD28^-$ suppressor T (Ts) cells, which are induced upon repetitive stimulation of PBMC with allogeneic antigen presenting cells. Anergic $CD8^+$ T cells induced by DC-10 express CD28 and do not express FOXP3, indicating that are distinct from $CD8^+CD28^-$ Ts cells. Interestingly, DC-10-primed $CD8^+$ T cells resemble the $CD8^+$ Treg cells generated by stimulating $CD8^+$ T cells with CD40-ligand activated plasmacytoid DC[16], which are hypo-responsive and secrete low levels of IFN-γ upon re-stimulation with the same allo-Ag used for their priming. It has still to be defined whether anergic $CD8^+$ T cells induced by DC-10 suppress allo-proliferation of

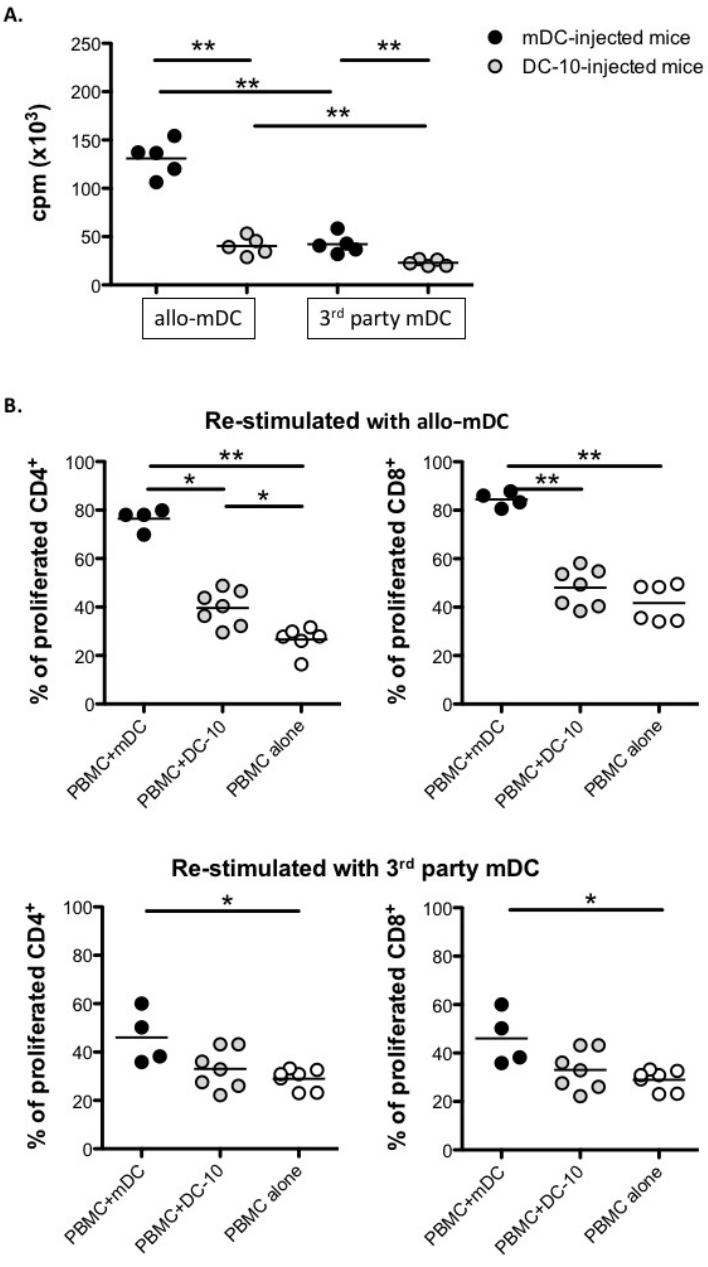
autologous naïve $CD8^+$ T cells, similar to Treg cells induced by activated pDC.

DC-10 suppression of mDC-stimulated $CD8^+$ T cell demonstrate that DC-10 not only modulate $CD8^+$ T cell in primary response, but also affect an ongoing response. The inhibition mediated by DC-10 is not dependent on competition between mDC and DC-10 for $CD8^+$ priming, as shown by transwell experiments. Moreover, the latter experiments demonstrate that DC-10-mediated suppression of activated $CD8^+$ T cells is contact-independent. Differential expression levels of soluble HLA-G in presence or absence of DC-10 in the CD8/mDC co-culture suggest that HLA-G can be the key molecule mediating DC-10 suppressive activity on activated $CD8^+$ T cells. We are currently testing whether inhibition of HLA-G-mediated signalling into $CD8^+$ T cells by blocking ILT-2, one of the HLA-G receptors, could revert $CD8^+$ T cell proliferation. Another possible candidate involved in DC-10-mediated inhibition of activated $CD8^+$ T cells is CD163, which is expressed at high levels on DC-10 cell membran [4] (Comi et al., manuscript in preparation). It has been reported that CD163 can be cleaved by proteases and released as soluble form[17] which significantly inhibit T cell proliferation upon phorbol-ester (TPA) stimulation[18]. We will investigate whether DC-10 release sCD163 from their membrane at steady state or upon activation and if this molecule in involved

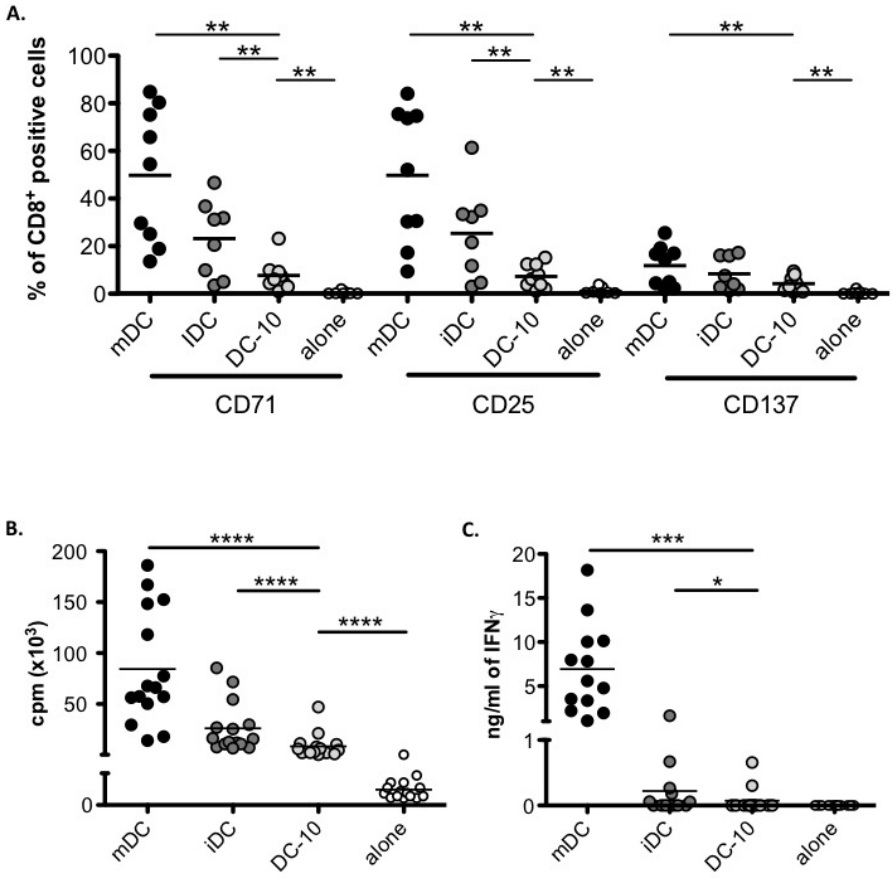
in DC-10 tolerogenic activity. Addition of exogenous VEGF during T cell stimulation with anti-CD3 antibody and IL-2 reduced proliferation[19]. VEGF is secreted by DC-10 at steady state and upon TLR stimulation (data not shown), thus, we are planning to define whether VEGF could be responsible for DC-10 mediated suppression of $CD8^+$ T cells. It cannot be excluded that the above mentioned mechanisms could cooperate in DC-10-mediated suppression of $CD8^+$ T cells.

In summary, DC-10 modulate allogeneic $CD8^+$ T cells *in vitro* and *in vivo*, overall limiting their proliferation and anergizing them towards allo-Ags, strongly support the potential use of DC-10 as DC-based cell therapy. In allogeneic transplantation, DC-10 ability to anergize T cells in primary immune response would be a valuable tool to generate a favourable milieu for establishment of a long-term tolerance, mediated by Tr1 induction. Conversely, dampening of mDC-stimulated $CD8^+$ T cell response demonstrates DC-10 ability to modulate an ongoing immune response, and thus the possibility to use DC-10 as cell therapy in autoimmune disorders.

FIGURES



Comi M. et al., Figure 1



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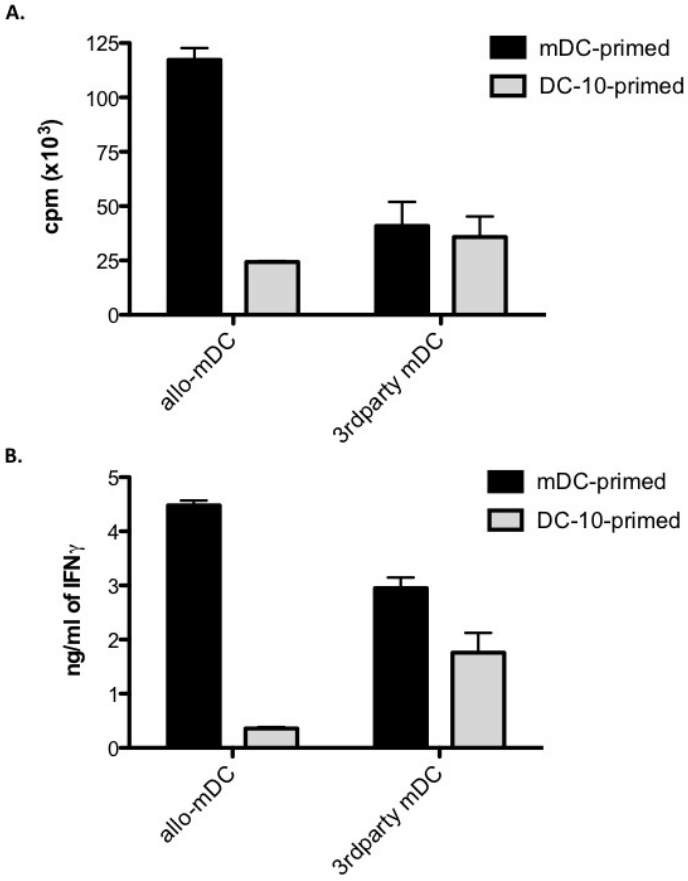

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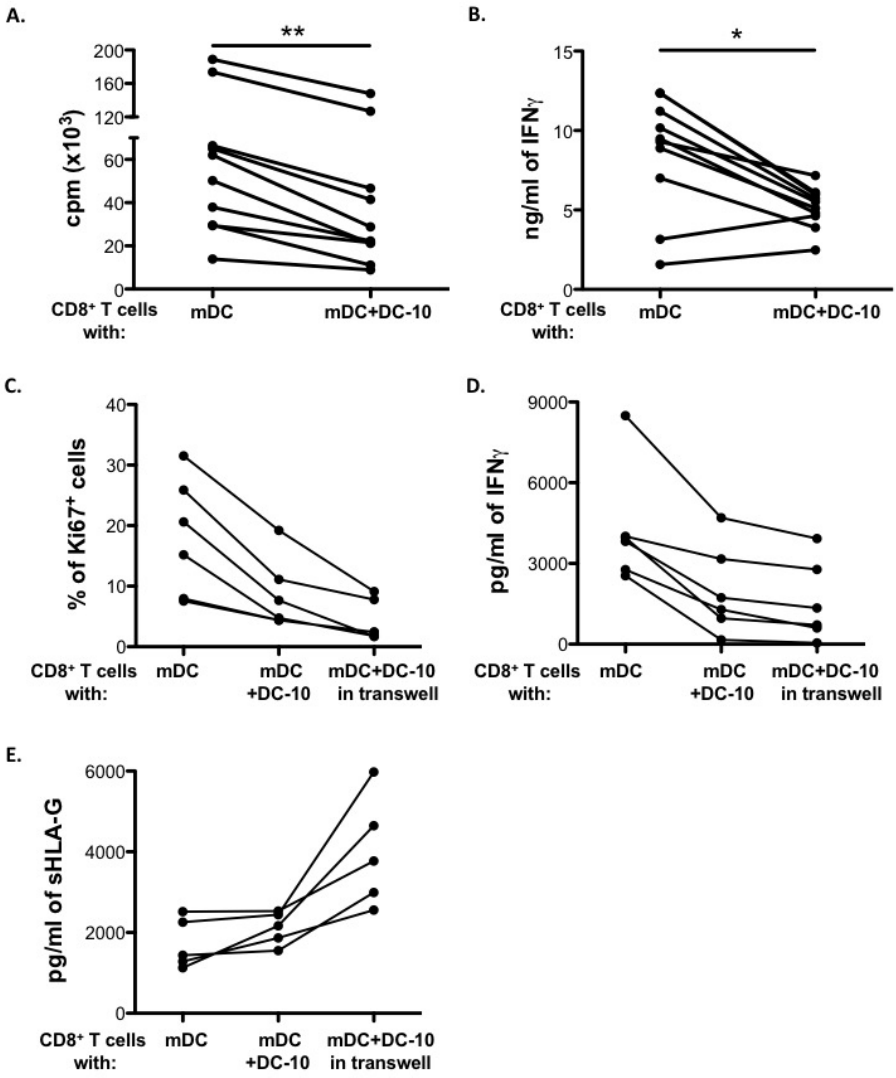

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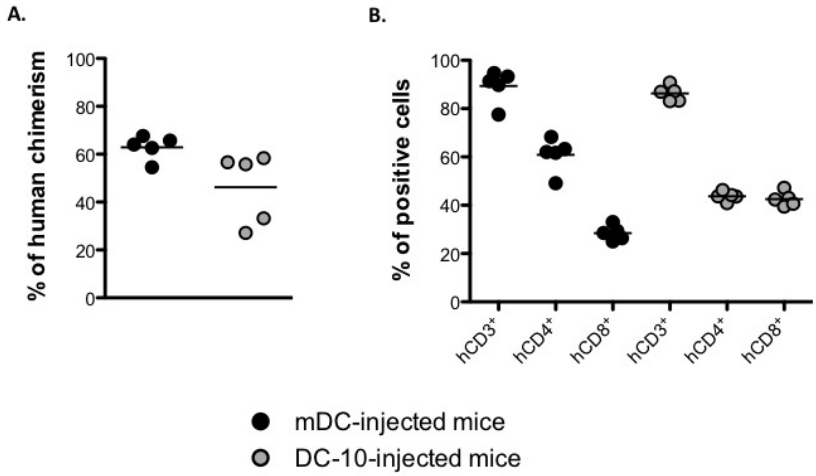

Comi M. et al., Figure 1s

FIGURE LEGENDS

Figure 1. DC-10 induce hypo-responsiveness in *in vivo* primed T cells. NSG mice were intraperitoneally injected with PBMC (10^7 cells) and allogeneic mDC or DC-10 (10^6 cells). After 14 days mice were sacrificed and total splenocytes were re-challenged *in vitro* with mDC of the same donor of DC injected in mice (allo-mDC) or third party mDC. **A.** Proliferative responses were evaluated after 4 days of co-culture by radioactive thymidine (^{3}HThy) incorporation in the last 16 hours of culture. One out of three independent experiments is shown. **P<0.01. **B and C**. Proliferative responses were evaluated after 3 days of co-culture by proliferation dye diluition. Percentages of proliferated human $CD4^+$ and $CD8^+$ in response to allo-mDC (B) or third party mDC (C) are indicated. Each dot represents a single mouse and lines indicate mean. *P<0.05, **P<0.01

Figure 2. DC-10 induce low activation and proliferation of allogeneic $CD8^+$ T cells. $CD8^+$ T cells were cultured alone or in the presence of allogeneic mDC, iDC or DC-10 at 10:1 ratio for 4 days. **A.** Activation of $CD8^+$ T cells was evaluated by CD71, CD25 and CD137 staining. Numbers indicate percentages of

positive cells gated on $CD8^+$ T cells. Each dot represents a single donor and lines indicate mean. Only statistics against DC-10 are indicated in the plot. *P < 0.05. **B.** Proliferative responses were evaluated by ^{3}HThy incorporation in the last 16 hours of culture. Each dot represents a single donor and lines indicate mean. Only statistics against DC-10 are indicated in the plot ****P < 0.0001 **C.** IFN-γ in culture supernatants were measured by ELISA. Each dot represents a single donor and lines indicate mean. Only statistics against DC-10 are indicated in the plot ***P < 0.001 *P <0.05

Figure 3. DC-10 anergize $CD8^+$ T cells in allo-specific manner. Naïve $CD8^+$ T cells were cultured with allogeneic mDC or DC-10 at 10:1 ratio for 10 days and then CD8-primed cells were re-stimulated either with allogeneic mDC from the same donor for 2 days, or with third party mDC for 4 days. **A.** Proliferative responses were evaluated by ^{3}HThy incorporation in the last 16 hours of culture. One representative donor out of 3 tested is shown. **B.** IFN-γ production was quantified in co-culture supernatants. One representative donor out of 3 tested is shown.

Figure 4. DC-10 suppress mDC-stimulated $CD8^+$ T cells response. **A and B.** $CD8^+$ T cells were cultured with allogeneic mDC in presence or absence of DC-10, autologous to mDC, for 5 days. Proliferative responses (A) were evaluated by ^{3}HThy incorporation in the last 16 hours of culture and IFN-γ (B) in culture supernatants was measured by ELISA. Each dot represents a single donor. *P <0.05, **P< 0.01. **C, D and E.** $CD8^+$ T cells were cultured with allo-mDC for 5 days in three different conditions: alone, in the presence of DC-10 or in the lower chamber of transwell, with $CD8^+$ T cells and DC-10 in the upper chamber of transwell. Proliferation (C) was measured by intracellular staining for Ki67, IFN-γ (D), and sHLA-G (E) in culture supernatants were measured by ELISA. Each dot represents a single donor.

Supplementary Fig.1 Spleen composition of NSG mice injected with PBMC and mDC or DC-10. NSG mice were intraperitoneally injected with PBMC (10^7 cells) and allogeneic mDC or DC-10 (10^6 cells). After 14 days mice were sacrificed and spleen composition was analysed at flow cytometry. **A.** Human chimerism is calculated as %human$CD45^+$/(%human$CD45^+$+%murine$CD45^+$)*100. **B.** Percentages of CD3, CD4 and CD8 positive cells are quantified

in humanCD45^{+} (hCD45^{+}) gate. Each dot represents a single mice and lines indicate mean.

REFERENCES

1. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **392,** 245–52 (1998).

2. Naranjo-Gómez, M. *et al*. Comparative study of clinical grade human tolerogenic dendritic cells. *J. Transl. Med*. **9,** 89 (2011).

3. Ten Brinke, A. *et al*. Clinical Use of Tolerogenic Dendritic Cells-Harmonization Approach in European Collaborative Effort. *Mediators Inflamm*. **2015,** 471719 (2015).

4. Gregori, S. *et al*. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–44 (2010).

5. Amodio, G. *et al*. HLA-G expression levels influence the tolerogenic activity of human DC-10. *Haematologica* **100,** 548–57 (2015).

6. Bacchetta, R. *et al*. Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–43 (2010).

7. Amodio, G. & Gregori, S. Human tolerogenic DC-10: perspectives for clinical applications. *Transplant. Res*. **1,** 14

(2012).

8. Petrelli, A. *et al*. Generation of Donor-specific T Regulatory Type 1 Cells From Patients on Dialysis for Cell Therapy After Kidney Transplantation. *Transplantation* **99,** 1582–9 (2015).

9. Rizzo, R. *et al*. Defective production of soluble HLA-G molecules by peripheral blood monocytes in patients with asthma. *J. Allergy Clin. Immunol.* **115,** 508–13 (2005).

10. Shimizu, Y., Geraghty, D. E., Koller, B. H., Orr, H. T. & DeMars, R. Transfer and expression of three cloned human non-HLA-A,B,C class I major histocompatibility complex genes in mutant lymphoblastoid cells. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 227–31 (1988).

11. Park, G. M. *et al*. Soluble HLA-G generated by proteolytic shedding inhibits NK-mediated cell lysis. *Biochem. Biophys. Res. Commun.* **313,** 606–611 (2004).

12. Groux, H., Bigler, M., de Vries, J. E. & Roncarolo, M. G. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J. Immunol.* **160,** 3188–93 (1998).

13. Kim-Schulze, S. *et al*. Recombinant Ig-like transcript 3-Fc modulates T cell responses via induction of Th anergy and differentiation of CD8+ T suppressor cells. *J. Immunol.* **176,**

2790–8 (2006).

14. Steinbrink, K., Graulich, E., Kubsch, S., Knop, J. & Enk, A. H. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* **99,** 2468–76 (2002).

15. Liu, Z., Tugulea, S., Cortesini, R. & Suciu-Foca, N. Specific suppression of T helper alloreactivity by allo-MHC class I-restricted CD8+CD28- T cells. *Int. Immunol.* **10,** 775–83 (1998).

16. Gilliet, M. & Liu, Y.-J. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J. Exp. Med.* **195,** 695–704 (2002).

17. Droste, A., Sorg, C. & Högger, P. Shedding of CD163, a novel regulatory mechanism for a member of the scavenger receptor cysteine-rich family. *Biochem. Biophys. Res. Commun.* **256,** 110–3 (1999).

18. Högger, P. & Sorg, C. Soluble CD163 inhibits phorbol ester-induced lymphocyte proliferation. *Biochem. Biophys. Res. Commun.* **288,** 841–3 (2001).

19. Ziogas, A. C. *et al.* VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2. *Int. J. cancer* **130,** 857–64

(2012).

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

Dendritic cells play a key role in immune system: they stimulate immune responses to pathogens and non-self antigens (Ags), and induce and maintain tissue homeostasis and immunological tolerance towards self or non-harmful Ags. This dual activity is achieved by specific functional segregation in pro-inflammatory and tolerogenic DC. Pro-inflammatory DC differentiated *in vitro* have been already applied in the clinic as cell therapy to promote active immune responses, typically in tumours[1]. Conversely, tolerogenic DC have become only recently a promising tool to suppress immune responses in T-cell mediated diseases, including transplantation and autoimmune disorders[2,3]. The development of methods to generate clinical grade products allowed the application of tolerogenic DC-based therapy, and thus far, clinical trials demonstrated the safety and feasibility of this approach. However, one of the open issues for improving the safety and the efficacy of a successful DC-based cell therapy is the stability of infused DC and the maintenance of their tolerogenic properties once they are introduced in an inflammatory environment. Gene expression profile data presented in this thesis demonstrates that DC-10 maintain a stable transcriptome upon activation: only 505 genes were differentially expressed between DC-10 in steady state and upon LPS stimulation. Moreover, LPS-activated DC-10 maintain their

cytokine production profile, the ability to poorly stimulate both $CD4^+$ and $CD8^+$ T cells (data not shown), and to promote the induction of anergic allo-specific Tr1 cells[4]. In addition, upon activation *via* different TLRs, DC-10 never acquired the ability to secrete IL-12, but produce high levels of IL-10 (data not shown). *In vivo* data confirmed the stability of the tolerogenic functions of DC-10: injected DC-10 are able to prime *in vivo* allogeneic T cells, limiting their proliferation and anergizing both $CD4^+$ and $CD8^+$ T cells towards allo-Ags. These findings strongly support the potential use of DC-10 as DC-based cell therapy to prevent allograft rejection in transplantation. In this setting, DC-10 administered concomitantly with the transplant would modulate primary allo-immune responses, allowing the establishment of a long-term tolerance by the induction of allo-specific $CD4^+$ Tr1 cells. In addition, it can be foreseen the use of DC-10 as cell product to restore tolerance in autoimmune diseases. In this clinical condition, it can be envisaged that DC-10 would be administered to patients with active autoimmune diseases, therefore, once injected, they should be able to inhibit an ongoing immune response and restore the correct balance between immunity and regulation by inducing Ag-specific Tregs. We demonstrated that addition of DC-10 in mDC-induced allogeneic T cells, either total PBMC (data not shown) or $CD8^+$ T cells, suppress proliferation and modulate T cell responses. These findings support the modulatory activity of DC-10 not

only during primary responses but also during an ongoing immune response. To restore autoAg-specific tolerance, DC-10 would both selectively control Ag-specific effector T (Teff) cell responses and support the induction and/or expansion of Ag-specific Tregs. To confer Ag-specificity to DC-10 different approaches can be envisaged and are currently under development. The first one is *in vitro* pulsing of DC-10 with a selected autoAg, similar to the current approach used in the clinical trial in rheumatoid arthritis and multiple sclerosis with tolerogenic DC generated with vitamin D3 and Dexamethasone[5–7]. Alternatively, to confer stable expression of autoAg by DC-10, we are currently testing the use of lentiviral vector-mediated gene transfer. If successful, the latter approach will lead to the generation of stable Ag-specific tolerogenic DC-10.

DC-10 are central determinant in the IL-10-mediated tolerance: they modulate the microenvironment towards long-lasting and self-feeding tolerance through the *in vivo* generation of Ag-specific Tr1 cells. Thus far, the lack of specific biomarkers allowing DC-10 detection *in vivo* limits their study. The identification of CD141 and CD163 as specific markers that in combination with CD14 and CD16 allow DC-10 detection and selection *in vivo* provides a powerful tool either for tracking these cells or for investigating their tolerogenic activity *ex vivo*. The possibility to track and identify DC-10 will make it possible

to study their *in vivo* localization and, therefore, their contribution in promoting tissue and organs homeostasis. Moreover, the enumeration of DC-10 *in vivo* will be critical for defining their role in physiological and pathological conditions. We are currently investigating whether the frequency and phenotype of DC-10 vary in peripheral blood in healthy conditions by analysing donors at different age. Moreover, we have already started the analysis of the frequency of DC-10 in peripheral blood and tissues of patients affected by autoimmune diseases, including Type 1 Diabetes (T1D), Celiac Disease (CD) or patients underwent allogeneic hematopoietic stem cell transplantation for β-thalassemia who developed tolerance associated with Tr1 cells. In the context of T1D, we are defining whether DC-10 frequency in peripheral blood may be used as biomarker to stage disease development, whereas in CD we are testing whether DC-10 frequency increased under gluten-free diet sustaining the induction of Tr1 cells. Results will be critical important for better define the role of *in vivo* occurring DC-10 in tolerance induction.

Crossing transcriptome and miRNome data, we will define specific pathways up- or down-regulated in DC-10 involved in their cell identity and functions. The analysis of expressed genes and their post-transcriptional regulation will allow the identification of key molecules, which can be used to induce

"super" DC-10. We already set up a protocol for high efficient DC-10 transduction with lentiviral vectors and the over-expression of tolerogenic molecule through this platform will result in increasing or further stabilizing DC-10 activity. Among molecules known to confer potent tolerogenic activity of DC-10, we are currently testing the effect of HLA-G over-expression in DC-10. Indeed, based on the presented results, DC-10 expressing high levels of HLA-G more efficiently promote the induction of Tr1 cells *in vitro*.

Overall, results from this thesis improved the knowledge on the biology of *in vitro* differentiated DC-10 and, the use of identified markers it makes it possible to better characterize *in viv*o occurring DC-10.

REFERENCES

1. Bol, K. F., Schreibelt, G., Gerritsen, W. R., de Vries, I. J. M. & Figdor, C. G. Dendritic Cell-Based Immunotherapy: State of the Art and Beyond. *Clin. Cancer Res.* **22,** 1897–906 (2016).

2. Rosborough, B. R., Raïch-Regué, D., Turnquist, H. R. & Thomson, A. W. Regulatory myeloid cells in transplantation. *Transplantation* **97,** 367–79 (2014).

3. Ten Brinke, A. *et al*. Clinical Use of Tolerogenic Dendritic Cells-Harmonization Approach in European Collaborative Effort. *Mediators Inflamm*. **2015,** 471719 (2015).

4. Amodio, G. & Gregori, S. Human tolerogenic DC-10: perspectives for clinical applications. *Transplant. Res*. **1,** 14 (2012).

5. Bell, G. M. *et al*. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann. Rheum. Dis*. **73,** no pagination (2016).

6. Benham, H. *et al*. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci. Transl. Med*. **7,** 290ra87-290ra87 (2015).

7. Mansilla, M. J. *et al*. Beneficial effect of tolerogenic

dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. *CNS Neurosci. Ther*. **21,** 222–30 (2015).