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Master Thesis

Investigation of the molecular mechanisms that are involved in the anti-inflammatory function of CTLA-4-treated dendritic cells

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Εργασία Μεταπτυχιακού Τίτλου Ειδίκευσης

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Abstract

DCs are the connecting step between innate and adaptive immunity and their major role is to present antigens to T cells. A mixture of instructions that reach naïve T cells affects their activation and leads them to acquire different effector phenotypes. These instructions are primarily derived by DCs which go through variable transcriptional and metabolic alterations. DCs produce three types of signals to stimulate T cell differentiation. Antigen presentation is the first signal (signal 1) but only in combination with co-activation signals (signal 2) they can induce T cell activation. One of the most essential secondary signals is promoted by the binding of CD80/86 on DC membrane with CD28 protein on T cell surface. CTLA-4, which is expressed by T cells, binds on CD80/86 to regulate T cell activation. A soluble form of CTLA-4 (CTLA-4–Ig) is used for treatment of autoimmune inflammatory diseases such as rheumatoid arthritis (RA). Also, the secretion of cytokines by DCs affects the polarization of T cells as well (signal 3).

In parallel, DCs are also capable to preserve homeostatic conditions upon harmless or self-antigens by providing tolerogenic signals to T cells. Tolerogenic DCs are generated both *in vivo* and *in vitro* upon a variety of different agents, such as dexamethasone, and they are able to inhibit T cell responses via different mechanisms. Failure of self-tolerance drives overactivation of lymphocytes which leads to the development of autoimmunity. Drugs that promote anti-inflammatory properties of DCs are another therapeutic approach for autoimmune inflammation in diseases like RA. Recent studies, including ours, have noted that soluble CTLA-4 (CTLA-4 Ig is an approved treatment for RA) is able not only to block T cell stimulation but it induces reverse signaling in DCs and promotes anti-inflammatory properties.

Hence, in order to further investigate the molecular mechanisms that drive the CTLA-4–mediated tolerogenic effects on DCs, we used an *ex vivo* setup where healthy human monocyte-derived DCs (moDCs) were cultured with CTLA-4–Ig. To address this, we analyzed the immunogenic profile of CTLA-4–Ig-treated moDCs. For the study of signal 1, we measured the levels of HLA-DR on moDCs surface which were elevated. T cell activation needs also signal 2 which is derived from co-stimulatory molecules. Among these, CD80 and CD86, through which CTLA-4 exerts its cell-extrinsic effects, were downregulated. The CD83 maturation marker was upregulated upon CTLA-4–Ig treatment. For the investigation of signal 3, we analyzed the mRNA of genes that encode important secreted factors. The expression of the pro-inflammatory *IL-6* and *IL-12b* genes and of the anti-inflammatory *IDO* and *IL-10* genes were inhibited in CTLA-4–Ig-treated moDCs. Interestingly, the IL-6 protein levels were found elevated.

Moreover, tolerogenic characteristics of DCs are related to changes in cellular metabolism. To better characterize this, we performed RNAseq analysis of human moDCs treated with CTLA-4–Ig, which revealed 575 differentially expressed genes.

Among them, the most deregulated pathways were those associated with mitochondria and oxidative phosphorylation. Also, a molecular pathway that is implicated in both metabolism and inflammatory responses is the Akt/mTOR-dependent pathway was found differentially regulated. It has been previously demonstrated that CTLA-4–Ig treatment on DCs activates the above pathway driving the induction of anti-inflammatory responses and the inhibition of autophagy, an important process for antigen presentation. Our results indicated an increase in mTOR protein expression while that of Akt was decreased.

In summary, our findings support that CTLA-4–Ig is able to induce tolerogenic DCs via downregulation of co-stimulatory molecules and inhibition of pro-inflammatory cytokines and these changes are associated with deregulation of essential metabolic pathways.

Περίληψη

Τα δενδριτικά κύτταρα (DCs) είναι ο συνδετικός κρίκος μεταξύ εγγενούς και προσαρμοστικής ανοσίας και βασικός τους ρόλος είναι η παρουσίαση αντιγόνων στα Τ κύτταρα. Ο συνδυασμός των «οδηγιών» που φτάνουν στα πρώιμα Τ κύτταρα επηρεάζει την ενεργοποίησή τους και οδηγεί την απόκτηση διαφορετικών δραστικών φαινοτύπων. Αυτές οι οδηγίες προέρχονται πρωτίστως από DCs τα οποία υφίστανται ποικίλες μεταγραφικές και μεταβολικές αλλαγές. Τα DCs παράγουν τρία είδη σημάτων για να προκαλέσουν τη διαφοροποίηση των Τ κυττάρων. Το πρώτο σήμα (σήμα 1) είναι η αντιγονοπαρουσίαση η οποία δεν μπορεί από μόνη της να προκαλέσει τη διαφοροποίηση των Τ κυττάρων αλλά πρέπει να συνδυαστεί με συν-ενεργοποιητικά σήματα (σήμα 2). Ένα από τα σημαντικότερα δευτερογενή σήματα είναι η πρόσδεση των CD80/86 των DCs με την πρωτεΐνη CD28 στην επιφάνεια των Τ κυττάρων. Ο CTLA-4, ο οποίος εκφράζεται από τα Τ κύτταρα, προσδένεται επίσης στα CD80/86 για τη ρύθμιση της ενεργοποίησης των Τ. Η διαλυτή μορφή του CTLA-4 (CTLA-4–Ig) χρησιμοποιείται για τη θεραπεία αυτοάνοσων φλεγμονωδών νοσημάτων, όπως η ρευματοειδής αρθρίτιδα (PA). Επίσης, και η έκκριση κυτοκινών από τα DCs επηρεάζει την πόλωση των Τ κυττάρων (σήμα 3).

Παράλληλα, τα DCs έχουν την ικανότητα να διατηρούν ομοιοστατικές συνθήκες υπό την επίδραση ακίνδυνων ή αυτό- αντιγόνων παρέχοντας ανοσοκατασταλτικά σήματα στα Τ κύτταρα. Ανοσοκατασταλτικά DCs παράγονται *in vivo* και *in vitro* από μια σειρά διαφορετικές θεραπείες, όπως η δεξαμεθαζόνη, και μπορούν να καταστείλουν τις αποκρίσεις των Τ κυττάρων μέσω διαφόρων μηχανισμών. Η κατάρρευση της ανοχής του εαυτού οδηγεί στην υπερενεργοποίηση των λεμφοκυττάρων και προκαλεί την ανάπτυξη αυτοάνοσων διαταραχών. Φάρμακα που προωθούν τις αντι-φλεγμονώδεις ιδιότητες των DCs είναι μια άλλη θεραπευτική προσέγγιση της αυτοάνοσης φλεγμονής σε ασθένειες όπως η PA. Πρόσφατες μελέτες, μεταξύ των οποίων και δικές μας, έχουν υποδείξει ότι η διαλυτή CTLA-4 (CTLA04-Ig) όχι μόνο εμποδίζει την ενεργοποίηση των Τ κυττάρων αλλά προκαλεί ανάστροφη σηματοδότηση στα DCs προάγοντας αντι-φλεγμονώδεις ιδιότητες.

Ως εκ τούτου, προκειμένου να μελετήσουμε περαιτέρω τους μοριακούς μηχανισμούς που οδηγούν σε παραγωγή ανοσοκατασταλτικών DCs υπό την επίδραση της CTLA-4, καλλιεργήσαμε *ex vivo* με CTLA-4–Ig DCs προερχόμενα από μονοκύτταρα (moDCs) υγιών ανθρώπων. Για να το διερευνήσουμε, αναλύσαμε το ανοσολογικό προφίλ των moDCs μετά τη δράση του CTLA-4–Ig. Για τη μελέτη του σήματος 1, υπολογίσαμε τα επίπεδα του HLA-DR στην επιφάνεια των moDCs τα οποία βρέθηκαν αυξημένα. Η ενεργοποίηση των Τ κυττάρων απαιτεί και το σήμα 2, το οποίο προέρχεται από συν-ενεργοποιητικά μόρια. Ανάμεσα σε αυτά, οι CD80 και CD86, μέσω των οποίων ο CTLA-4 προκαλεί τις εξωκυτταρικές του δράσεις, ήταν μειωμένα. Ο παράγοντας ωρίμανσης CD83 βρέθηκε αυξημένος μετά τη δράση του CTLA-4–Ig. Για τη διερεύνηση του σήματος 3, αναλύσαμε τα επίπεδα mRNA των γονιδίων που κωδικοποιούν σημαντικούς εκκρινόμενους παράγοντες. Η έκφραση των προ-

φλεγμονωδών γονιδίων *IL-6* και *IL-12b* και των αντι-φλεγμονωδών γονιδίων *IDO* και *IL-10* καταστάλθηκε στα moDCs υπό την επίδραση του CTLA-4–Ig. Ενδιαφέρον εύρημα ήταν ότι τα πρωτεϊνικά επίπεδα της IL-6 βρέθηκαν αυξημένα.

Επιπλέον, ανοσοκατασταλτικά χαρακτηριστικά των DCs σχετίζονται με αλλαγές στον κυτταρικό μεταβολισμό. Με σκοπό την περαιτέρω μοριακή ανάλυση, έγινε ανάλυση γονιδιακής έκφρασης (RNAseq) η οποία αποκάλυψε 575 γονίδια με διαφορετική έκφραση υπό την επίδραση του CTLA-4–Ig. Ανάμεσά τους, περισσότερα απορυθμισμένα μονοπάτια σχετίζονταν με τα μιτοχόνδρια και την οξειδωτική φωσφορυλίωση. Επίσης, ένα μοριακό μονοπάτι που εμπλέκεται και στο μεταβολισμό και στις φλεγμονώδεις αντιδράσεις είναι το μονοπάτι Akt/mTOR. Έχει δειχθεί από παλαιότερες μελέτες ότι η καλλιέργεια DCs με CTLA-4–Ig ενεργοποιεί το προαναφερθέν μονοπάτι επάγοντας αντι-φλεγμονώδεις δράσεις και καταστέλλοντας την αυτοφαγία που είναι σημαντική για την αντιγονοπαρουσίαση. Τα αποτελέσματά μας δείχνουν μια αύξηση στην πρωτεϊνική έκφραση του mTOR και μια μείωση στην έκφραση του Akt.

Συνολικά, τα ευρήματά μας υποστηρίζουν ότι ο CTLA-4–Ig μπορεί να επάγει ανοσοκατασταλτικά DCs μέσω της μειωμένης έκφρασης συν-ενεργοποιητικών μορίων και της καταστολής προ-φλεγμονωδών κυτταροκινών ενώ αυτές οι αλλαγές συνδέονται με απορρύθμιση σημαντικών μεταβολικών μονοπατιών.

Introduction

Dendritic cells shape immune responses

Dendritic cells (DCs) are raised in the bone marrow from multipotent hematopoietic stem cells (HSCs), sharing a common progenitor path with monocytes. HSCs differentiate, firstly, into common myeloid precursor and, subsequently, to monocyte-DC progenitor before they give rise to common monocyte progenitors, that produce pre-monocytes, or to common DCs progenitors, that lead to production of fully differentiated plasmacytoid DCs (pDCs) and pre-classical DCs (pre-cDCs). Recent evidence suggest pDCs can rise also from a lymphoid precursor, contributing to the heterogeneity of this subset (1). Pre-cDCs migrate through the blood to peripheral organs, where they differentiate to cDCs (2,3). The development and differentiation of DC subsets in the bone marrow is dependent on the cytokine FMS-like tyrosine kinase 3 (Flt3L) although the expression of important transcription factors, such as Basic leucine zipper transcription factor ATF-like 3 (Baft3), Interferon Regulatory Factor 8 (IRF8) and IRF4, defines their fate (4). cDCs are the most abundant DCs in human organism and they are the main defense against pathogens in almost every tissue, while pDCs are characterized by anti-viral properties and, recently, it was described an important role of them in auto-immune responses (5). Under inflammatory conditions, circulating monocytes can also reach to organs and differentiate into another subtype of DCs, named monocyte-derived DCs (moDCs). These DCs are also found in draining lymph nodes (2). *In vitro,* monocytes can differentiate into moDCs upon treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) (6). All these subpopulations of DCs differ in gene expression, morphology and surface factors.

DCs are of paramount importance in the immune system activation, since they provide a crucial link between innate and adaptive immune responses. DCs are professional Antigen-Presenting Cells (APCs) providing antigens to T cells, but, in parallel, they shape immune responses via secondary signals (7). A key feature of DC function is their ability to migrate from infected tissues to lymph nodes where they can stimulate antigen-specific naïve T cells and determine the intensity of the immune response (4,7). Transcriptional plasticity of DCs supports the need for differential responses upon the encounter of numerous antigens. The variety of different instructions derived from DC responses, points out the cardinal role of DCs in regulation of the immune system. The interaction between DCs and cluster of differentiation 4-positive T cells ($CD4^+$ T) cells is dependent not only on antigen presentation by type II major histocompatibility complex (MHC-II) to T cell receptor (TCR), often termed signal 1, but on additional signals as well (*Figure 1*). A co-stimulatory signal is also transmitted through CD28 upon binding CD80/86 on DC

surface (signal 2) and the production of cytokines by DCs is important for shaping T cell activation (signal 3) (8,9).

At steady state, DCs guard tissues and continually sample local antigens for presentation to T cells. Different types of antigens follow different intracellular pathways of processing and antigen-presentation. Specifically, intracellular antigens, including parasites, viruses or even tumor proteins, are presented after cytoplasmic proteolysis and they are loaded onto MHC-I molecules in the endoplasmic reticulum. After that, they are transported to the plasma membrane and the MHC-I/peptide complexes are presented to $CD8^+$ T cells to develop cytotoxic immune responses (2). MHC-I molecules are expressed by almost every living cell. APCs also present extracellular antigens, following internalization and processing, upon loading onto MHC-II molecules. The MHC-II/peptide complex on membrane activates $CD4^+$ T helper cells, which, subsequently, trigger other cell types such as macrophages and B lymphocytes. Also, when APCs encounter cellular compartments from surrounding cells, which were infected by pathogens, they are able to perform "cross-presentation", a unique process through which they present extracellular proteins on MHC-I in order to drive $CD8^+$ T cell-mediated cytotoxic responses (10). The MHC/peptide complex is recognized by T cells upon interaction with T cell receptor (TCR) which is responsible for signal 1 transportation (8).

The procedure of DC activation from antigen encounter and environmental cues is termed "maturation". The maturation program triggers alterations in DC morphology associated with increase of cellular projections and enhancement of migratory capacity. Additionally, maturation amplifies the DC internalization ability in order to stimulate the proper T cell response. DCs, depending subpopulation type and their maturation state, uptake antigens via phagocytosis, endocytosis or micropinocytosis. Right after the recognition of pathogens, DCs upregulate the MHC molecules which are implicated in antigen-presentation together with essential costimulatory molecules, such as CD80 and CD86 (11). The presentation of extracellular antigens by DCs to $CD4^+$ T cells is mediated by MHC-II molecules, which are encoded by three isotypes, Human Leukocyte Antigen – DP isotype (HLA-DP), HLA-DQ and HLA-DR. HLA-DR intracellular assembly and trafficking is the most well studied among MHC-II molecules. Intriguingly, MHC-II are also expressed upon other immunological stimuli, such as interferon γ (IFNγ) and tumor growth factor β (TGF-β) (10).

The recognition of the MHC class II/peptide complexes alone on the APCs surface cannot induce the variable $CD4^+$ T cell responses. To complete T cell activation, a second costimulatory signal is delivered by DCs. In parallel with DC morphological changes, activation via toll-like receptors (TLRs) signaling leads to enhancement of antigen-presentation capacity and elevation of costimulatory protein levels in cell surface, including CD80, CD86 and CD40 (11). The majority of co-signaling molecules, i.e. both co-stimulatory and co-inhibitory, are members of the immunoglobulin (Ig) superfamily and tumor necrosis factor receptor (TNFR) superfamily. CD28 is a co-stimulatory receptor constitutively expressed on T cell

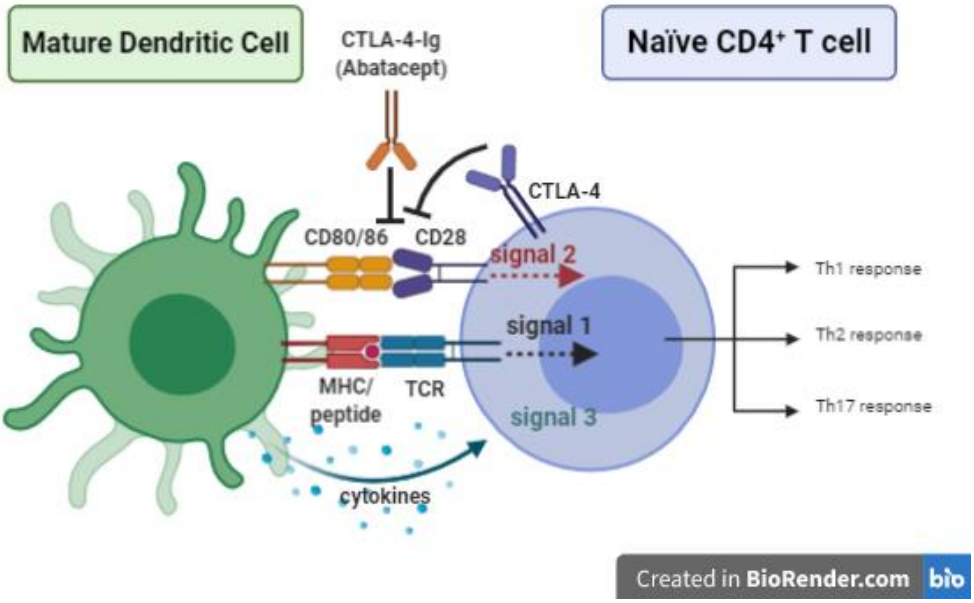

Figure 1: DCs, upon antigen encounter and maturation, provide three important signals to naïve $CD4^+$ T cells in order to drive their differentiation and shape immune responses. Signal 1: Antigen presentation of the foreign peptide on MHC-II to TCR. Signal 2: Secondary co-activation signal after interaction of CD80/86 molecules with CD28 on T cell surface. CTLA-4 and Abatacept bind on CD80/86 to inhibit co-activation. Signal 3: Soluble factors (cytokines) are secreted from DCs and recognized by T cells. Created with BioRender.com.

surface which provides T cell growth and survival signals upon binding on CD80 and CD86 (also known as B7-1 and B7-2, respectively). The expression levels of CD80 and CD86 on DCs, and, generally, on APCs, are low on inactive cells and, upon stimulation, such as infection, stress or cytokine cues, their transcription, translation and cell surface transportation are upregulated (8). The CD28-mediated activation of T cells is negative regulated by the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) molecule which is also on T cell membrane. CTLA-4 acts primarily via removal of CD80/86 from APCs surface (12). The mechanism of this inhibition is described in a later section.

Another important surface marker associated with DC function is CD40. CD40 is a co-stimulatory receptor, part of the TNFR family and has a pivotal role in the initiation of intracellular pathways of DCs (13). Expression of CD40 is connected with cell survival while downstream signaling can promote activation, proliferation and cytokine production (14). Specifically, binding of CD40-ligand (CD40L), also called CD154, enhances MHC-II and CD80/CD86 production. Together, T cells via CD40L expression can produce CD40 signaling in DCs and the expression of IL-12 by the latter, a cytokine that can skew the polarization of $CD4^+$ cells towards a Th1 phenotype. Opposite to CD40, DCs also express co-inhibitory markers (signal 2) to restrict inflammatory responses. The function of PD-L1 and other co-inhibitory markers is described later.

A marker of activation, although it is emerging as a promising regulator, which is used by immunologists on APCs membrane, is CD83. CD83 belongs to the

immunoglobulin (Ig) superfamily and is expressed in membrane bound (mCD83) or soluble form (sCD83). Mature DCs highly express mCD83 which is induced upon TLR or TNF engagement. Sites for the binding of the critical transcriptional factors nuclear factor κ-light-chain-enhancer of activated B (NF-κB) and specificity protein 1 (SP-1) have been found in the promoter region of the human CD83 gene (15). In APCs and, as a result, in DCs, the upregulation of mCD83 can drive the upregulation of surface MHC class II (MHC-II) and CD86 (16). This is attributed to its transmembrane region which inhibits an enzyme that mediates the internalization and degradation of MHC-II (16). Different from the expression, the signaling via mCD83 can possibly lead to suppressed or regulatory functions by DCs. *In vivo*, antibody or homotypic binding by DC cell lines, which express CD83, reduced maturation and secretion of proinflammatory cytokines, a MAPK signaling-associated feature (17). sCD83 has been reported to be elevated in patients with autoimmunity, including RA sera and synovial fluid, and, also, to have suppressive functions in hematological malignancies (15).

Signals from pathogens, allergens, or harmless antigens, influence the expression of DC factors which determine $CD4^+$ T cell activation towards the appropriate effector T-helper cell (Th). Briefly, signaling through pattern-recognition receptors (PRRs), such as TLRs, together with IFN-γ production by cells infected from viruses or other pathogens, and even self-antigens, can induce IL-12 and IL-27 upregulation by DCs. The production of these cytokines by DCs facilitates the polarization of $CD4^+$ T cell towards a Th1 phenotype. In other situations, PRR signaling and IFN-I production, often triggered from allergens, inhibit IL-12 expression by DCs and lead to Th2 polarization. In different cases, including self-antigens, on which PRR engagement drives IL-6 and TGF-β production by DCs, Th17 differentiation is promoted. Finally, under steady environment, DCs are conditioned into a tolerogenic phenotype which is connected to TGF-β and retinoic acid production that support T-regulatory (Tregs) generation (7). Despite the cytokine expression profile, the changes of DC transcriptional profile upon activation involve also changes of their metabolic profile. Specifically, the TLR-mediated activation of DCs promotes the increase of aerobic glycolysis by these cells (18). Commitment to glycolysis is essential for DC effector functions, including antigen presentation and T cell stimulatory capacity and loss of glycolytic capability impairs these activities (19).

DCs are essential for self-tolerance maintenance

DCs are remarkably immunogenic under inflammatory conditions, but, to properly moderate immune responses, they also promote a diametrically opposed effect, the induction and maintenance of self-tolerance and homeostasis. Tolerogenic mechanisms are of paramount importance in order to avoid overactivation of immune system upon encounter with harmless antigens, such as those derived from commensals which are present in many organs. Balance between immunogenicity (to non-self) and tolerance (to self) is necessary to achieve immune homeostasis. This is exemplified by investigating anatomical sites with constant exposure to foreign antigens. Skin, which

is the largest barrier against pathogens, together with lungs and intestine are continually in contact with foreign antigens. At the same time, these tissues are habituated by a plethora of commensal bacteria. Therefore, DCs are burdened with the role of immuno-regulators, promoting T cell activation upon foreign microorganisms and peptides while they inhibit T cells upon self-peptides and beneficial microbes (20).

DCs are also implicated in the induction of central tolerance in thymus where, together with other cells, they drive the production of thymic Tregs via positive selection to prevent anti-self responses. Tregs are a $CD4^+$ T cell subset which expresses the master transcriptional repressor forkhead box P3 (FOXP3) and they are able to control self-reactive peripheral T cells. In parallel, DCs interact with T cells extrathymically and ameliorate or deplete immune responses in the periphery (20,21). DCs which have acquired a tolerogenic phenotype and are able to establish immune tolerance, are called tolerogenic DCs (tol-DCs). This type of DCs is generated *in vivo* under steady state conditions, including absence of pathogens and basal or anti-inflammatory signals from surrounding cells, such as engulfment of apoptotic – and not necrotic – cells. Thus, although these tol-DCs do not encounter inflammatory signals to drive their differentiation, they do not remain immature and they acquire an anti-inflammatory phenotype (22).

Tol-DCs with variable characteristics can be generated, both *in vivo* and *in vitro*, through multiple pathways. Regardless of the different pharmaceutical options for *in vitro* tol-DCs generation, these cells share some common pathways of action. Tol-DCs are usually exceptional in antigen uptake and processing but they migrate via the circulatory and lymphatic system only to antigen-present (signal 1) in the absence of co-stimulatory molecules (signal 2). One of the hallmarks of tol-DCs is the reduction of co-stimulatory markers expression. At the same time, these cells secrete low levels of IL-12 while they produce high levels of anti-inflammatory IL-10 and TGF-β cytokines (22). The combination of all these signals derived by tol-DCs can halt T cell activation and determine their fate by inducing deletion, i.e. antigen-specific apoptosis, or anergy, i.e. clonal inactivation, or regulatory polarization (11,22) (*Figure 2*). Deletional signals are useful for the removal of potentially autoreactive naïve T cells. Central tolerance is mainly achieved via a deletional process against highly reactive T cells. The most common signals that drive T cell apoptosis are the interaction of DC's Fas ligand (FasL) with T cell's Fas (22), the interference with T cell tryptophan catabolism through indoleamine 2,3-dioxygenase (IDO) expression by DCs (23) and TRAIL interactions with TRAIL receptors (22). *In vitro*, T cell anergy can be induced by the CTLA-4 binding of T cells on CD80/86 of DCs (24), a pathway which is described in details in a later section.

The mechanism of CTLA-4-CD80/86 pathway is also implicated in the induction of Treg polarization. The induction of polarization towards Tregs is primarily driven by TGF-β and retinoic acid secretion by tol-DCs, in combination with low co-stimulatory signals. Subsequently, Tregs drive inactivation of immune responses upon presence of harmless antigens via interaction with self-reactive $CD4^+$ T cels (21). The

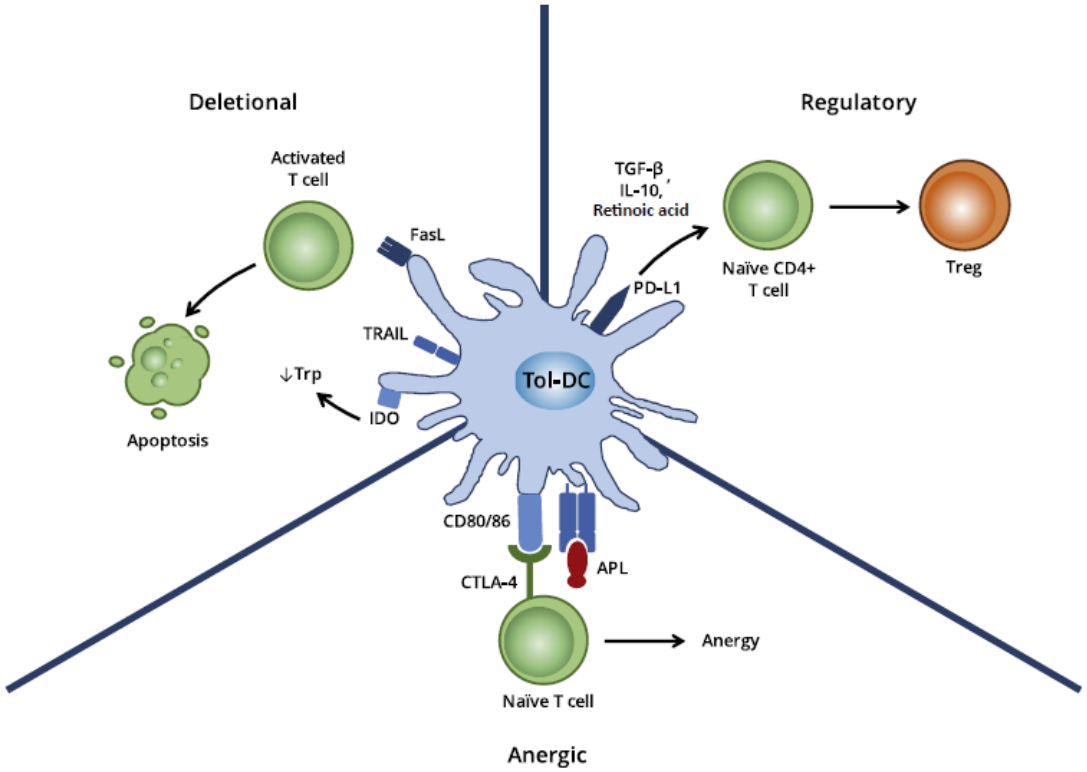

Figure 2: Tol-DCs inhibit T cell activation via different mechanisms. Binding of CD80/86 molecules with CTLA-4 on the surface of T cells drives to inactivation of T cells (anergic). Expression of PD-L1 by tol-DCs in combination with TGF-β, IL-10 and retinoic acid secretion trigger the polarization of T cells towards Treg phenotype (regulatory). Finally, interactions of FasL and TRAIL on tol-DCs with T cells promote apoptotic pathways (deletional). Limiting tryptophan (Trp) availability upon IDO secretion is implicated with deletional properties too. *Figure from: Horton C. et al., Biomed J (2017)*

anti-inflammatory cytokine IL-10 also plays a pivotal role in Treg induction. $CD4^+$ T cells conditioned with tol-DCs, which are generated under IL-10 effect, require the presence of secreted IL-10 by tol-DCs to acquire a regulatory phenotype (25). Another immunomodulatory pathway which promotes *Foxp3* expression in naïve T cells is the PD-L1/PD-1 axis. Programmed death ligand-1 (PD-L1) is a protein expressed on DCs surface which binds programmed death-1 (PD-1) on T cell membrane, which negatively regulates TCR signaling. Moreover, PD-L1 antagonizes CD28 for binding on CD80, an interaction which increases the mechanism complexity due to inhibition of both PD-1 and CTLA-4 axes (26). The activation of T cells can also induce the expression of inducible T cell co-stimulator (ICOS). DCs constitutively express its ligand, B7h, which has also been designated as ICOS-L, and which has proved to implicate in Tregs generation (20).

Strategies for DCs manipulation are currently under investigation in order to exploit their immunoregulatory potential for the development of tolerance-inducing drugs. Examples of agents used for *in vitro* generation of tol-DCs (usually derived from isolated monocytes) are dexamethasone (Dex), vitamin D3 (VitD3), rapamycin,

minocycline, GM-CSF, IL-10 etc (25,27-29). Thus far, the generation of tol-DCs is available via *ex vivo* procedures and tol-DCs are loaded into the organism upon generation in the lab. Nevertheless, similar agents are also prescribed to patients for achieving immunosuppression in many inflammatory disorders but, unfortunately, they come along with numerous side effects. Allergies and autoimmune diseases can be initiated upon breakdown of tolerance regulation systems and presentation of modified self-proteins, or even inappropriate activation signals, by DCs to T cells (5). For instance, in the context of RA, it has been proved that DCs recognize autoantigens and promote the activation of self-reactive T cell populations, which secrete pro-inflammatory cytokines, leading to activation of other immune cells (30). Based on data from animal models, several clinical trials are on-going for the treatment of autoimmune diseases, such as rheumatoid arthritis (RA), type 1 diabetes, Crohn's disease and multiple sclerosis (31), with the usage of tol-DCs.

Metabolic changes promote tolerogenic phenotype

Metabolism is determined by a plethora of biochemical reactions that primarily lead to energy production and regulate essential cellular procedures. For the maintenance of cell homeostasis, energy is produced in the form of ATP by living organisms. Non-proliferating cells metabolize glycose via aerobic glycolysis (with oxygen presence) towards pyruvate. Oxidative phosphorylation (OXPHOS) is the biochemical procedure through which pyruvate is oxidized for the production of the bulk amount of ATP in most cells. However, when rapid ATP production is needed, e.g. under stress conditions, where oxygen is limiting, cells breakdown glucose to ATP via anaerobic glycolysis and lactate production. A metabolic shift towards anaerobic glycolysis (and away from OXPHOS) has been described by Warburg effect on tumor cells (32). In the context of immune system, nutrients and metabolites demands are dependent on the function of immune cells.

Growing evidence indicate that metabolic reprogramming can regulate immune cell differentiation. Metabolism seems to act as a switch for the determination between immunogenic or tolerogenic cell fates in human peripheral blood leukocytes. Naïve T cells are quiescent and, due to low energy demands, they are dependent on OXPHOS for ATP production. Signaling via TCR receptor drives activation and elevation in glycolytic metabolism which is preserved in Th1, Th2 or Th17 effector cells that acquire strong glycolytic metabolism as well. Contrastingly, Tregs and memory T cells functions are supported by OXPHOS and fatty acid oxidation (FAO) (33). Another paradigm for the role of metabolism in immune system comes from APCs and, specifically, macrophages. Similar to the Warburg effect, the M1 polarization of macrophages is connected with anaerobic glycolysis and lactate release. On the contrary, M2 macrophages employ aerobic glucose metabolism and OXPHOS pathway (34).

DCs acquire changes on their metabolic profile during their development from precursors to mature immunogenic cells. Firstly, DCs progenitors are characterized by

glycolysis which shifts towards OXPHOS during DC differentiation. This comes together with an increase of reactive oxygen species (ROS) production, expression of respiratory mitochondrial enzymes, and antioxidant capacity. A second metabolic shift happens at the level of DC maturation. More specifically, upon TLR stimuli, the AKT pathway is promoted and it activates hexokinase 2, an important enzyme for glycolysis initiation and, as a result, for citric acid cycle. Moreover, the NF-κB signaling pathway is activated leading to stimulation of fatty acid synthesis (35). The overexpression of inducible nitric oxide synthase (iNOS) and downregulation of 5' adenosine monophosphate-activated protein kinase (AMPK) drive OXPHOS and FAO inhibition, respectively (32).

Contrastingly, tol-DCs show a distinct profile with increased expression of OXPHOS-related genes. At the same time, tol-DCs are characterized by high mitochondrial activity and ROS production and this increased mitochondrial function elevates FAO and triglycerides consumption (36). In other words, tol-DCs have a catabolic and highly energetic profile which might be associated with increased energy requirements for active suppression. Based on this data, in combination with the essential role of DCs in shaping immune responses, the manipulation of DC metabolism is a significant target for therapeutic purposes. This is exemplified by the usage of rapamycin for induction of catabolic metabolism and tol-DCs generation. Rapamycin inhibits the mammalian target of rapamycin (mTOR) which is a significant glycolysis regulator and coordinates DCs metabolic changes upon immunological signals (32). Activation of mTOR, and of upstream PI3K/Akt axis, happens upon variable immunological stimuli and usually leads to a long-term commitment of DCs to glycolytic metabolism in a NO-dependent manner. In parallel, mTOR inhibits DC activation (37), implicates in lipid metabolism and expression of costimulatory molecules and cytokines (18). Moreover, TLR signaling downregulates initiation of autophagy, which is an inextricable part of antigen presentation, via mTOR activation (18). Finally, an mTOR-dependent human tol-DC phenotype has been described upon VitD3 treatment, pointing out the significance of mTOR and metabolism in the regulation of DC immune functions (38).

Dexamethasone induces tol-DCs

The discovery of glucocorticoids (GCs) and their effects by E. C. Kendall, T. Reichstein and P. S. Hench was awarded with the Nobel Prize for Physiology or Medicine in 1950 (39). Thus far, therapeutic GCs are widely used for the treatment of rheumatic diseases, such as RA, and other inflammatory disorders, although their mechanisms of action are not fully understood. In humans, cortisol is the most essential to life GC, which is synthesized from cholesterol. All the synthetic GCs which are used in the clinic, including Dex, are similar in structure to cortisol with modest modifications. Endogenous GCs are known to bind two cytoplasm receptors, the GC receptor (encoded by *NR3C1*) and the mineralocorticoid receptor (encoded by *NR3C2*). From the *NR3C1* gene locus two main variants are transcripted, GRα and GRβ. The

latter does not bind GCs directly but it functions as a negative regulator of GRα. Dex does not bind the mineralocorticoid receptor (40).

The binding of GC on GRa leads to transportation into the nucleus where it binds DNA on DNA GC response elements (DNA GREs). GC receptor signaling is complicated and still poorly understood. In general, GC signaling exert its actions primarily via trans-activation or trans-repression mechanisms, although other pathways exist. Usually, trans-activation is mediated by receptor homodimers (GRα-GRα) which bind to GREs and culminate in increase of gene transcription. Monomers of GC receptor promote trans-repression, i.e. they limit transcription, either indirectly via the interaction with factors that prevent binding to DNA or directly via binding to "negative" GREs that subsequently downregulate transcription (*Figure 3*). Essential anti-inflammatory targets of GCs in numerous cell types are NF-κB (*Figure 3*), activator protein 1 (AP-1), annexins, specific phosphatases, glucocorticoid-induced leucine zipper and microRNAs, but they act in a combinatory way and none of these factors seems to have a dominant role in regulation of inflammation and (40).

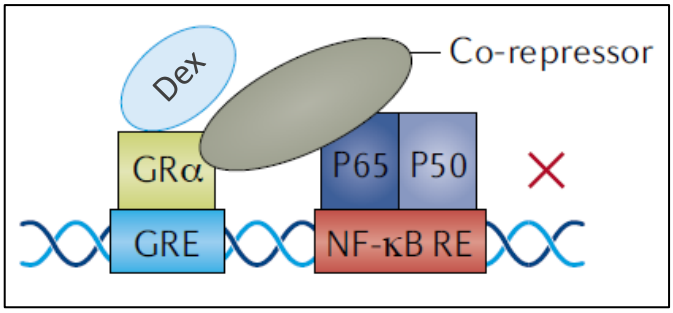

Figure 3: Dexamethasone (Dex) exerts its anti-inflammatory via trans-repression of *NF-κB* genes expression. Dex binds a GR monomer which subsequently binds to DNA on a GRE. A co-repressor is attracted to the area and the expression of *NF-κB* genes is inhibited.
Figure from: Hardy et al., Nat Rev Rheum (2020)

The GCs, including Dex, are used for the induction of anti-inflammatory and immunosuppressive effects in clinical application. In part, these actions are mediated by the generation of tol-DCs. The effect of Dex, and generally of therapeutic GCs, on DCs is associated with decreased antigen presentation, cytokine production and maturation, in parallel with increased apoptosis (41). DCs generated in the presence of Dex (Dex-DCs) acquire a normal dendritic morphology but they have differences concerning their membrane phenotype, designated as semi-mature phenotype (42-44). For instance, when Dex-DCs are generated from monocytes, they express lower CD1a levels and higher CD14 and CD16 levels compared to untreated mo-DCs (44). Overall, Dex-DCs are characterized by low expression levels of MHC-II molecules and co-stimulatory proteins, high IL-10 production and low IL-12 secretion (42,44-46). Important secondary co-stimulatory molecules which are found downregulated on these cells are CD86, CD40 and PD-L1 (43,44,46,47). IL-10 production by mature Dex-DCs is dependent on extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases (MAPK) phosphorylation. IL-10 antibody neutralization increases partially the Dex-DCs maturation while they keep low CD83 and CD86 levels (46). The secretion of pro-inflammatory IL-6 is also limited (47,48). As regards to metabolism, treatment of DCs with Dex seems to inhibit iNOS expression and NO production and, as a result,

OXPHOS is promoted (49). Also, Dex-DCs have limited capacity to stimulate $CD4^+$ T cell proliferation in MLR while they favor Treg induction (45). Tolerogenic effects of Dex on DCs *in vitro* can last at least 5 days upon Dex removal from culture and Dex is categorized as a long acting GC (45,46).

CTLA-4-Ig is a candidate inductor of DC tolerogenicity

CD80 and CD86 on APCs are essential for positive co-stimulatory signal (signal 2) that drives full T cell activation after binding to the CD28 receptor on T cell surface (8). Approximately 2 days after activation, T cells upregulate CTLA-4 levels (also referred as CD152) on their surface, provoking negative regulation of T cell-mediated responses. This negative costimulatory molecule expressed on the T cell membrane binds CD80, with relatively high affinity, and CD86, with lower affinity, on the surface of APCs, including macrophages and DCs (50). CTLA-4 interacts with both molecules with higher affinity than CD28 and, therefore, antagonizes CD28-induced costimulation and leads to T cell inhibition (24,51). It has been demonstrated that CTLA-4 on the surface of T cells acts in a T cell-extrinsic manner by removing CD80/86 from the APCs surface, including DCs (12). The co-stimulatory molecules are transferred inside the $CTLA\text{-}4^+$ cells through trans-endocytosis, resulting in downregulation of costimulation via CD28.

Almost 90% of CTLA-4 is found intracellularly of Tregs or conventional T cells due to the continuous endocytosis of the molecule (52). The CTLA-4 endocytosis is probably a regulatory mechanism of its cell-extrinsic functions. The essential role of CTLA-4 is pointed out on CTLA-4 knockout mice or in mice with Treg-specific depletion of CTLA-4 which both develop spontaneous fatal lympho-proliferative disease while Tregs' suppressive function is impaired (53,54). In parallel, in the context of rheumatoid arthritis, CTLA-4 signaling on Tregs is significantly reduced compared to healthy individuals (55). Moreover, several studies support that CTLA-4 signaling regulates both T cells and DCs by inhibiting the activation of the former and by reducing the inflammatory function and enhancing the tolerogenic phenotype of the latter (50).

In autoimmune diseases, the remarkable clinical benefit upon CTLA-4–Ig (Abatacept marketed as Orencia; Bristol-Myers Squibb) treatment in patients with RA, juvenile idiopathic arthritis and kidney transplantation confirms the significance of CTLA-4 in the immune system regulation. Abatacept is composed by the extracellular domain of human CTLA-4 fused with the Fc region of human immunoglobulin G1 (IgG1) and blocks CD28-CD80/86 pathway, acting as a negative regulator of T cell activation (*Figure 1*) (56). The Fc portion has also been modified to minimize antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. CTLA-4–Ig was the first biological agent that aimed to modulate T cell activation in chronic inflammatory disorders. Thus, $CD4^+$ T cells receive only signal 1 while signal 2 is blocked by Abatacept driving T cells to polarize towards anergic or unresponsive phenotype limiting autoreactive responses.

As it was aforementioned, a T cell-extrinsic role of CTLA-4 was revealed via the trans-endocytosis mechanism. Other T cell-extrinsic roles of CTLA-4, which are referred as "reverse signaling", seem to initiate different intracellular pathways on APCs through CD80 and CD86 binding (50,57-59). As a result, CTLA-4–Ig probably induce reverse effects on APCs, although its main usage in clinic is associated with T cell activation blockade. In 2002, it was indicated that, in mouse-derived DCs, CTLA-4–Ig interacted with CD80/86 and promoted IDO induction (59), a result that was reported again by another publication (60). IDO is a tryptophan-catabolizing enzyme that metabolizes tryptophan to kynurenine with consequent tryptophan depletion from T cell's microenvironment leading to inhibition of T cell proliferation (23). IDO production in the 2002 study was reported to be depended on NF-κB and p38 mitogen-activated protein kinase (p38 MAPK) activation and IFNγ upregulation (59). However, a more recent publication demonstrated that CTLA-4–Ig was not able to induce IDO activity on murine bone marrow-derived DCs and splenic DCs (61). In humans, the significance of IDO on CTLA-4-Ig-mediated immunosuppression in murine models it remains debatable as well. Studies have reported an induction of IDO activity upon ligation of CD80/86 by CTLA-4-Ig (62) but there are others that claim no changes in IDO levels upon CTLA-4–Ig treatment (63).

The function of DCs is also connected with cellular metabolic processes, as it was described above. Recently, it was pointed out that $Foxp3^+$ Tregs exert their regulatory function in mouse bone marrow-derived DCs by affecting autophagy in a CTLA-4-dependent way (57). The amelioration of autophagy was achieved via the upregulation of the PI3K/Akt/mTOR axis. The activation of this pathway promoted the nuclear exclusion of Forkhead Box O1 (FOXO1) transcription factor leading to downregulation of *Lc3b* transcription and a subsequent downregulation of autophagy. Formation of autophagosomes is an essential function of DCs in order to efficiently present antigens on MHC-II molecules. This phenomenon seems also to exist in moDCs from healthy donors after Abatacept therapy (57).

With this study, the effect of CTLA-4 reverse signaling on DCs is further investigated. For this purpose, human moDCs were isolated and cultured upon the effect of CTLA-4–Ig and were compared to Dex-derived DCs for tolerogenic properties. The changes which were found are associated with all the important signaling pathways of APCs, i.e. antigen-presentation, co-stimulation and cytokine secretion. In parallel, metabolic alterations were discovered on the Akt/mTOR signaling and on major metabolic pathways.

Aim of the study

DCs are considered as professional APCs and they are the connecting step between innate and adaptive immunity. These cells are capable of changing their transcriptional and metabolic profile upon the effect of different agents such as pathogens, self-antigens or cytokines. This plasticity is required for interactions with T cells and elicits from them the suitable response for each antigen. Under steady state conditions, or under the effect of harmless antigens, the establishment of immune unresponsiveness is also one of DCs roles, a condition described as immune tolerance. The failure of self-tolerance mechanisms can lead to aberrant activation of lymphocytes, culminating in the development of autoimmune disorders. Deregulated signals by DCs are able to drive T or B cell-mediated auto-reactive responses. Therefore, DCs can be really important targets for immunotherapy.

Tolerogenic DCs (tol-DCs) are *in vivo* or *in vitro* generated DCs upon a variety of different treatments which are able to inhibit T cell responses via different mechanism. A very common drug that can be used for tol-DCs induction both *in* vivo and *in vitro* is the synthetic corticosteroid dexamethasone. This drug is known for its anti-inflammatory properties and it is also used for the treatment of autoimmune inflammation in diseases like rheumatoid arthritis (RA). CTLA-4 is a co-stimulatory molecule expressed in the surface of T cells which acts by binding and removing CD80/86 of the DCs surface. CTLA-4–Ig, another therapeutic approach in RA and a soluble form of CTLA-4 seems to deliver reverse signal through CD80/86 on DCs. Furthermore, it has been demonstrated by different studies that CTLA-4–Ig treatment on DCs upregulates IDO induction and inhibits autophagy via an Akt/mTOR-dependent pathway, two responses that are implicated in the downregulation of DC immunogenic potential.

Hence, the aim of this study is to characterize the molecular mechanisms that drive the CTLA-4–mediated tolerogenic effects on DCs. This aim is better described by the following objectives:

- Investigation of the effect of CTLA-4–Ig on major features of antigen presenting DCs.
- Investigation of the effect of CTLA-4–Ig on the expression of pro- and anti-inflammatory genes in DCs.
- Characterization of the DCs metabolic changes after CTLA-4–Ig treatment (Akt/mTOR pathway).
- Comparison of the effect of CTLA-4–Ig on DCs with an established tol-DCs model induced upon dexamethasone treatment

Methods

Monocyte isolation from peripheral blood

Heparinized blood (18-24ml) was collected from healthy donors from General University Hospital of Heraklion. Peripheral blood mononuclear cells (PBMCs) were isolated after density centrifugation with ficoll for 30minutes at 1800rpm. Then, PBMCs' layer was collected and cells were washed with PBS in order to remove plasma or ficoll remnants. Erythrocytes were eliminated by hypotonic lysis (500μl ddH_2O for 35 seconds and 500μl 1.8% NaCl). Monocyte population was magnetically isolated, using the CD14 cell surface marker and purity was evaluated by flow cytometry ($CD14^+$ cells/total live cells).

Monocyte-derived dendritic cell differentiation

The isolated monocytes were cultured in 6-well plates (~$2x10^6$ cells/well) in complete RPMI 1640 medium, containing 10% FBS, 1X penicillin/streptomycin, 10mM HEPES, 50μM 2-mercaptoethanol, in the presence of GM-CSF and IL-4 (both 100ng/ml) to induce dendritic cell differentiation (moDCs) (6). At day 3, 500μl of the medium were removed and refreshed by 1ml of complete medium with GM-CSF and IL-4 (50ng/ml). Dexamethasone (Dex) (10^{-6}M, i.e. ~472ng/ml) was also added, at day 3, in some wells as a positive control of tolerogenicity since it can transform DCs towards a tolerogenic phenotype (tol-DCs) (64). At day 6, moDCs were activated with LPS (mature DCs) or cultured alone (immature DCs) and treated with CTLA-4–Ig or IgG1 control for 16 hours. Dexamethasone was added again to moDCs which were already pre-exposed to dexamethasone. Cells were plated in 96-well plates ($3x10^5$ cells/well) and kept at 37°C and 5% CO_2. The process of moDCs and tol-DCs generation is described in *Figure 4*. Culture supernatants and cells were collected and stored at -80°C.

RNA isolation, cDNA synthesis and Real-Time PCR

Cell pellets from moDCs, mature, immature or tol-DCs, and with or without the effect of CTLA-4, underwent RNA extraction. Total RNA was isolated either with the NucleoZOL (supplied by MACHEREY-NAGEL) or with the TRIzol (supplied by Invitrogen) protocol. Both protocol are using guanidium thiocyanate and a phenol-based reagent. For the NucleoZOL protocol, cells were firstly lysed with the NucleoZOL reagent. RNase-free water was added for DNA, proteins and polysaccharides precipitation and, after 15-minute incubation and centrifugation (12000g, 15min, RT), RNA remained solubilized in the supernatant. For the TRIzol protocol, the TRIzol reagent was added to cells and a 5-minute incubation allowed the dissociation of the nucleoproteins complex. Chloroform was added for DNA, proteins and polysaccharides precipitation and, after 3-minute incubation and centrifugation

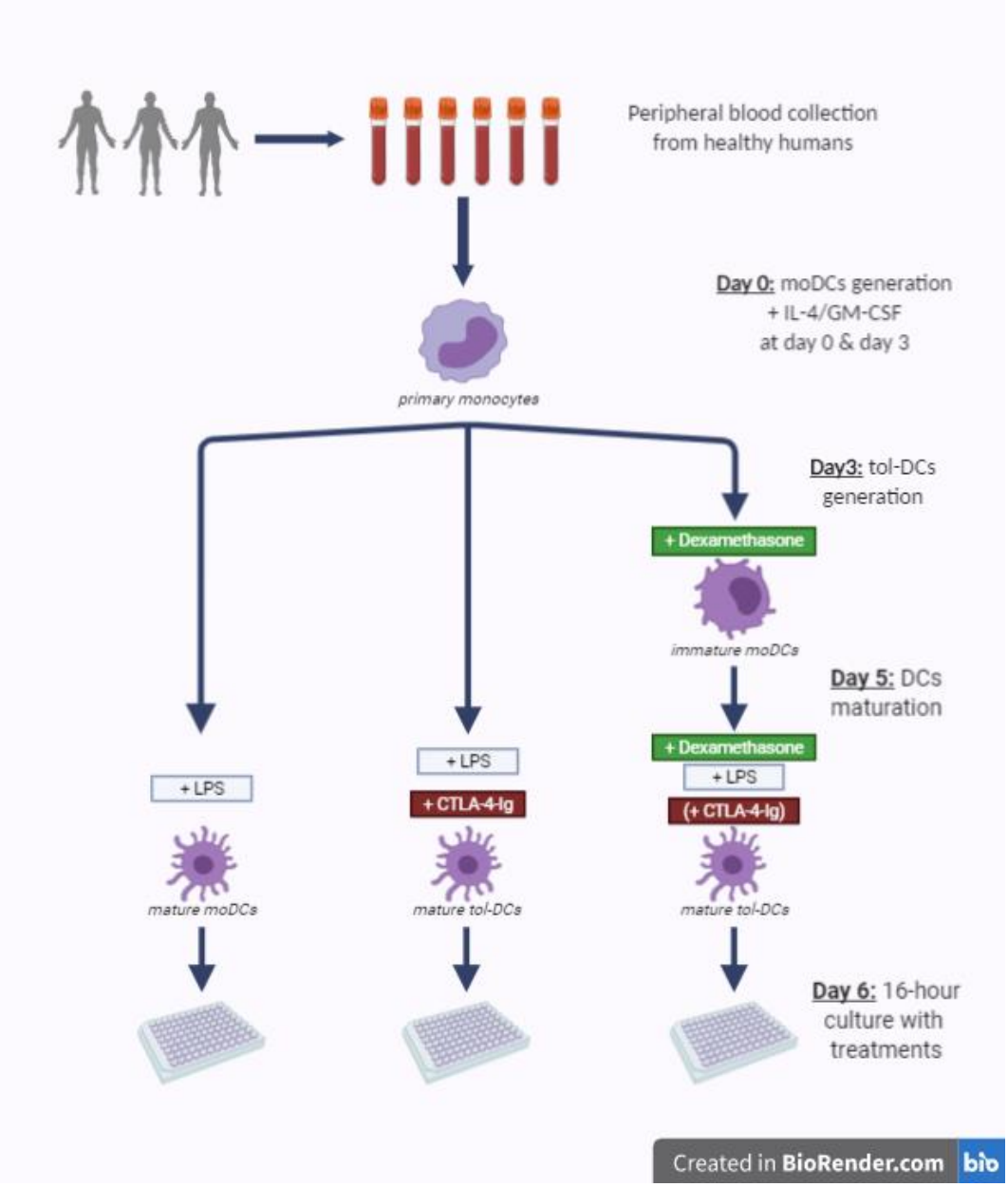

Figure 4: Step by step the processes which were used in this study for the generation of moDCs and tol-DCs with different properties. Created with BioRender.com.

(12000g, 15min, 4ºC), RNA remained solubilized in the supernatant. For both protocols RNA supernatants were transferred in fresh tubes, isopropanol was added and a 10-minute incubation followed. To wash the RNA, two centrifugations with 75% ethanol were performed (5min, 8000g) and the RNA pellet left to dry for 5-10 minutes.

RNase-free water was used for the reconstitution of RNA. The RNA concentrations were counted with the NanoDrop 2000 spectrophotometer after an incubation with DNase. Complementary DNA (cDNA) library from the isolated RNA samples was synthesized upon reverse transcription with the PrimeScript RT Reagent Kit (Perfect Real-Time). To degrade RNAs from RNA/DNA duplexes and, subsequently, allow an efficient dsDNA formation, an RNaseH treatment at 37ºC for

20 minutes followed. For the quantification of transcripts, KAPA SYBR FAST qPCR Master Mix Kit was used with specific primers in order to study the amplification of pro- and anti- inflammatory genes, such as IL-6, IL-10, IL-12b and IDO. The relative fold gene expression was calculated with the delta-delta-C_T ($\Delta\Delta C_T$) method with Gapdh mRNA levels.

Flow Cytometry

Single-cell suspensions of undifferentiated monocytes, mature or immature DCs, dexamethasone treated and CTLA-4–Ig treated moDCs were prepared and cells were stained for surface or intracellular markers in order to compare the different stages of differentiation, maturation and function. For surface markers, cells were washed with phosphate-buffered saline (PBS) supplemented with 10 % fetal bovine serum (FBS), called FACS buffer, and were incubated with fluorochromes, isothiocyanate (FITC), Phycoerythrin (PE), Allophycocyanin (APC) or Peridinin-Chlorophyll-protein-Cyanine5.5 (PerCp-Cy5.5) conjugated with antibodies against proteins of interest for 20 min at 4 ^oC, in dark. FITC is excited by lasers around 490nm leading to fluorescence emission around 525nm, PE is maximally excited at 565nm and emits at 573nm, APC is excited at 594nm and 633nm and its fluorescence emission peak is at 660nm, while PerCp is maximally excited at 482nm and its fluorescence emission peak is at 690nm. After incubation, cells were washed again with FACS buffer in order to stop the reaction.

For intracellular staining, cells were incubated with 1,5% formaldehyde for 10min at room temperature, in dark, in order to fix the cells. After wash centrifugation, cells were incubated with ice-cold methanol for cell membrane permeabilization. At this step, cells could be stored at -20^oC for up to 4 weeks. The day of flow cytometry, cells were undergone washing centrifugation twice with a PBS buffer supplemented with 1% bovine serum albumin (BSA). Subsequently, samples were incubated for 30 minutes with antibodies conjugated with fluorochromes for intracellular staining in PBS/BSA 1% buffer at room temperature, in dark. A final wash with the PBS/BSA 1% buffer was performed before cells' resuspension in FACS buffer. Flow cytometry data were analyzed using FlowJo (TreeStar Inc., Ashland, OR, US).

Detection of cytokines (ELISA)

The levels of secreted proteins, such as cytokines, were measured by performing enzyme-linked immunosorbent assay (ELISA). Mature moDCs, immature moDCs, tol-DCs or CTLA-4–Ig-treated moDCs were cultured and the supernatants (sups) of these cells were collected for ELISA. At first, an ELISA 96-well plate was coated with 100μl of anti-human IL-6 Capture antibody diluted in Coating Buffer (mix 1 part of PBS and 10 parts of dH_2O), and the plate was incubated overnight at 4^oC. Capture antibody is used for the immobilization of antigen of interest. After incubation, wells were washed with Wash Buffer (1X PBS, 0,05% Tween-20) and a blocking step followed with 200μl of ELISA diluent (constructed according to manufacturer's instruction) for 1h at room temperature while shaking. Another wash was performed after blocking. To make the

standard curve, dilutions of the top standard were performed according to manufacturer's instruction. Samples were diluted in ELISA diluent and were added in duplicates. The plate was incubated for 2 hours at room temperature, while shaking, and it washed with Wash Buffer. 100μl of biotin-conjugated anti human IL-6 Detection antibody (diluted in ELISA diluent) was subsequently shared to every well and the plate was incubated for 30minutes at room temperature, while shaking. The wells were rinsed with Wash Buffer. For indirect detection of the antigen, 100μl Streptavidin-Horseradish Peroxidase (HRP) were added in each well. Streptavidin recognizes biotin in the surface of the detection antibody and the complex forms the strongest known non-covalent interaction (Kd = 10^{-15}M). HRP is able to catalyze the oxidation of chromogenic substrates, such as TMB. The plate was incubated for 30minutes at room temperature and it was washed with Wash Buffer. Finally, 100μl of tetramethylbenzidine (TMB) solution were added, followed by a 15-minute incubation in dark at room temperature. In the presence of HRP, TMB is converted into a blue liquid. The conversion of TMB into colored product was paused after the addition of 100μl 2N H_2SO_4. The absorbance of each well was measured at 450nm. The standard curve was used for the quantification of the samples' absorbance.

Results

Monocytes were differentiated to immature moDCs and tol-DCs

To investigate alterations in DCs, monocytes had to be isolated from total blood samples of healthy individuals with the use of magnetic beads for the CD14 surface marker (day 0). The percentage of $CD14^+$ cells in the isolated population needed to be examined before performing subsequent experiments in order to confirm the purity of this monocyte population. Thus, the percentage of $CD14^+$ isolated cells was 96,7% (*Figure 5a*).

Next, monocytes were cultured with GM-CSF and IL-4 in order to be differentiated into moDCs. A number of these cells was also cultured with dexamethasone in order to acquire the phenotype of tol-DCs. The proper differentiation of monocytes was examined via the expression of the following surface markers: CD14 which is specific for monocytes, CD1c and CD11c which are considered DC markers, and HLA-DR, a necessary marker for antigen-presentation, which is expected to be found on both monocytes and DCs. At day 5, moDCs had low expression of CD14 (*Figure 5b*), high expression of CD1c (*Figure 5c*) and CD11c (*Figure 5d*) while the expression of HLA-DR did not change at all (*Figure 5e*) compared to undifferentiated monocytes (day 0). The tol-DCs downregulated CD14 (*Figure 5b*) and upregulated

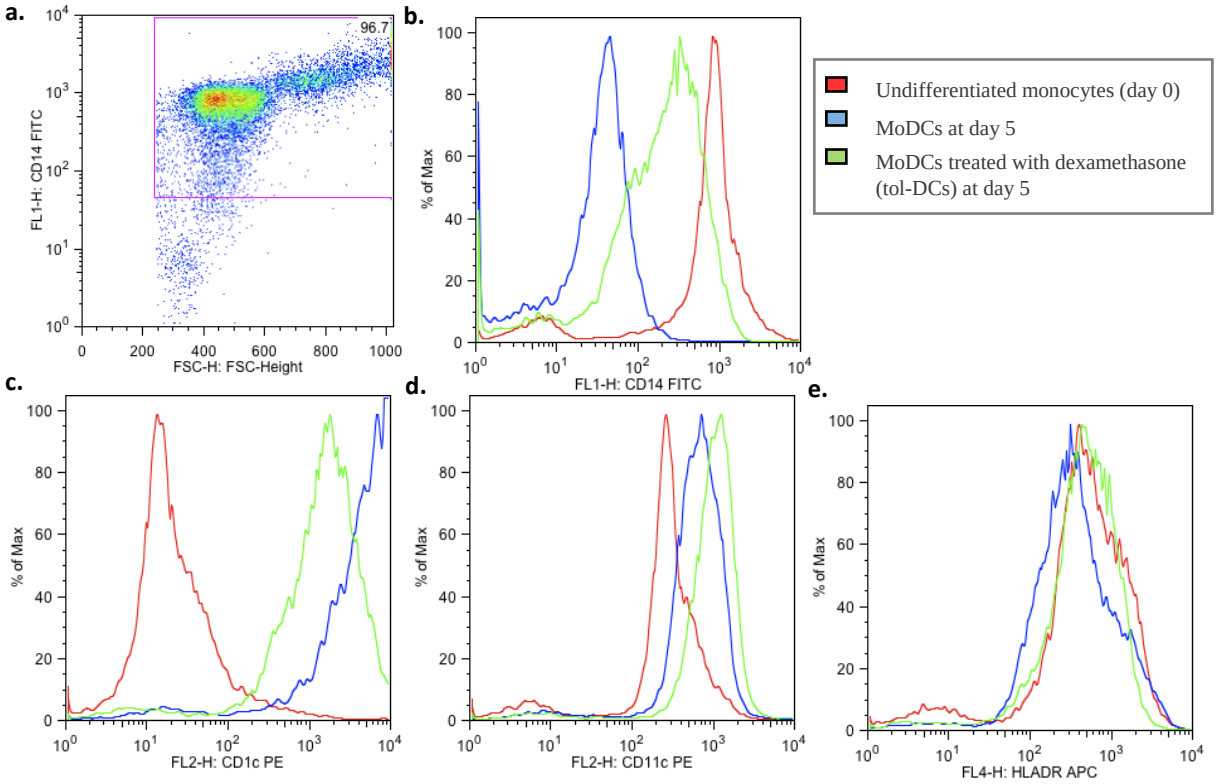

Figure 5: Expression of surface markers on undifferentiated and differentiated monocytes. **(a)** The percentage of $CD14^+$ cells in the PBMCs determines the purity of isolated monocytes. **(b, c, d, e)** Histograms of fluorescence emission peaks of monocytes (day 0) and moDCs (day 5) after extracellular staining for **(b)** CD14, **(c)** CD1c, **(d)** CD11c and **(e)** HLA-DR markers.

CD1c (*Figure 5c*). The expression of these markers on tol-DCs was intermediate compared with that on monocytes and moDCs. In contrast, the expression of CD11c was higher compared to moDCs (*Figure 5d*), while the expression of HLA-DR was stable (*Figure 5e*).

Objective 1: CTLA-4–Ig downregulated the expression of co-stimulatory markers and upregulated maturation and antigen-presentation molecules on DCs surface

DCs have the ability to shape immune responses via interaction with T cells, either by direct contact (signals 1 & 2) or cytokine secretion (signal 3). In order to study changes on DCs which could affect their function and DC-T cell contact, the expression of surface markers implicated in antigen presentation and co-stimulation/co-inhibition were examined. Antigen-presenting molecules, such as HLA-DR, are necessary to present antigen to T cells and to provoke their differentiation, but without receiving co-stimulatory signals by CD80/86 molecules or CD40 they are unable to polarize towards an effector phenotype. In parallel, the expression of co-inhibitory molecules, e.g. PD-L1, can downregulate the immunogenic function of T cells. Also, the activation state of DCs can be investigated by the expression of DC-specific molecules, including CD83, which have been shown to be expressed after DC maturation upon treatment with lipopolysaccharide (LPS) or other antigens. In the current study, the levels of surface markers were estimated by using flow cytometry with fluorochromes conjugated with antibodies against the marker under investigation.

Maturation of DCs is essential for their function and *in vivo* it usually happens after encounter with foreign proteins. To achieve maturation *in vitro*, LPS was administered to DCs. In this regard, CTLA-4–Ig effect was evaluated in moDCs upon maturation with LPS. Untreated moDCs, i.e. without treatments, and immature moDCs treated with only CTLA-4–Ig were used as controls for the significance of LPS conditioning. CTLA-4 and, subsequently, CTLA-4–Ig bind on CD80 and CD86 molecules, two well-known markers for their co-stimulatory effect. The expression of both CD80 and CD86 on mature moDCs were found inhibited upon CTLA-4–Ig treatment (*Figure 6a,b*). The inhibition of CD80 expression on LPS/CTLA-4–Ig-treated moDCs was statistically significant in comparison with LPS-treated moDCs. The tol-DCs (Dex/LPS-treated mo-DCs) had a small downregulation of CD80 marker but the expression of CD86 was totally abolished. The analysis for CD40 expression, another co-stimulatory marker, showed no effect on mature moDCs upon CTLA-4–Ig treatment, although tol-DCs overexpressed CD40 (*Figure 6c*). HLA-DR levels were also measured since it is a characteristic antigen presentation molecule. HLA-DR tended to be slightly upregulated upon CTLA-4–Ig treatment on mature moDCs and a similar increase observed on tol-DCs (*Figure 6d*). CTLA-4–Ig treatment had almost no effect on expression of co-inhibitory PD-L1 on mature moDCs while tol-DCs repressed PD-L1 expression (*Figure 6e*). Finally, CTLA-4–Ig tended to upregulate the expression of CD83 maturation marker on mature moDCs (*Figure 6f*). Contrastingly, tol-DCs had lower CD83 levels.

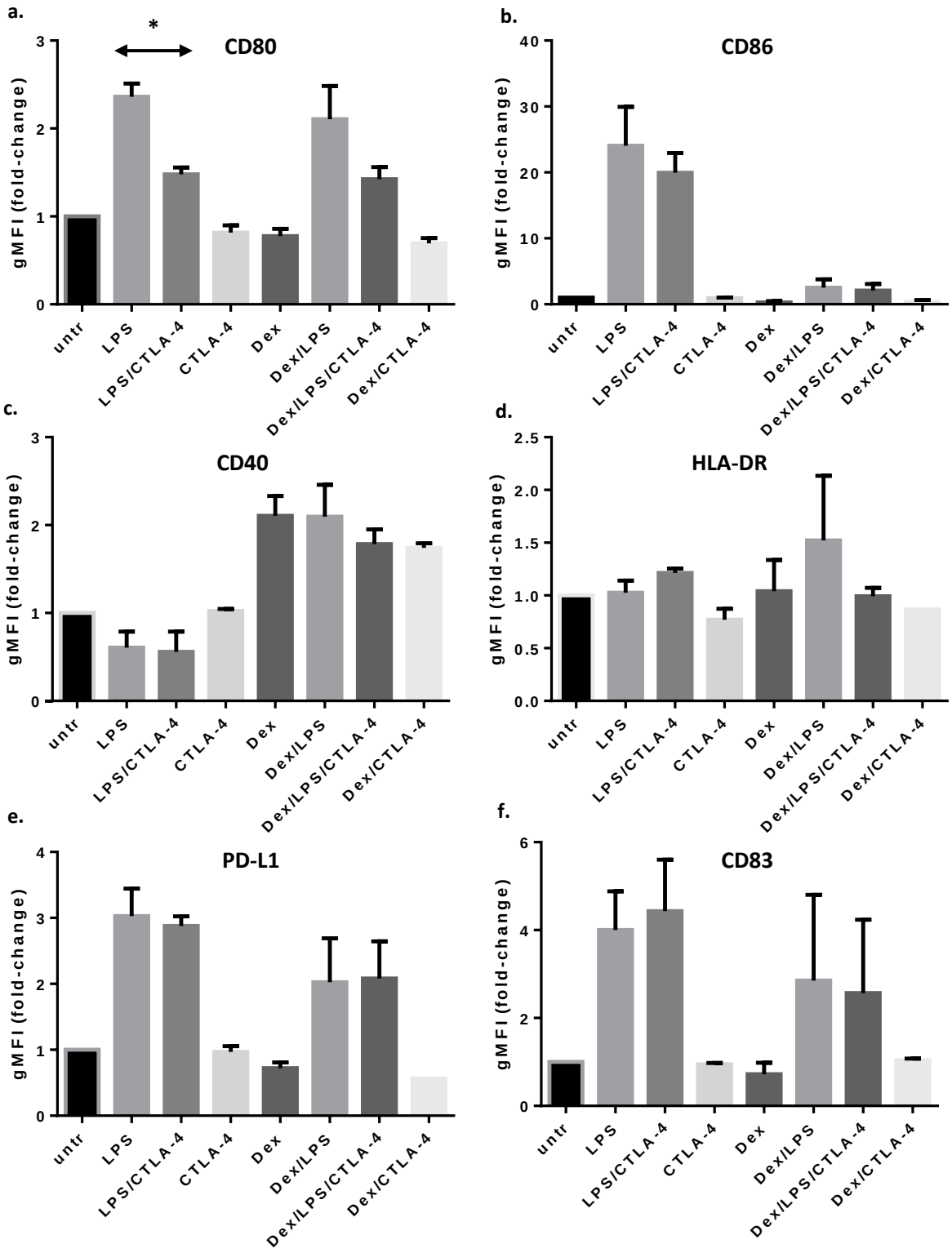

Figure 6: *Relative e*xpression of extracellular markers on moDCs upon maturation with LPS, CTLA-4–Ig treatment, LPS/CTLA-4–Ig treatment and after the same conditions on tol-DCs. **(a, b, c)** Expression of surface co-stimulatory **(a)** CD80, **(b)** CD86, and **(c)** CD40 markers. **(d)** Expression of antigen-presentation molecule HLA-DR. **(e)** Expression of co-inhibitory molecule PD-L1. **(f)** Expression of maturation molecule CD-83.

16h culture of moDCs with treatments. 3-day moDCs culture with dexamethasone for tol-DCs generation. Results were expressed as mean ± SEM. n=3-4 healthy donors per group. No statistically significant difference was detected among LPS and LPS/CTLA-4–Ig treated moDCs unless it is noted differently. *: $p<0.05$ statistically significant difference among LPS and LPS/CTLA-4–Ig treated moDCs.

<u>Objective 2:</u> CTLA-4–Ig downregulated the expression of both pro- and anti-inflammatory genes on DCs

As it is aforementioned, DCs influence T-cell responses through 3 interaction signals, i.e. antigen-presentation, co-stimulation and secretion profile. Secreted agents act in combination with the other two signals. However, they are categorized alone as anti-inflammatory, such as IL-10 and IDO, due to their implication in inflammation restriction, or pro-inflammatory, such as IL-6 and IL-12b, because they promote inflammation. A real-time PCR analysis was performed in order to study the expression of both anti-and pro-inflammatory agents in transcriptional level (*Figure 7*). No

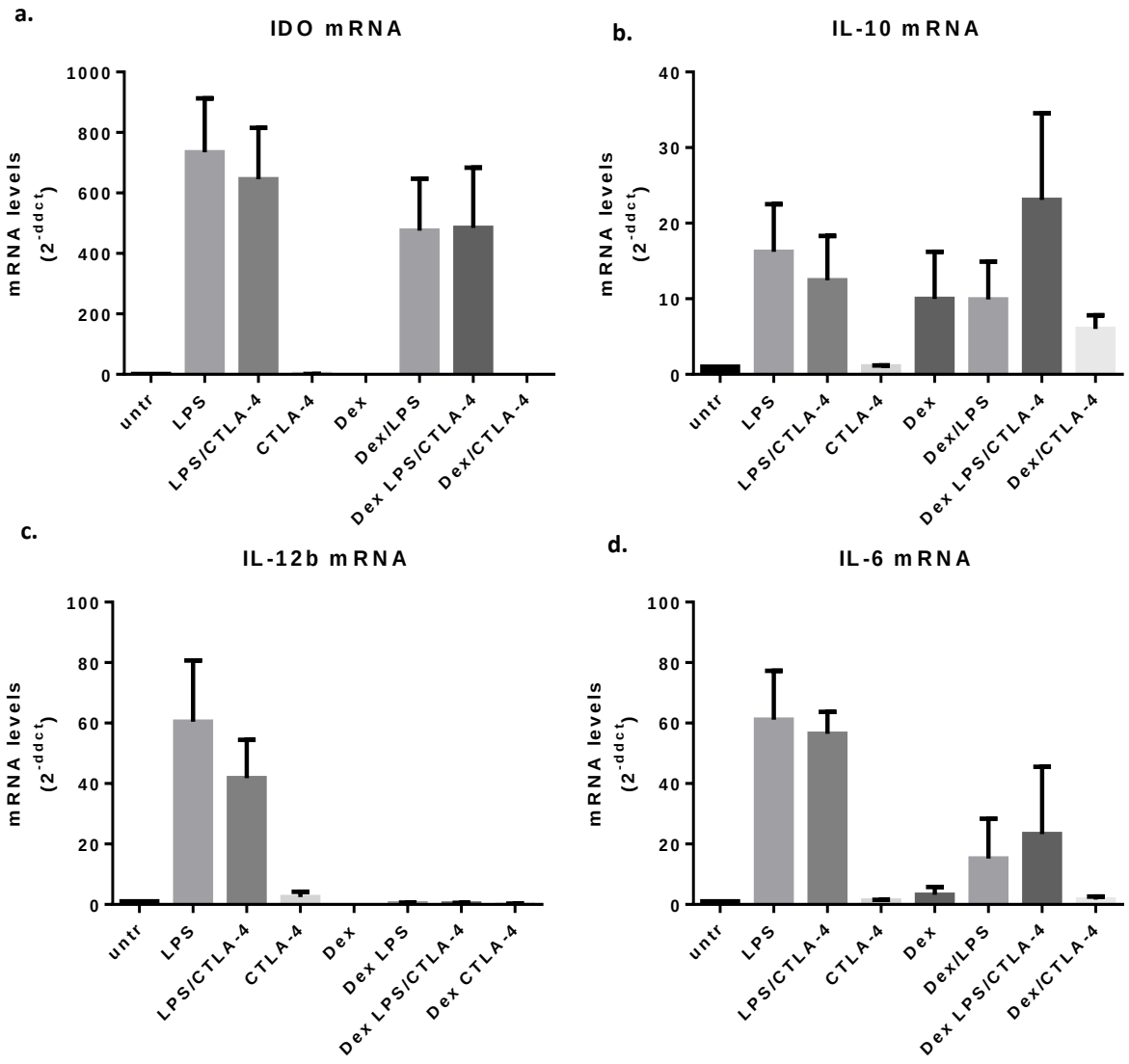

Figure 7: Relative mRNA expression of inflammation-associated genes in moDCs upon maturation with LPS, CTLA-4–Ig treatment, LPS/CTLA-4–Ig treatment and after the same conditions on tol-DCs. **(a, b)** Relative mRNA expression of anti-inflammatory **(a)** *IDO* and **(b)** *IL-10* genes. **(c, d)** Relative mRNA expression of pro-inflammatory **(c)** *IL-12b* and **(d)** *IL-6* genes.
16h culture of moDCs with treatments. 3-day moDCs culture with dexamethasone for tol-DCs generation. Results were expressed as mean ± SEM. n=3 healthy donors per group. No statistically significant difference was detected among LPS and LPS/CTLA-4–Ig treated moDCs.

statistically significant results were detected, although CTLA4-Ig–treated cells tended to have a lower transcriptional expression of *IDO*, *IL-10*, *IL-12b* and *IL-6* mRNA levels compared to mature moDCs. Similar results were also pointed out for tol-DCs, meaning downregulation of all mRNAs compared to mature moDCs.

Additionally, the protein levels of secreted IL-6 were estimated by performing an ELISA assay (*Figure 8*). No statistically significant results were detected, although CTLA4-Ig–treated cells tended to have higher levels of IL-6 cytokine in their supernatants compared to mature moDCs. Tol-DCs inhibited IL-6 cytokine secretion.

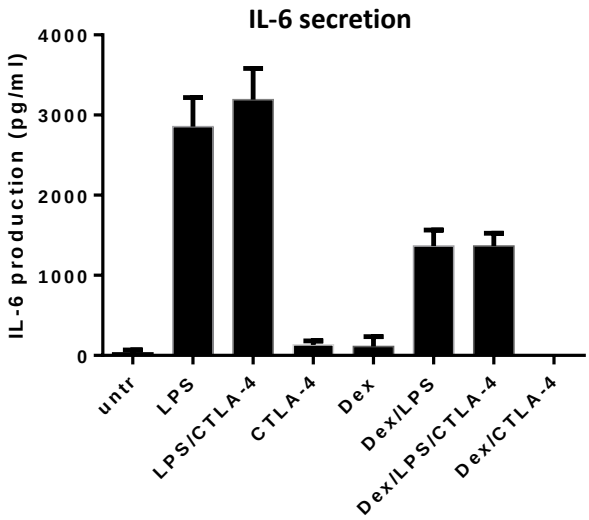

Figure 8: Secreted protein levels of pro-inflammatory IL-6 by moDCs upon maturation with LPS, CTLA-4–Ig treatment, LPS/CTLA-4–Ig treatment and after the same conditions on tol-DCs.
16h culture of moDCs with treatments. 3-day moDCs culture with dexamethasone for tol-DCs generation. Results were expressed as mean ± SEM. n=3 healthy donors per group. No statistically significant difference was detected among LPS and LPS/CTLA-4–Ig treated moDCs.

<u>Objective 3:</u> The mTOR pathway was upregulated on the CTLA-4–Ig-treated DCs

The mTOR/Akt pathway has been demonstrated to be implicated in CTLA-4–Ig effects on DCs. mTOR is also an upstream regulator of glycolytic metabolism and coordinator of DCs metabolic changes upon immunological signals. Akt is a protein kinase that signals upstream of mTOR. To clarify changes in metabolic signaling, moDCs were also undergone expression analysis for phosphorylated mTOR (p-mTOR) and Akt (p-Akt) via flow cytometry for intracellular proteins (*Figure 9*). The expression levels of p-mTOR tended to be upregulated and p-Akt downregulated upon CTLA-4-Ig treatment compared with the LPS/IgG1-treated control. Tol-DCs tended to have increased p-mTOR and lowered p-Akt levels as well.

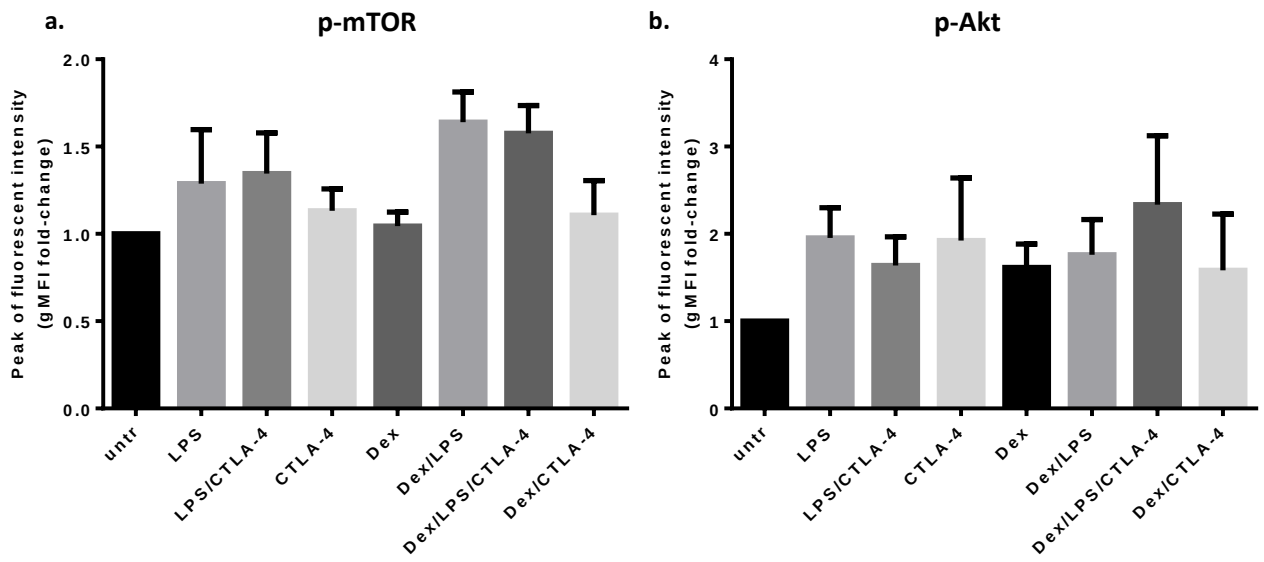

Figure 9: Relative *e*xpression of **(a)** p-mTOR and **(b)** p-Akt on differentiated mo-DCs after maturation with LPS, CTLA-4–Ig treatment, LPS/CTLA-4–Ig treatment and after the same conditions on dex-derived tol-DCs.
16h culture of mo-DCs with treatments. 3-day mo-DCs culture with dexamethasone for tol-DCs generation. Results were expressed as mean ± SEM. n=3 healthy donors per group. No statistically significant difference was detected among LPS and LPS/CTLA-4–Ig treated mo-DCs.

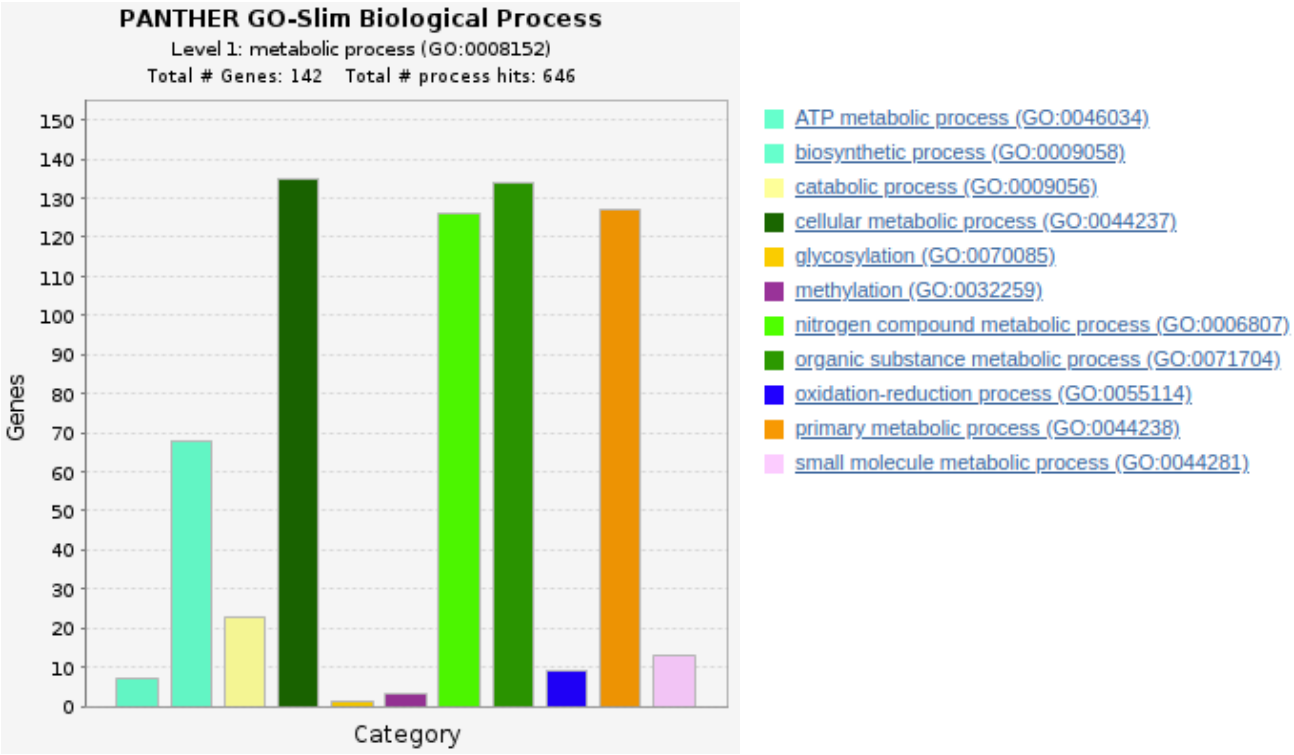

Figure 10*:* Top 11 biological processes enriched in the set of deregulated genes on activated DCs treated with CTLA-4–Ig .
p-value: <0.05, counts: <10

Objective 4: CTLA-4–Ig deregulated important biological processes of DCs

To investigate the molecular mechanisms through which CTLA-4–Ig mediated the tolerogenic phenotype on human DCs, we performed RNA sequencing (RNAseq) on activated DCs and activated DCs exposed to CTLA-4–Ig (5 experiments). The analysis of RNAseq revealed 575 genes that were differentially expressed on CTLA4-treated DCs. Interestingly, the mainly processes that found to be deregulated upon CTLA-4–Ig was the mitochondrial process and the oxidative phosphorylation (*Figure 10*).

Discussion

DCs are an inextricable part of immune system considering their function as professional APCs. Illustratively, they are able to provide instructions to naïve T cells affecting T cell activation and, as a result, shaping immune responses (7). DC-derived instructions to T cells can be described by three categories of signals, antigen presentation, co-stimulation/co-inhibition and cytokine production (8,9). Interaction of CD28 protein on T cells with CD80/86 molecules on APCs is one of the most essential co-stimulatory signals for promoting T cell activation. CTLA-4, another molecule on T cell surface, usually expressed by Tregs, is able to interact with CD80/86 with higher affinity than CD28 and leads to T cell inhibition by blocking a positive signal from CD28 (24,51). Although the mechanism of CTLA-4-mediated negative regulation is still poorly understood, it is proved that CTLA-4 has a cell-extrinsic function of removing CD80/86 of the APCs surface, including DCs, through trans-endocytosis (12). In parallel, evidence in literature have pointed out additional cell-extrinsic mechanisms of CTLA-4 molecule on DCs. The soluble form of CTLA-4 (CTLA-4–Ig) is an approved therapy for RA and, although it was developed to target the CD28-CD80/86 pathway on T cells, it also interacts with CD80/86 on DCs and promotes anti-inflammatory characteristics via the co-called "reverse signaling" (57-59) . Thus, CTLA-4–Ig can be a useful tool to study the cell-extrinsic actions of CTLA-4. In fact, our *in vitro* setup shows that CTLA-4–Ig affected moDCs and was able to induce many anti-inflammatory characteristics.

Under steady state conditions, DCs acquire a tolerogenic phenotype and they promote either Treg polarization or T cell anergy. Furthermore, in order to provide different signals upon different pathogens, DCs are equipped with plasticity of their cellular processes, such as transcription and metabolism. In many situations, especially in autoimmunity, DCs fail to supply T cells, and other immune cells, with tolerogenic signals leading to the development of inflammatory disorders. DCs with tolerogenic properties can be induced upon pharmaceutical treatment, e.g. dexamethasone, and this is exploited to cure many diseases. Antigen presentation is the first and most important signal in this process. The increase of HLA-DR levels on moDC surface upon CTLA-4–Ig treatment demonstrates that the antigen presentation is upregulated on these cells and, intriguingly, tol-DCs, generated with dexamethasone treatment, upregulate HLA-DR too. The magnitude of this upregulation is limited, a fact that could be explained by the generally high expression levels of HLA-DR by healthy monocytes while lower expression is associated with disease development (65). This also seems to happen here since the freshly isolated blood monocytes have high surface HLA-DR, as it is suggested by flow cytometry, and this expression is not abrogated after 5 days of differentiation to moDCs.

The DC surface markers CD80, CD86 and CD40 are significant inducers of the second co-activation signal to T cells while PD-L1 is one of the most known co-

inhibitors in the surface of APCs. Moreover, CD80 and CD86 are the molecules upon which CTLA-4–Ig binds to exert its effects to DCs (12,54,56,57,59,62). Our findings demonstrate that the co-stimulatory function of moDCs were downregulated upon CTLA-4–Ig treatment. The expression of CD80 and CD86 were inhibited upon CTLA-4–Ig incubation, similar to the tol-DCs. Although a CTLA-4-dependent downregulation of CD80/86 has already been referred by previous works at the level of DC-T cell crosstalk (54,66), the extent of CTLA-4 trans-endocytosis contribution is not clarified yet and, therefore, other mechanisms of CTLA-4 cannot be excluded. Additionally, it should be noted that low levels of CD80 and CD86 might be associated with poor binding of the flow cytometry antibodies due to the previous binding of CTLA-4–Ig molecules. If this is the case, it can also explain the robust downregulation of CD80 compared to the moderate inhibition of CD86 expression because CTLA-4–Ig binds CD80 stronger than CD86 (50). To exclude this effect, an antibody-independent method, such as real-time PCR analysis, could be a useful tool.

As regards CD40 expression, there was no change on moDCs upon CTLA-4–Ig treatment. Previous studies in mice have demonstrated that there is not a CTLA-4-dependent pathway which can affect CD40 levels on DCs surface (54,66). On the same time point (16h) CD40 was overexpressed on tol-DCs although, according to literature, we were expecting a downregulation of CD40 upon dexamethasone treatment (43). This could be related to the experimental conditions, given that the expression of surface markers on dexamethasone-generated tol-DCs is usually evaluated after 24-hour maturation with LPS. Nevertheless, the time point of 16-18 hours used by our lab was based on previous experiments with CTLA-4–Ig. The case for the co-inhibitory PD-L1 expression was almost no change on moDCs upon CTLA-4–Ig treatment but it was inhibited on tol-DCs. Although tol-DCs tend to overexpress co-inhibitory markers expression, dexamethasone seems to actually downregulate PD-L1 on moDCs (47). Finally, treatment with CTLA-4–Ig promoted the maturation of moDCs. This result is based on the upregulation of CD83 expression upon CTLA-4–Ig. Usually, tol-DCs are characterized as semi-mature cells, i.e. they express low to intermediate maturation markers, something that was observed on our tol-DCs control and also on other studies with human DCs (42,43). However, the maturation state of DCs does not determine their immunogenic potential but maturation happens with and promotes a concomitant change in expression of co-stimulatory markers (67).

Another essential instruction to naïve T cells is promoted by the secretory profile of DCs upon antigen encounter which is usually formed by a combination of secreted cytokines (signal 3). Hence, in order to extend our knowledge on DCs' alterations caused by CTLA-4–Ig treatment we assessed the expression of pro- and anti-inflammatory genes on moDCs. Our results revealed that CTLA-4–Ig inhibited the expression of both pro- and anti-inflammatory genes. IDO is an important anti-inflammatory agent in tolerance which inhibits T cell proliferation when secreted by DCs (23). In our experiments, IDO expression was downregulated on CTLA-4–Ig-treated moDCs, such as on the tol-DCs. A 2003 publication demonstrated that CTLA-4–Ig was not able to induce IDO activity on murine bone marrow-derived DCs and

splenic-derived DCs (61). However, other studies in mice have implicated CTLA-4–Ig reverse signaling with increased IDO secretion only by DCs and not other APCs (59,60). In human moDCs, the significance of IDO in CTLA-4-Ig-mediated immunosuppression remains debatable since there are publications which report induction of IDO activity upon ligation of CD80/86 by CTLA-4-Ig (62) and others that claim no changes in IDO levels upon CTLA-4–Ig treatment (63).

The gap between CTLA-4 reverse signaling and DC cytokine secretion has not been totally filled because the majority of the studies has focused on DCs' surface co-stimulatory markers. Here, it is indicated that CTLA-4–Ig, the soluble form of CTLA-4, is able to change the gene expression profile of secreted cytokines. Specifically, the expression of anti-inflammatory IL-10 was downregulated. In parallel, tol-DCs expressed low IL-10 results although it was expected to have increased IL-10 levels based on the literature (42,46). As aforementioned, tol-DCs are generated upon 24-hour incubation with LPS or other maturation agents meaning that this difference in time point must be considered for our control. As regards to pro-inflammatory cytokines, the gene expression of IL-6 and IL-12b were repressed upon CTLA-4–Ig and the same was found for tol-DCs. Although both anti- and pro-inflammatory genes were downregulated in transcriptional level, the inhibition of pro-inflammatory IL-6 and IL-12b can be capable for the induction of DC tolerogenic phenotype. To extend these findings, the protein levels of secreted IL-6 were estimated and found upregulated despite of the mRNA downregulation. IL-6 is an important regulator of inflammatory conditions and for this reason it is regulated by cells in many levels after transcription (68). CTLA-4 might influence a post-transcriptional pathway to result in upregulated secretion. However, more replications of this experiments are needed since there were slight differences which could be affected from random factors. Also, the secretion levels of these cytokines could be calculated. This can be useful because transcriptional changes are not always leading to changes in protein production. The results for tol-DCs for both IL-6 and IL12b agree with the literature (46-48).

Despite the cytokine expression profile, the function of DCs is also associated to their cellular metabolism. After maturation, immunogenic DCs shift their metabolism towards glycolysis, while tolerogenic DCs increase oxidative phosphorylation and fatty acid oxidation (36,69). From our RNAseq analysis it is obvious that CTLA-4–Ig promotes major alterations in metabolism-associated genes. For this reason, it is important to investigate the molecular pathways which are activated (or repressed) upon CTLA-4–Ig treatment. Pathways initiated upon mTOR expression have been implicated in human moDCs pro- and anti-inflammatory functions (70). Also, previous work from Dr. Verginis' lab revealed that CTLA-4–Ig was able to upregulate the Akt/mTOR pathway on human moDCs leading to autophagy inhibition (57). Our results indicate an increase in mTOR expression while Akt expression was decreased with a similar effect on tol-DCs. For this experiment, cells were fixed and incubated with methanol in order to be stored at -20^{o}C for a few days. During the flow cytometry analysis it was obvious that the number of cells per sample was small after this method (~5-10*10^4 cells) compared to the initial population

(~$300*10^4$) and possibly the fixing and freezing of moDCs limited the number of intact cells per sample and affected the quality of our results. This experiment should be repeated without the step of freezing the cells to avoid cell lysis.

In summary, our data confirm that CTLA-4–Ig can induce reverse signaling and promote tolerogenic characteristics by DCs. Important contributors of this modulation are the CD80 and CD86 co-stimulatory molecules. In parallel, IL-12b and IL-6, essential pro-inflammatory cytokines for T cell activation, are downregulated and this can contribute to the induction of tolerance. Finally, CTLA-4–Ig promotes changes in regulation of metabolism-related genes that favor anti-inflammatory phenotype. Akt/mTOR pathway, which can lead to downregulation of DC autophagy and, subsequently, their immunogenic potential, seems to be implicated in metabolism alterations. These results are further investigated by our lab and future experiments are needed to elaborate discrepancies with published studies. An important step to increase the significance of these data is to further clarify the intracellular mediators of CTLA-4–Ig signaling and, especially, the consequences on DC metabolism.

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