MASTER THESIS

"The effect of abatacept (CTLA4-Ig) on the tolerogenic potential of human dendritic cells"

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GRADUATE PROGRAM IN
THE MOLECULAR BASIS OF HUMAN DISEASE

ΜΕΤΑΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ

« Η επίδραση του abatacept (CTLA4-Ig) στη λειτουργία των ανθρώπινων δενδριτικών κυττάρων»

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ΜΠΣ « Μοριακή βάση των νοσημάτων του ανθρώπου»

Επιστημονική επιτροπή

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Τμήμα Ιατρικής

Πανεπιστήμιο Κρήτης

"…το 'πε κι ο Μαρξ, όσα περισσότερα χρωστάει κανείς τόσο πιο πλούσιος είναι…"

Ο Παντελής Μπουκάλας για τον Άγγελο Ελεφάντη,

«Minima memoralia», Η Καθημερινή, 30-05-2008

«Χρωστάω» για τη δουλειά αυτή:

στον Πάνο Βεργίνη που σχεδίασε όλο το project, ανέχτηκε τα λάθη και τις παραλείψεις της απειρίας μου και με καθοδήγησε

σε όλα τα παιδιά του εργαστηρίου Ρευματολογίας και το προσωπικό της Ρευματολογικής Κλινικής για την πολύτιμη βοήθειά τους

στον Καθηγητή μου κο Μπούμπα που συγχωρεί ακόμα τα «αμφίσημα σήματα» (ambivalent signals) που εκπέμπω

στη γυναίκα μου

SUMMARY

T-cell activation and effector function is regulated by a delicate fine-tuning of antagonistic positive and negative signals carried out by costimulatory and coinhibitory molecules respectively, in conjunction to the signal provided by the T-cell receptor. Probably the most important inhibitory molecule on the surface of T-cells is CTLA-4, a molecule structurally homologous to CD28, which binds to B7 ligands (CD80/CD86) on the surface of professional antigen-presenting cells (APCs) to terminate T- cell responses. CTLA-4 knockout mice develop an uncontrolled deleterious immune response and eventually succumb to a serious T-cell lymphoproliferative disorder. The ability of CTLA-4 to block the activation of T-cells has been therapeutically exploited for the attenuation of excessive immune system activation in autoimmune diseases. Abatacept is a recombinant protein consisting of the extracellular domain of human CTLA-4 fused to the Fc portion of human IgG1 (CTLA4-Ig) and was developed as a soluble CD28 antagonist. Since December 2005, abatacept is the first FDA approved T-cell costimulation modulator for the treatment of patients with rheumatoid arthritis.

Dendritic cells constitute a heterogeneous group of cells with multiple subsets that are able, under different circumstances, to induce either immunity or tolerance in the periphery. While a unique tolerogenic “signature” for DCs has not yet been established and is not restricted to one specific DC subset, it is generally accepted that an immature state, high net expression of co-inhibitory molecules and production of tolerogenic enzymes, such as indoleamine 2,3 deoxygenase (IDO), are important, though not the sole, prerequisites for a tolerogenic DC phenotype.

Although competition with CD28 for binding to CD80 and CD86 is considered to be the major mechanism by which abatacept blocks T-cell activation, its complete mode of action still remains to be elucidated. The aim of the present study was to investigate the ability of abatacept to influence the tolerogenic properties of human DCs both in healthy subjects as well as in RA settings. Specifically, we examined whether abatacept can induce DCs with a tolerogenic phenotype by assessing the ability of abatacept-treated DCs to block the proliferation of responder T-cells and attempted to delineate the mechanism of this inhibition.

In our study, treatment of human DCs with abatacept was insufficient to halt the proliferation of allogeneic T-cells in a mixed leukocyte reaction. Abatacept was unable to induce either IDO upregulation inside DCs or changes in the expression of potential tolerogenic surface markers of DCs, such as ICOS-L or PD-L1.

ΠΕΡΙΛΗΨΗ

Η πλήρης ενεργοποίηση και διαφοροποίηση των Τ-κυττάρων ρυθμίζεται από μια λεπτή ισορροπία μεταξύ θετικών και αρνητικών σημάτων, τα οποία παρέχονται από συν-διεγερτικά και συν-ανασταλτικά μόρια αντίστοιχα, σε συνδυασμό με το σήμα που παρέχεται από τον υποδοχέα των Τ-κυττάρων (TCR). Το σημαντικότερο ανασταλτικό μόριο στην επιφάνεια των Τ-κυττάρων είναι το CTLA-4, ένα μόριο δομικά ομόλογο με το CD28, το οποίο συνδέεται με Β7 συνδέτες (CD80/CD86) στην επιφάνεια των επαγγελματικών αντιγονοπαρουσιαστικών κυττάρων για να τερματίσει τις Τ-κυτταρικές αποκρίσεις. Ποντίκια στα οποία λείπει το CTLA-4 αναπτύσσουν μια ανεξέλεγκτη ανοσιακή αντίδραση και τελικά υποκύπτουν σε μια σοβαρή λεμφουπερπλαστική νόσο από Τ- κύτταρα. Η ικανότητα του CTLA-4 να μπλοκάρει την ενεργοποίηση των Τ-κυττάρων έχει αξιοποιηθεί θεραπευτικά για την αντιμετώπιση της υπερβολικής ενεργοποίησης του ανοσολογικού συστήματος στα αυτοάνοσα νοσήματα. Το abatacept είναι μιία ανασυνδυασμένη πρωτεΐνη που αποτελείται από το εξωκυττάριο τμήμα του CTLA-4 ενωμένο με το Fc τμήμα της ανθρώπινης IgG (CTLA4-Ig) και κατασκευάστηκε ως ένας διαλυτός ανταγωνιστής του CD28. Απ' το Δεκέμβριο του 2005, το abatacept είναι το πρώτο φάρμακο που στοχεύει εκλεκτικά τη συν-ενεργοποίηση των Τ-κυττάρων, εγκεκριμένο για τη θεραπεία ασθενών με ρευματοειδή αρθρίτιδα.

Τα δενδριτικά κύτταρα αποτελούν μια ετερογενή ομάδα κυττάρων με πολλαπλές υποομάδες, τα οποία μπορούν υπό διαφορετικές προϋποθέσεις να προάγουν είτε ανοσία είτε ανοχή στην περιφέρεια. Παρά το ότι σαφή, συγκεκριμένα χαρακτηριστικά των κυττάρων αυτών που προάγουν την ανοχή δεν έχουν ταυτοποιηθεί, είναι γενικά παραδεκτό ότι ο μειωμένος βαθμος ωριμότητας, η έκφραση ανασταλτικών μορίων επιφανείας και η παραγωγή «ενζύμων ανοχής», όπως το IDO, είναι σημαντικά χαρακτηριστικά για ένα φαινότυπο ανοχής.

Παρά το ότι ο ανταγωνισμός με το CD28 για τα μόρια CD80 και CD86 θεωρείται ο κύριος μηχανισμός δράσης του abatacept, ο ακριβής τρόπος δράσης του χρήζει περαιτέρω διερεύνησης. Ο σκοπός της παρούσας μελέτης ήταν η εκτίμηση της δυνατότητας του abatacept να δημιουργήσει ανθρώπινα δενδριτικά κύτταρα με ικανότητες προαγωγής ανοχής τόσο σε υγιείς όσο και σε ασθενείς με ΡΑ. Συγκεκριμένα, εξετάστηκε η ικανότητα δενδριτικών κυττάρων, επωασθέντων

προηγουμένως με abatacept, να αναστέλλουν τον πολλαπλασιασμό αλλογενών Τ-κυττάρων και δευτερευόντως εξετάστηκε ο μηχανισμός αυτής της αναστολής.

Σύμφωνα με τα αποτελέσματα μας, η χορήγηση abatacept δεν ήταν ικανή να «δημιουργήσει» δενδριτικά κύτταρα με τη δυνατότητα κατόπιν να μπλοκάρουν τον πολλαπλασιασμό αλλογενών Τ-κυττάρων σε μια μεικτή λεμφοκυτταρική αντίδραση. Το abatacept δε φάνηκε να προκαλεί ούτε αύξηση της παραγωγής IDO στα δενδριτικά κύτταρα αλλά ούτε και αλλαγές στην έκφραση μορίων επιφανείας των δενδριτικών κυττάρων, όπως τα ICOS-L και PD-L1, τα οποία θα μπορούσαν δυνητικά να προκαλέσουν ένα φαινότυπο ανοχής στα κύτταρα αυτά.

CONTENTS

1. INTRODUCTION

1.1 The "two-signal" model for T-cell activation

The human immune system has evolved in order to generate effective immune responses against foreign pathogens and tumor cells, while at the same time avoiding excessive self-tissue damage which may lead to autoimmunity ("self-non self" discrimination). This demanding feature of the immune system requires a very precise regulation which involves multiple cell subsets, surface receptors and soluble molecules (cytokines/chemokines).

The concept of T-cell costimulation has been one of the major advances in basic immunology over the past decades. It claims that naïve T-cells require two independent signals provided by "professional" antigen presenting cells (APCs), in order to achieve full activation and effector function. The first signal is the stimulation of the T-cell receptor (TCR) by an antigen bound to a major histocompatibility complex molecule (MHC) ("TCR-dependent signal"). This is necessary but insufficient to accomplish maximal T-cell activation. The second signal is independent of the TCR and requires the interaction of a costimulatory molecule on T-cells with its specific ligand of APCs. Stimulation of TCR without deliverance of this second signal renders the T-cells unresponsive or anergic[1]. On the other hand, costimulation in the absence of antigen has no effect on T-cells[2] **(Fig.1)**. Teleologically this is important for the maintenance of peripheral tolerance, because costimulation is a unique feature of professional APCs. Consequently, the development of unwanted immune responses is avoided, when potentially autoreactive T-cells encounter self-antigen bearing cells in peripheral tissues[3].

Extensive research has identified a number of different pairs of receptor-ligands that function as costimulatory molecules for T-cell activation[4]. Probably the most important T-cell costimulatory molecule is CD28, which interacts with its ligands CD80 (B7-1) and CD86 (B7-2) on the surface of APCs to initiate T-cell responses. CD28 is constitutively expressed as a homodimer on the surface of T-cells and its binding to B7 molecules, apart from providing the necessary second signal to T-cells, also enhances the effects of TCR signaling at the level of gene

transcription[5].

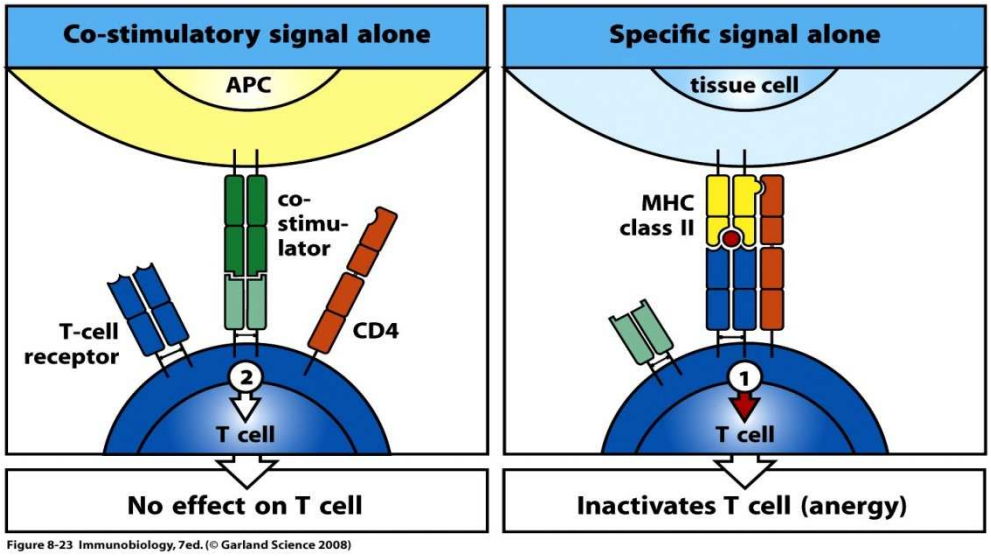


Fig.1 Two-signal model for T-cell activation. TCR signaling alone leads to T-cell inactivation, while costimulation in the absence of TCR engagement has no effect on T-cells. Adapted from *Janeway and Travers, Immunobiology, 7th ed, 2008*.

1.2 Coinhibitory molecules: the discovery of CTLA-4

Soon after the identification of CD28, a second molecule was isolated by means of subtractive cloning from the surface of activated CD8+ T-cells and it was named cytotoxic T cell antigen-4 (CTLA-4)[6]. The first implication that CTLA-4 might be involved in T-cell costimulation came from genetic studies which showed that the CTLA-4 gene is located at the same chromosomal region with CD28 and shares a high degree of sequence homology with the latter (20%, including the ligand binding domain), dictating the significant structural homology of the two proteins[7]. While initially thought that the novel protein would represent an additional costimulatory molecule, subsequent *in vitro* and *in vivo* experiments established its role as the most prominent coinhibitory receptor on the surface of T-cells. Firstly, soluble CTLA-4 was able to inhibit the proliferation of T-cells cultured *in vitro* with B7-expressing APCs [8]. Subsequent experiments examining the interactions between TCR, CD28 and CTLA-4 showed that, when all three receptors were cross-linked together, CTLA-4 greatly reduced T-cell proliferation driven by stimulation by CD28[9]. Finally, in the landmark paper of Tivol et al [10], the generation of CTLA-4 knockout mice showed that these animals succumb early to a massive T-cell lymphoproliferative disorder. This observation firmly established this protein as a negative regulator of T-cell

function and proliferation. Over the next years, additional stimulatory and inhibitory molecules were identified on T-cells and APCs[4], clearly indicating that the regulation of the immune response requires a delicate fine-tuning between "positive" and "negative" signals **(Fig.2)**.

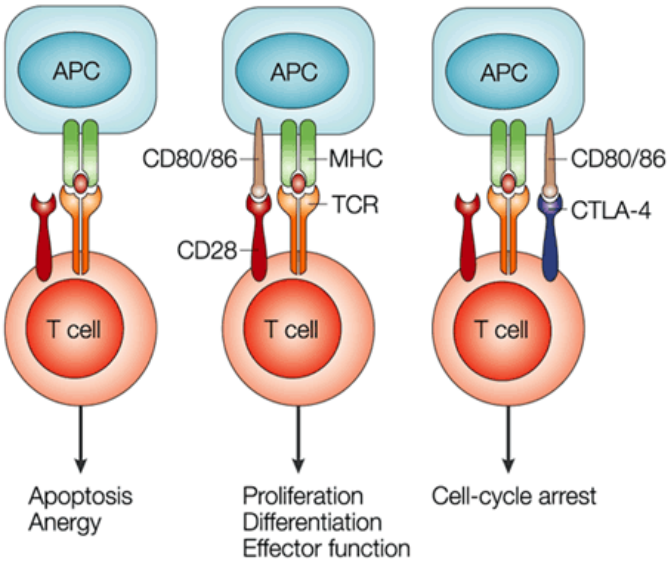


Fig.2 Opposing effects of CD28 and CTLA-4 on T-cell activation. CD28 and CTLA-4 on the surface of T-cells share the same ligands on APCs to deliver positive and negative signals respectively.

The relative interactions of CD28 and CTLA-4 with their B7 ligands, CD80 and CD86, on the surface of APCs provide useful insight into the temporal nature of an immune response and its regulation. Both CD28 and CTLA-4 exist on the cell surface as homodimers, but CD28 possesses a single ligand-binding domain, while CTLA-4 is able to bind to two B7 molecules through its extracellular V domain [11]. Soon after the identification of CTLA-4, experiments using soluble CTLA-4 proved that it can bind B7 molecules with 20-fold higher avidity than CD28 [8]. In contrast to CD28, which is constitutively expressed on resting T-cells, CTLA-4 is mainly expressed on activated T-cells and transcription of CTLA-4 mRNA is highly upregulated upon TCR stimulation[6]. Similarly, although both CD80 and CD86 are expressed on APCs and B-cells, CD86 has a more temporally stable expression while CD80 expression is significantly enhanced after APC activation [12]. Moreover, CD86 binds CD28 two- to threefold more effectively than CD80 [11], while CTLA-4 has a significantly higher affinity for CD80 than CD86 (this happens probably because CD80 exists as a homodimer, in contrast to CD86 which is monomeric)[8, 11]. Taken together, these observations suggest that there is a predilection for CD86 to bind to CD28 and conversely CD80 is "biased" towards binding CTLA-4. These differences in binding

avidity of CD28 and CTLA-4 for CD80 and CD86 have led to a “model” suggestion, whereby interactions between CD28 and CD86 predominate initially to drive the immune response, while the subsequent CTLA-4-CD80 strong ligation serves to provide inhibitory counterregulatory signals in order to terminate the T-cell responses [3, 13]. This model emphasizes the fact that opposite signals delivered through distinct receptor-ligand interactions provide the precise regulation that is needed for a normal outcome of an immune response and the avoidance of aberrant immune activation.

1.3 CTLA-4 structure, kinetics and signaling inside T-cells

The human CTLA-4 gene is located on the long arm of chromosome 2 (2q33), sharing significant sequence homology with CD28, and the CTLA-4 protein (CD152) is comprised of an extracellular V domain containing the ligand-binding motif MYPPY, a transmembrane domain and a cytoplasmic tail[13]. As mentioned above, transcription of the gene is highly upregulated upon T-cell activation[14] and CTLA-4 is found in the region of the immunological synapse within 48 hours of the interaction between APCs and T-cells[15]. However, the kinetics of this expression include a prior step of intracellular accumulation, after which CTLA-4 moves to the cell membrane and is subsequently internalized by a process dependent on clathrin-coated endocytosis[16-17]. A series of biochemical experiments showed that the phosphorylation of one of two tyrosine residues in the cytoplasmic tail of CTLA-4 by the kinases lck and ZAP-70 is essential for the retention of the molecule on the cell surface and the prevention of clathrin-associated internalization [18-20]. Additionally, the strength of the TCR-MHC/antigen interaction may also play a role in the active accumulation of CTLA-4 on the cell surface, providing another example of tight system regulation [21].

Once on the cell membrane, CTLA-4 functions in different ways to inhibit activation of T-cells. Its primary role was initially considered to be ligand competition with CD28 for B7 molecules, based on its higher avidity for these ligands. In this model, CTLA-4-B7 ligation simply acts by obstructing interaction with CD28, thus preventing the positive effects of CD28 signaling[22]. This was supported by the observation that a truncated CTLA-4 molecule that lacks the cytoplasmic tail was able to block T-cell activation [23]. However, subsequent studies provided evidence

that CTLA-4 also acts on intracellular pathways inside T-cells to provide negative signals. More specifically, the intracellular domain of CTLA-4 has no intrinsic catalytic activity; however it contains an SH2-domaIn that has been shown to interact with the phosphatases SHP-1 and SHP-2[20, 24]. Importantly, interaction with SHP-2 in particular results in dephosphorylation of the TCR complex ζ chain, resulting in termination of T-cell signaling [25]. Moreover, the cytoplasmic tail of CTLA-4 also interacts with the serine/threonine phosphatase PP2A [26-27]. This interaction confers inhibitory potential acts upon various intracellular pathways inside the T-cell, most notably the mitogen-activated protein kinase (MAPK), Akt and c-Jun-NH2-kinase pathways, all of which are triggered by the combined effect of TCR engagement and CD28 costimulation [28]. The end result is a reduction in cytokine secretion, especially IL-2 which is crucial for T-cell proliferation, and an arrest in the cell cycle at the G1 phase[29].

Fig.3 depicts the intracellular kinetics of CTLA-4 during an immune response and the intracellular pathways affected by the ligation of CTLA-4 with B7 molecules, especially CD80.

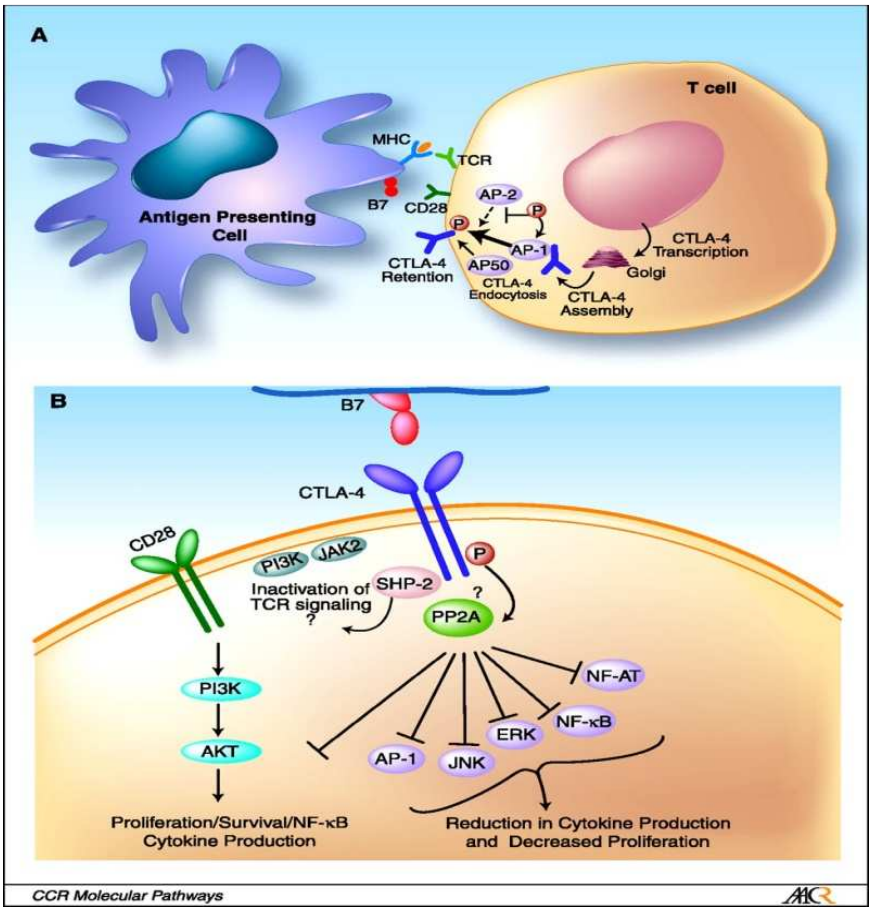


Fig.3 A. Kinetics of CTLA-4 upon TCR activation. Following TCR stimulation, CTLA-4 "travels" to the cell surface through the Golgi. TCR signaling also results in tyrosine phosphorylation of CTLA-4, thereby limiting its internalization.

B. CTLA-4 signaling inside T-cells. Phosphorylation of CTLA-4 results in downregulation of several signaling cascades that are triggered from TCR stimulation. The end result is a reduction in cytokine secretion and proliferation of T- cells. Adapted from ref. 29

1.4 CTLA-4 and regulatory T-cells (Tregs)

A very interesting aspect of CTLA-4 physiology is the role of this surface molecule in the subset of T-cells known as regulatory T-cells (Tregs). Initially described as T-cells with the capacity to suppress autologous T-cell proliferation, Tregs are now considered as primary mediators of peripheral tolerance, essential for normal homeostasis and the prevention of autoimmune diseases. Their importance is highlighted by the development of spontaneous generalized autoimmunity in rodents and humans bearing mutations in forkhead box P3 (FOXP3), a key transcription factor that is required for the development, maintenance and function of Tregs (scurfy mice and human IPEX syndrome, respectively). Two major subsets of Treg cells have been described: i)thymus-derived "naturally-occurring" $CD4^+CD25^+FoxP3^+$ Tregs, present under steady state conditions and ii) "adaptive" Tregs generated in the periphery, with the ability to produce anti-inflammatory cytokines, such as IL-10[30].

Two cardinal features of natural Tregs are that they constitutively express CTLA-4 on their surface (contrary to effector T-cells which upregulate CTLA-4 only after activation) and that Foxp3 controls this surface expression of CTLA-4[31-32]. However, the physiological role and importance of CTLA-4 on Tregs had not been definitely established until recently. Due to the suppressive nature of Tregs and the inhibitory potential of CTLA-4 it was initially tempting to speculate that CTLA-4 is required for normal Treg function. Indeed, early experiments showed that higher surface expression of CTLA-4 correlated with more efficient Treg function[33]. At the same time however, Tang et al provided evidence that mice lacking CTLA-4 contain a population of cells that express Foxp3 and are capable of suppressing autologous T-cell proliferation *in vitro*[34], suggesting that CTLA-4 may actually not be absolutely required for Treg suppression. To add more complexity, antibodies targeting CTLA-4 resulted in attenuation of Treg suppressive capacity *in vitro* in an mouse model of experimental colitis, pointing towards a physiologic role for surface CTLA-4 expression on Tregs[35]. Overall, these previous studies suggested that although CTLA-4 may not be indispensable for the development and suppressive function of Tregs, it probably constitutes an important component in the arsenal of these cells.

More recently, a series of *in vitro* and *in vivo* studies provided more definite evidence concerning the requirement of CTLA-4 for the normal inhibitory potential of Tregs. Transfection of CTLA-4 knockout mice, which develop generalized autoimmunity, with CTLA-4-competent wild-type Tregs resulted in significant survival benefit of these animals [36]. Similarly, when human T-cells were transfected to express CTLA-4 protein, they were able to suppress DC-driven proliferation of naïve $CD25^-$ T-cells in the context of an MLR, in a similar manner as natural Tregs[37]. Interestingly, Tregs from patients with rheumatoid arthritis were shown to express less amounts of CTLA-4 on their surface and this reduced expression correlated with attenuated ability of these cells to suppress cytokine production, notably IFN-γ[38]. This defect was restored when CTLA-4 was artificially expressed on the surface of these cells, indicating the necessity of its presence for Treg normal function. Finally, probably the most important observation came from the generation of mice bearing a conditional deletion of CTLA-4 in the Treg population [39]. This specific deficiency resulted in spontaneous development of systemic lymphoproliferation and a fatal T cell–mediated autoimmune disease, as these $CTLA\text{-}4^-$ Tregs exhibited significantly reduced suppressive capacity both *in vitro* and *in vivo*, illustrated by their inability to downregulate expression of B7 molecules on the surface of APCs [39].

Taken together, the available data from human and animal studies, although not entirely conclusive, provide sufficient evidence to support the concept that CTLA-4 constitutes an essential and physiologically important molecule on the surface of Tregs. Considering the difficulties found in the characterization of a specific suppressive “signature” for Tregs, these observations add valuable information in the elucidation of the function of this T-cell subset.

1.5 CTLA-4 in human autoimmune diseases

The importance of CTLA-4 as a negative regulator of T-cell responses, illustrated by the massive lymphoproliferation developed in CTLA-4 knockout mice, generated an early interest in the exploration of possible alterations in the CTLA-4 gene that might contribute to human autoimmunity. A significant number of genetic association studies has given support to this hypothesis, although no specific gene mutation has been linked to a certain human disease. The CTLA-4 gene consists of

four exons: exon 1 encodes a leader peptide, exon 2 encodes the ligand-binding domain, exon 3 encodes the transmembrane domain, and exon 4 encodes the cytoplasmic tail. A number of genetic linkage studies have identified polymorphisms within or near the CTLA-4 gene region associated with a wide variety of autoimmune diseases. A study of single-nucleotide polymorphisms (SNPs) within the CTLA-4 locus revealed an association between a SNP in exon 1 and both type 1 diabetes (T1D) and Graves' disease [40], although a link between these diseases and CTLA-4 was not confirmed in a subsequent association study [41]. The result of this SNP is an A–G substitution, which consequently leads to a Threonine-Alanine substitution in the translated leader peptide. The stable presence of this polymorphism in exon 1 has been subsequently documented in several association studies within families with T1D and Graves' disease [42-43]. Additionally, the same A-G transition was subsequently documented in a variety of other autoimmune diseases, including Addison's disease[44], autoimmune hypothyroidism[45], primary biliary sclerosis[46], SLE[47], and rheumatoid arthritis[48]. Although it would be tempting to speculate for a causative role of this substitution in the pathogenesis of the aforementioned diseases, such a functional significance has not been yet demonstrated (which is at least partly reasonable, since a single common pathogenetic disorder underlying multiple diseases would seem unlikely). Patients with Graves' disease harboring the above exon 1 transition did not differ in the proliferation of their T-cells, compared to controls, although indirect data supported a possible attenuated inhibitory effect of CTLA-4 in the former [49].

Additional sequence mapping studies have subsequently identified more SNPs within or near the CTLA-4 gene locus that are associated with human autoimmune diseases, although a clear impact of any of these genetic alterations on the expression or function of CTLA-4 remains to be established. Taken together, the abundance of data from genetic association studies concerning the CTLA-4 gene and human autoimmunity point towards a role for CTLA-4 as a potentially important autoimmune locus, although functional studies will be needed to provide more solid proof[13].

1.6 Therapeutic exploitation of CTLA-4 in cancer , autoimmunity and transplantation

Once the significance of CTLA-4 as the most prominent inhibitory receptor of T-cells was firmly established, the potential for manipulating this pathway for therapeutic purposes was soon recognized. A strenuous effort from both the academy and the industry has been undertaken in order to construct molecules that can interfere with the normal CTLA-4/B7 interaction in either of two opposite directions: inhibition or conversely enhancement of CTLA4-4 actions. Indeed, CTLA-4 can be therapeutically exploited in a dual manner. On the one hand, by using antibodies directed against CTLA-4, one may actually block the normal inhibitory function of this molecule, in order to enhance host immune responses, as in the case of tumor immunity. On the other hand, soluble agonist CTLA-4 molecules take advantage of the inhibitory potential of CTLA-4 as an approach to treatment of autoimmune diseases. As will be discussed below, the former approach has led to the use of CTLA-4 antibodies for human tumors, while the latter has resulted in the first T-cell based therapy for human autoimmune diseases with the development of abatacept, a soluble CTLA-4 agonist.

1.6.1 CTLA-4 blockade in tumor immunity

The potential of blocking CTLA-4 to boost tumor responses was initially demonstrated by studies in mice with various tumor models. Treatment of mice transplanted with cancer cells with antibodies directed against CTLA-4 resulted in rejection of established tumors[50], providing the first evidence that manipulation of this pathway may have major therapeutic implications. More specifically, injection of anti–CTLA-4 antibodies led to rapid rejection of both colon carcinoma tumors that were engineered to express B7 and of wild-type tumors. Administration of anti–CTLA-4 after tumor implantation also resulted in tumor rejection[50].

Despite the striking efficacy of CTLA-4 blockade in enhancing antitumor immune responses in the previous models, subsequent experiments showed that blocking CTLA-4 was not as efficacious in tumor models that are less immunogenic[29]. This observation has significant clinical relevance, since the majority of human tumors are considered relatively non-immunogenic. A major advance came with the finding that the combination of CTLA-4 blockade with either

GM-CSF-producing vaccines or a tumor vaccine can induce rejection of breast carcinoma or melanoma[51-52], implying that at least in non-immunogenic tumors the therapeutic effect of CTLA-4 inhibition requires a prior boost of the immune system. However, another interesting finding came with the observation that the anti-CTLA-4/GM-CSF combination, concomitantly with its antitumor boost effect, also resulted in autoimmune fur depigmentation of animals, as expressed by infiltration of the dermis by lymphocytes and destruction of melanocytes[52]. The latter finding provides yet another example of the delicate balance that is needed in order to avoid the cost of breaking self tolerance when promoting desirable immune responses.

Based on the above, CTLA-4 blockade subsequently progressed into human clinical trials, mainly in patients with melanoma as well as other types of tumors [53-56]. In most of these studies, significant biological activity was accompanied by a variety of autoimmune adverse effects. Bowel inflammation including episodes of bowel perforation was the most important autoimmune side-effect. In addition, cases of immunomediated hypophysitis, hepatitis, nephritis, and symptomatic skin inflammation have also been reported [53, 55]. Interestingly, in one clinical trial, the investigators observed that autoimmune toxicity correlated with the degree of clinical response [57].

Currently, several phase III clinical trials are under way in patients with melanoma, testing different dosing schedules as well as various combinations and adjuvants. Definite results pending, the existing data suggest that inhibition of CTLA-4/B7 pathway might provide a useful adjunctive therapy at least for some forms of malignancy.

1.6.2 CTLA4-Ig in the treatment of autoimmunity and in the quest for transplant tolerance

On the other end of the spectrum of CTLA-4 manipulation, the high avidity of this molecule for B7 ligands raised the possibility that developing a similar soluble form of CTLA-4 as a CD28 antagonist could serve as potential therapy for aberrant immune responses, commonly autoimmunity. By blocking CD28 costimulation of T-cells, soluble CTLA-4 provided an attractive candidate for promoting peripheral tolerance in cases where the latter is “broken”. This rationale led to the

development of CTLA4-Ig, a recombinant protein in which the extracellular B7-binding domain of CTLA-4 was fused to the Fc portion of immunoglobulin IgG[58].

Early studies in various animal models of autoimmunity clearly showed promising results for the suggested approach. In the NZ B/W mouse, the classic murine model of SLE, administration of CTLA4-Ig resulted in significant clinical responses, as demonstrated by the delay in the progression of renal disease and the prolongation of survival[59]. Subsequent experiments in rodent models of experimental autoimmune encephalomyelitis[60], type 1 diabetes and myasthenia gravis[61] showed similar encouraging results. Concomitantly, the same approach was tested in animal models of transplantation, the second major field of "tolerance induction". In various transplant settings, different groups showed significant success in graft survival and achievement of tolerance [62-63]. Enthusiasm was temporarily withheld when it was shown that stepping up to a primate non-human organism was not accompanied by similar effectiveness [64]. Nevertheless, the accumulation of data from rodents, especially from combination therapies manipulating the CTLA-4 together with an additional pathway like CD40-CD40L [62, 65], were sufficient for the upgrading of research in humans.

Based on the above encouraging observations, the extrapolation of CTLA4-Ig use in humans ultimately led to the development of abatacept, the humanized form of CTLA4-Ig. Abatacept is a recombinant protein consisting of the extracellular domain of human CTLA-4 fused to the Fc portion (hinge-CH2-CH3 domain) of human IgG1. The first significant results came from an early phase clinical trial in patients with psoriasis, an autoimmune skin disorder. In these patients, CTLA4-Ig treatment led to decreased infiltration of the dermis with T-cells and normalization of keratinocytes[66]. Subsequently, a series of large randomized controlled trials tested the efficacy and safety of abatacept in patients with active rheumatoid arthritis and showed impressive clinical responses, similar to the ones observed with anti-TNF agents, which represent the major therapeutic advance in the treatment of rheumatic diseases in the last years [67-69]. Equally important was the fact that the safety profile of abatacept administration was favorable, as reports of infections from these trials were minor. The end result was that, as of December 2005, abatacept is the first FDA approved selective T-cell costimulation modulator for the treatment of patients with rheumatoid arthritis. A similar molecule, bearing two

amino acid substitutions in the extracellular domain, was termed belatacept and has shown to be significantly effective in non-human primate transplantation settings [70], as well as in a randomized clinical trial in patients with renal transplantation [71]. This introduction will focus on abatacept, as the latter is the subject of the present research project.

1.7 CTLA4-Ig mode of action: more than competition with CD28 for B7 ligands

Although abatacept was originally engineered as a soluble antagonist of CD28 acting through competition with CD28 for B7 ligands on APCs, subsequent observations suggest that there might be more in this molecule than pure competition for CD80 and CD86. Indeed, the complete mode of action of abatacept has not been completely elucidated and interesting new insight has been provided by recent studies. More specifically, abatacept may i) directly inhibit osteoclast differentiation, thereby delaying progression of erosive arthritis in RA patients and more importantly ii) "signal back" inside dendritic cells to generate indoleamine 2,3 deoxygenase (IDO) in an action that potentially generates "tolerogenic" dendritic cells.

1.7.1 Inhibition of osteoclast differentiation

A long term study of abatacept in patients with RA attempted to examine the efficacy of this agent in the progression of structural damage of joints, as measured by radiographic assessments[72]. Interestingly, at 2 years of observation, a significant proportion of patients experienced no progression in articular bone erosions[72]. Clearly, this is of utmost importance, since abatacept seems to interfere with the natural history of the disease, rather than merely alleviating symptoms. Also, delaying the rate of joint destruction has major implications for quality of life, since the association between structural damage and impaired physical function has been firmly established.

Along with the above observation, a study of the effect of CTLA4-Ig on human osteoclast precursors provided a possible explanation for the attenuation of bone resorption with abatacept treatment. When CTLA-4 was added in cultures of human monocytes, which can be differentiated into osteoclasts in the presence of M-CSF and RANKL, this differentiation was halted [73]. Moreover, this effect of CTLA-4 was

T-cell independent. This *in vitro* observation was reinforced by the fact that, in a mouse model of TNF-driven inflammatory arthritis, addition of CTLA-4 led to a significant reduction in bone erosions, accompanied by a reduction in the number of osteoclasts formed in the joints of these animals [73]. Taken together, these elegant experiments may provide an attractive explanation for the bone-sparing effect of abatacept treatment, in a mode of action aiming directly at monocytes to negatively regulate osteoclast differentiation.

1.7.2 IDO induction and "tolerogenic" dendritic cells

Indoleamine 2,3 deoxygenase (IDO) is an intracellular enzyme expressed in various subsets of dendritic cells and its main function is the degradation of tryptophan, an essential amino acid for the survival of T-cells. Extensive research has convincingly shown that IDO represents one of the major characteristics of what are now called tolerogenic dendritic cells.

Indeed, dendritic cells constitute a heterogeneous group of cells with multiple subsets that were long considered to orchestrate the initiation of an immune response. However, a major progress in our understanding of DC biology during the last years is the discovery that DCs are able, under different circumstances, to induce either immunity or tolerance in the periphery. While a unique tolerogenic "signature" for DCs has not yet been established and is not restricted to one specific DC subset, it is generally accepted that an immature state, high net expression of co-inhibitory molecules and production of tolerogenic enzymes, such as IDO, are important, though not the sole, prerequisites for a tolerogenic DC phenotype[74]. In most circumstances, the way in which these DCs promote induction of tolerance is by enhancing regulatory T-cell development. In this regard, both major subsets of human and rodent DCs, plasmacytoid (pDCs) and myeloid DCs (mDCs), are able to promote IDO expression under distinct circumstances. Different ligands acting on pDCs, such as TLR 9 ligands, ligands of CD200 receptor, CD40 ligand and glucocorticoid-inducible TNF receptor-related protein (GITR) ligand, may all contribute to IDO-mediated Treg generation by murine pDCs (reviewed in [75]). Moreover, human pDCs require IDO to drive Treg cell development[76], while a recent study from our group showed that in patients with RA responding to anti-TNF therapy, pDCs are able to induce interleukin-10–

producing Treg cells in an IDO dependent manner[77]. It is now clear that tryptophan catabolism by IDO is a major mechanism employed by DCs in the quest for peripheral tolerance Induction.

The first evidence that CTLA-4 may promote IDO induction in DCs came from studies in murine transplantation, which showed that CTLA-4Ig treatment of DCs induced the expression and activation of IDO both *in vitro* and *in vivo*, leading to immunosuppression and long-term transplant survival [78]. A subsequent study identified certain subsets of murine DCs (namely CD8 DCs, pDCs and NK DCs) that are capable of IDO upregulation upon CTLA4-Ig treatment [79]. These observations were extremely important, since they introduced the concept of "reverse signaling" of CTLA-4 inside APCs and the implication that receptors and ligands may sometimes change roles. Moreover, through back signaling inside DCs, soluble CTLA-4 may promote tolerogenic properties in DCs in a T-cell independent manner. This potential contribution of CTLA4-Ig in the development of tolerogenic dendritic cells could aid in the quest for tolerance induction in autoimmunity and transplantation, through the application of "cell-based" therapy.

In contrast to the studies in rodents, the role of CTLA-4Ig in reverse signaling through CD80/86 in human DCs remains controversial. Consecutive studies using human monocytes-derived DCs (moDCs) yielded conflicting results. Initially, Mellor et al showed that treatment of human moDCs with CTLA4-Ig upregulates IDO expression, however in the subsequent study of Terness et al, the same DCs were not found to express IDO [80]. Similarly, a recent study failed to demonstrate induction of IDO with abatacept treatment or the ability of 1-methyltryptophan (1-MT, a selective inhibitor of IDO) to reverse the abatacept-mediated inhibition of T-cell proliferation [81]. The latter study is important because the authors used abatacept in their experiments, in contrast to the previous studies which utilized other forms of commercially available CTLA4-Ig bearing subtle differences in the Fc portion of IgG with effects on effector function.

Taken together, the above data indicate that in humans the clinical relevance of IDO upregulation for the mode of action of abatacept remains debatable. Additional studies are needed to provide more convincing answers for the potential tolerogenic effects of this molecule in human settings.

2. OBJECTIVES

In an attempt to add more information concerning the mechanism of action of abatacept in RA, the aim of the present study was to investigate the ability of abatacept to influence the tolerogenic properties of human DCs both in healthy subjects as well as in RA settings. Specifically, we examined whether abatacept can induce DCs with a tolerogenic phenotype. To validate this hypothesis, experiments were performed, based on the following specific aims:

Specific aim 1

Investigate the ability of abatacept-treated DCs of healthy subjects and RA patients to block the proliferation of naïve $CD4^+CD25^-$ T-cells

To address the first objective, monocyte-derived DCs (moDCs) were generated from peripheral blood mononuclear cells (PBMCs) of healthy volunteers and RA patients. Differentiated mo-DCs were matured with recombinant human CD40L, either i) in the presence or ii) in the absence of abatacept for 36 hours. *In vitro* generated mo-DCs were used due to the capability of their generation in sufficient numbers for functional experiments, since *in vivo* DCs (pDCs or myeloid DCs, mDCs) are isolated in only small numbers from peripheral blood.

Mature abatacept-pretreated moDCs were co-cultured with CFSE-labeled allogeneic naïve $CD4^+$ T-cells in a mixed leukocyte reaction (MLR). The activation and proliferation of T cells was determined based on surface CD69 expression (an early activation marker of T-cells) and CFSE dilution by flow cytometry, respectively. MoDCs matured in the absence of abatacept were used in co-culture with T-cells as a positive control (strong proliferation), while the same moDCs will be co-cultured with T-cells in the additional presence of 100 μg/ml abatacept as a negative control (weak proliferation). If pretreatment of DCs with abatacept could halt the proliferation of T-cells, it would be attributed to an acquired property of these DCs. Additionally, culture supernatants were collected in order to be assessed for the

presence of different cytokines, characteristic for distinct T-cell polarization (IL-10, IFN-γ).

Specific aim 2

Delineate the mechanism by which abatacept induces tolerogenic DCs

The latter aim was addressed by the assessment of the expression of both intracellular molecules, such as IDO, as well as surface molecules on DCs that could confer regulatory-inhibitory properties. A recent study showed that human pDCs are able to polarize naïve T-cells in the regulatory direction (Tregs) by upregulation of the molecule inducible costimulator ligand, ICOS-L[82]. Another candidate molecule examined was the member of the B7 family of molecules programmed death ligand-1(PD-L1), which is also present on human DCs and binds to programmed death-1 (PD-1), another inhibitory receptor on the surface of T-cells[4].

To address the second objective, RNA was extracted by moDCs matured in the presence or absence of abatacept, as well as from the same moDCs before receiving the maturation stimulus. Both RA patients and healthy controls were evaluated. IDO expression was quantified by quantitative real-time PCR.

Concomitantly with IDO evaluation, DCs were examined by flow cytometry to assess the effect of abatacept on the expression of ICOS-L and PD-L1 on their surface, along with common markers of activation (HLA-DR, CD80, CD86). Potential upregulation of either ICOS-L or PD-L molecules by abatacept pretreatment would explain the tolerogenic “shift” of these cells.

3. MATERIALS AND METHODS

3.1 Media and reagents

The following materials were used:

- Fluorescent antibodies for flow cytometry:
  - CD45–phycoerythrincyanine-5 (PC-5)(clone J33), CD69–phycoerythrin (PE)(clone TP1.55.3), and CD4–PC-5 (clone 13B8.2): Beckman-Coulter, Miami, FL
  - CD11c-FITC (clone 3.9), programmed death ligand 1 (PDL-1)–PE (clone MIH1), inducible costimulator ligand (ICOS-L)–PE (clone MIH12), CD80-PE (B7-1, clone 2D10), CD86-PE (B7-2, clone IT2.2) and HLA–DR–PE (clone LN3): eBioscience, San Diego, CA
  - CD25-FITC (clone M-A251) and CD62L-FITC (clone Dreg-56): BD Biosciences PharMingen, San Diego, CA
- CFSE proliferation kit: Invitrogen, Eugene, OR
- RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 g/ml), and 2 m*M* L-glutamine for cell cultures: all from Gibco, Carlsbad, CA
- Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF): Peprotech, London, UK
- Recombinant human IL-4: :
- Anti-human CD40L: Pepro Tech
- Anti-human purified CD3 (OKT-3 clone): eBioscience
- Soluble anti-human purified CD28 (clone CD28.3): eBioscience
- Ficoll-Histopaque: Sigma Aldrich
- CD14 magnetic microbeads: Miltenyi Biotec
- CD4 and CD25 magnetic microbeads: Miltenyi Biotec
- Human IFN-γ and IL-10 ELISA kits: eBioscience
- TRIzol reagent: Sigma-Aldrich
- RNeasy Mini Kit: Qiagen, Chatsworth, CA
- ThermoScript Reverse Transcriptase: Invitrogen
- SYBR Green Master Mix: Bio-Rad, Richmond, CA
- Abatacept: Bristol-Myers Squibb, New York, NY (Orencia©)

3.2 Cell isolation

Peripheral blood mononuclear cells (PBMCs) from RA patients or healthy volunteers were isolated by Ficoll-Histopaque density-gradient centrifugation and aspiration of buffy coats in heparinized venous blood aspirates, immediately after peripheral blood draw.

For monocyte isolation, PBMCs from RA patients or healthy volunteers were magnetically separated using CD14 magnetic microbeads, following the manufacturer's instructions. Briefly, PBMCs were incubated with CD14 beads for 15 minutes in 4^{o} C , washed with PBS and loaded on a magnetic column to positively select all $CD14^{+}$ cells (monocytes).

For naïve $CD4^{+}CD25^{-}$ T-cell isolation, PBMCs from healthy volunteers were magnetically separated using the $CD4^{+}CD25^{+}$ regulatory T-cell isolation kit, following the manufacturer's instructions. Initially, PBMCs were incubated with a $CD4^{+}$ biotin antibody cocktail (10 min, 4^{o}C) and afterwards with magnetic anti-biotin beads (15min, 4^{o}C), washed with PBS and then loaded on a magnetic column to negatively select all $CD4^{+}$ T-cells. $CD4^{+}$ T-cells were subsequently incubated with CD25 magnetic microbeads (15min, 4^{o}C), washed and loaded on a column for negative selection of CD25- T-cells. To participate in the MLR, $CD4^{+}CD25^{-}$ T-cells were subsequently resuspended in CFSE labeling buffer (PBS + 0,1% BSA) and labeled with CFSE 2μM for 10 min in 37^{o}C.

3.3 Generation of monocyte-derived dendritic cell (moDCs)

To generate moDCs, CD14+ monocytes were cultured in 6-well flat-bottomed plates, in the presence of 50 ng/ml IL-4 and 50 ng/ml GM-CSF, for 7 days. On day 3 and day 6, half of the medium was changed and replaced with fresh medium containing the same concentration of cytokines. On day 7, cells were gently harvested. For maturation, cells were subsequently incubated in 96-well flat-bottomed plates for 24 hours in the presence of 1 μg/ml CD40L, either with or without the concomitant administration of 100 μg/ml abatacept.

3.4 Flow cytometry analysis for surface markers of moDCs

For flow cytometry analysis, moDCs from healthy volunteers and RA patients were extracellularly stained for the following markers: HLA-DR, CD80 (B7-1), CD86 (B7-2), ICOS-L and the coinhibitory molecule PD-L1. Three different conditions were examined: i) moDCs after their differentiation from monocytes, but before maturation with CD40L, ii) moDCs matured with CD40L and iii) moDCs matured with CD40L in the presence of abatacept.

All stainings with the above fluorescent antibodies were conducted in phosphate buffered saline (PBS)/5% fetal calf serum for 20 minutes at 4^{o}C. Matched IgG isotypes were used as negative controls. After incubation, cells were washed and flow cytometry was performed with an Epics Elite model flow cytometer (Beckman-Coulter). Data were analyzed using the WinMDI software.

3.5 MLR experiments

For MLR experiments, 10^4 mature moDCs from healthy volunteers or RA patients were cocultured with $2x10^4$ allogeneic CFSE-labeled $CD25^-$ T cells in round-bottomed 96-well plates for 7 days, in the presence of 50 ng/ml soluble anti-human CD3. On day 7, cells were harvested and the activation and proliferation of T cells were determined based on surface CD69 expression (an early activation marker of T-cells) and CFSE dilution by flow cytometry, respectively .

Three different conditions were used in the MLRs, as mentioned in the Objectives section: i) MLR of moDCs with $CD25^-$ T-cells, ii) MLR of moDCs with $CD25^-$ T-cells, in the additional presence of 100 μg/ml abatacept and iii) MLR of abatacept-pretreated moDCs with $CD25^-$ T-cells.

On day 7, culture supernatants were also collected and stored in -80^{o}C for cytokine detection (see below).

3.6 Quantification of IDO expression by real-time PCR

moDCs (2 x 10^5) isolated from five RA patients and two healthy controls were resuspended in TRI reagent and total RNA extraction was performed using the RNeasy Mini Kit. RNA was then reverse-transcribed into cDNA using ThermoScript Reverse Transcriptase. cDNA was stored at -20° C until further use.

Quantitative expression of IDO enzyme by moDCs was determined by real time-PCR using gene-specific primers for the human gene IDO: 5'-CATGCTGCTCAGTTCCTCCAG-3' (forward), 5'-CAGAGCTTTCACACAGGCGTC-3' (reverse) with the use of GAPDH as a reference gene (gene-specific primers for GAPDH: 5'-CATGTTCCAATATGATTCCACC-3' (forward) and 5'-GATGGGATTTCCATTGATGAC-3' (reverse)). PCR was run with 5 μl undiluted cDNA template in 20μl reactions in duplicate on an Prism 7000 SDS (Applied Biosystems) using 1x iTaq SYBR Green master mix (Biorad) and IDO primers at a concentration of 0.4μM or 0.25μM. The temperature profile consisted of an initial 95° C step for 10 minutes (for Taq activation), followed by 40 cycles of 95° C for 15 sec and 56° C for 1 min. Standard curve was determined by serially diluting a control cDNA sample. Relative quantification method using a standard curve was used to evaluate the expression of IDO gene after normalization to the reference gene GAPDH.

3.7 Cytokine determination by enzyme linked immunosorbent assay (ELISA)

Culture supernatants of the aforementioned MLR experiments were assessed for measurement of IFN-γ and IL-10 production, using the respective human cytokine ELISA kits, following the manufacturer's instructions. Briefly, a sandwich ELISA was performed. A standard curve was constructed using serial dilutions of a sample of known concentration. Each dilution of recombinant standard or sample was assayed in duplicate .Supernatants were initially incubated for two hours in 96-well plates pre-coated with antibody to each respective cytokine (100 μl/well). After washing five times, a biotinylated detection antibody was added to each plate for one hour incubation. After another five washes, avidin – HRP was added in the plates as enzyme-conjugated secondary antibody and plates were incubated for 30 minutes . After a total of seven more washes, 100 μl/well of substrate solution was added to

each well and the plate was sealed and incubated at room temperature for 15 minutes. The blue colour produced by enzymatic activity was converted into yellow by adding 50 µl of stop solution to each well. The procedure was completed by reading the plate at 450 nm in an ELISA microplate reader (Biorad). The detection limits were 15,65pg/ml for IFN-γ and 16,25pg/ml for IL-10.

Principles of sandwich ELISA are shown in Fig.4.

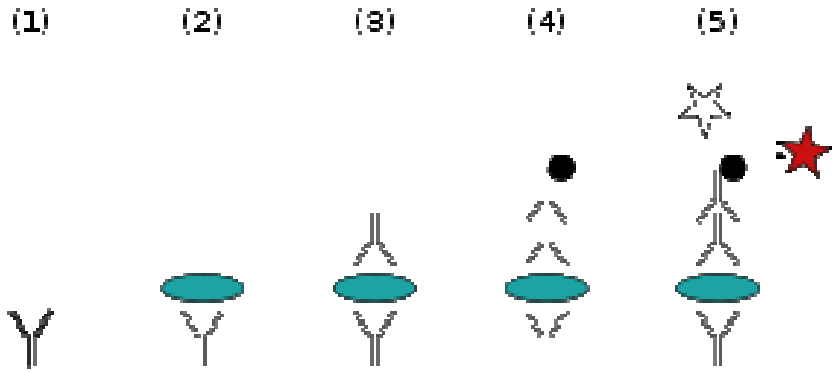
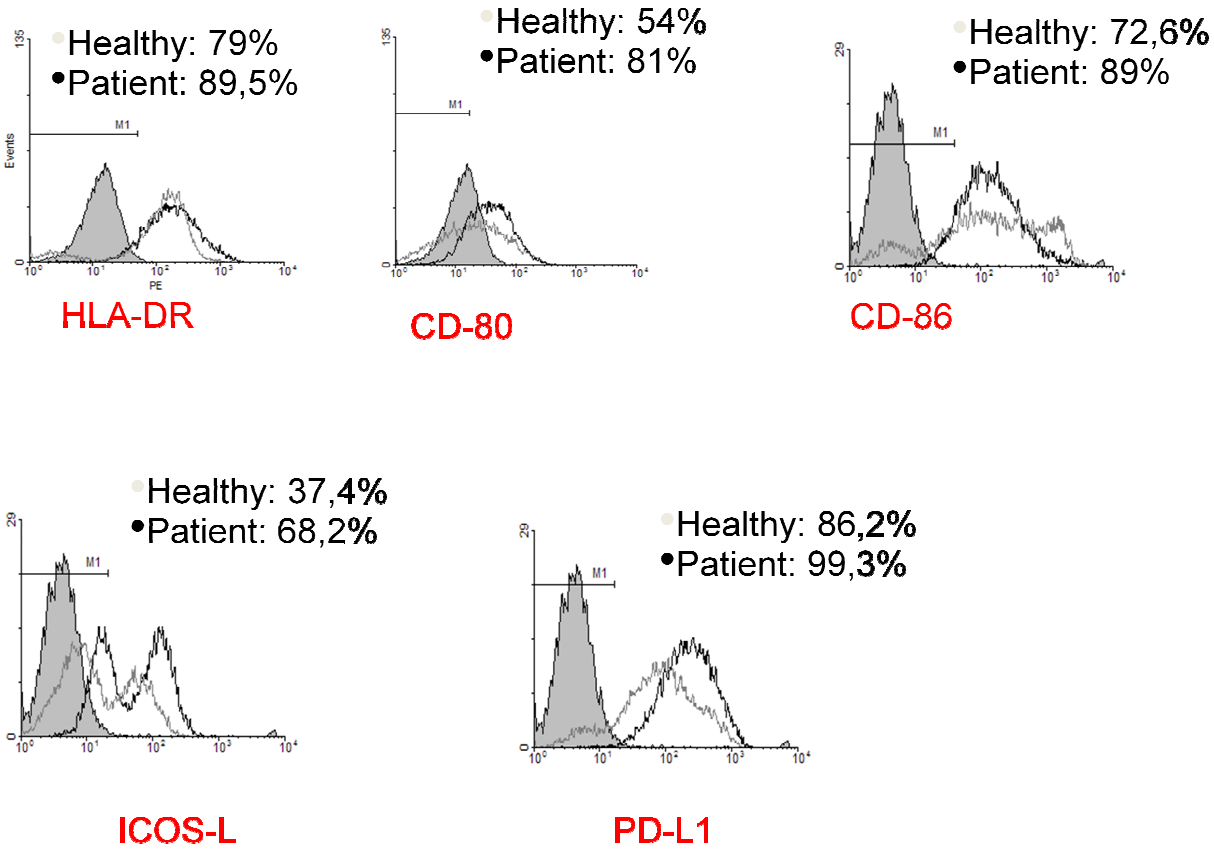


Fig.4 A sandwich *ELISA*. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.

4. RESULTS

4.1 Phenotype of mature human moDCs in healthy subjects and RA patients

Initially, we evaluated *in vitro* generated moDCs from healthy volunteers and RA patients receiving abatacept in the clinic for baseline surface expression of common markers, such as HLA-DR, CD80 and CD86, as well as inhibitory molecules ICOS-L and PD-L1, after their maturation with CD40 and without any abatacept treatment. As expected and shown in **Fig. 5**, mature moDCs universally expressed high levels of common activation markers, HLA-DR, CD80 and CD86, with CD80 showing a somewhat lower expression in some, but not all, healthy compared to patients. Similarly, PD-L1 was also highly expressed on mature moDCs of both groups (more than 80% positivity). Interestingly, there were two cell populations concerning ICOS-L surface expression in both healthy and patients (Fig.5, ICOS-L), with the latter generally showing higher expression than the former.



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4.2 Protocol of MLR

To test our hypothesis that abatacept may promote a "tolerogenic shift" in human DCs, MLRs were set up using three different conditions: the expected strong proliferation of naïve CD25- T-cells, when cocultured with mature DCs in the presence of anti-CD3, was used as a positive control. Conversely, addition of abatacept in the previous reaction was expected to halt the proliferation of responder T-cells (even if the sole mechanism would be antagonism with CD28 for B7 molecules), and this condition provided the negative control of the experiment. Finally, the potential acquired tolerogenic DC phenotype was assessed by performing an MLR with DCs that were pretreated with abatacept concomitantly with their maturation stimulus.

In **Fig. 6** the protocol of the MLRs is shown:

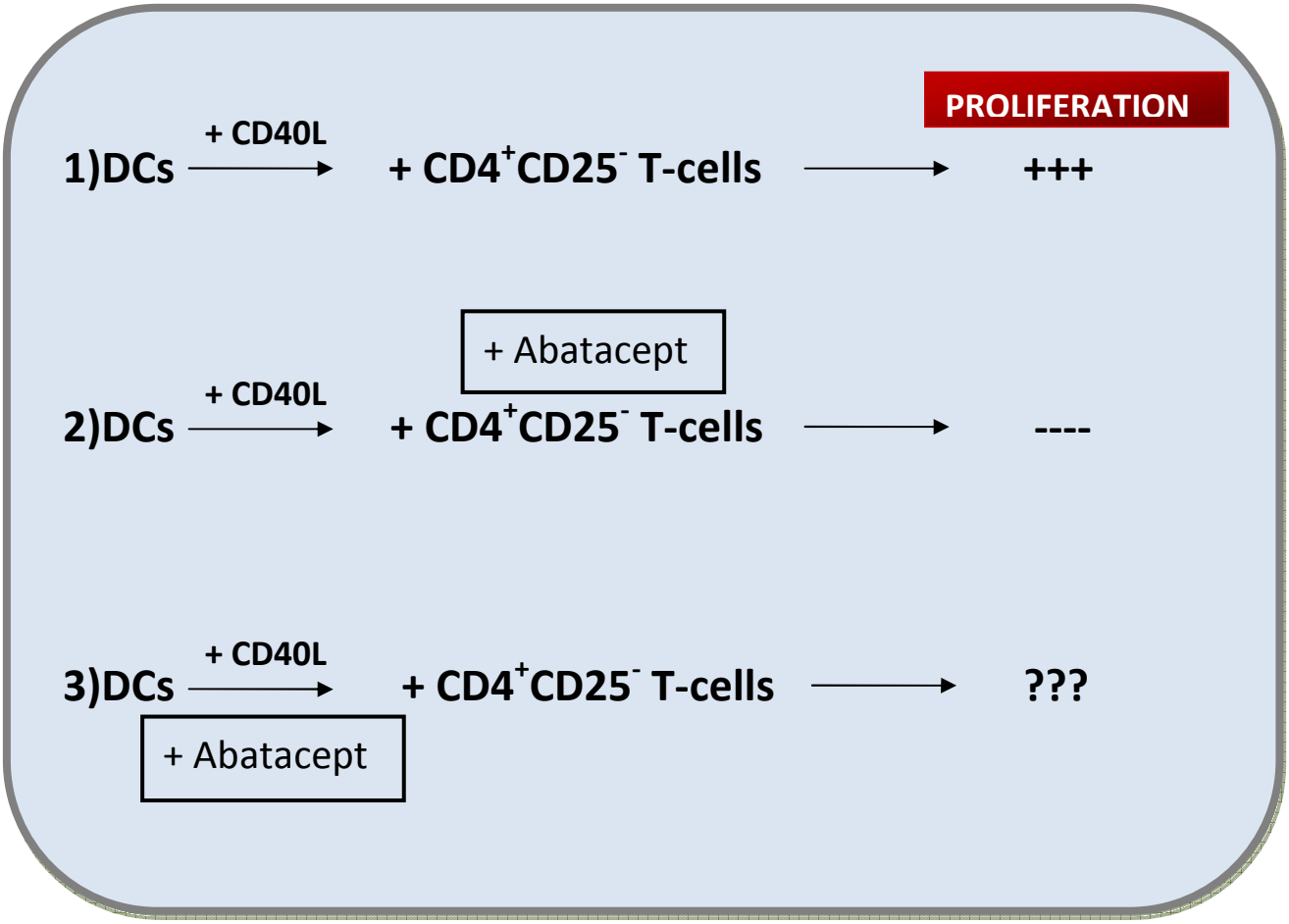


Fig.6 MLR protocol.

4.3 DC-driven proliferation, activation and cytokine secretion of CD25⁻ T-cells

As expected, coculture of DCs with allogeneic responder T-cells in the presence of agonist CD3 antibody resulted in robust proliferation of T-cells by day 7 of culture (**Fig7a**). Activation of T-cells was also assessed based on CD69 expression, an early T-cell activation marker (**Fig. 7b**). Finally, measurement of IFN-γ secretion from culture supernatants showed production of significant amounts of this cytokine, consistent with the enhanced activation of T-cells (**Fig. 7c**).

Results were similar for healthy subjects and RA patients. In Fig.7 an MLR from a healthy subject is depicted.

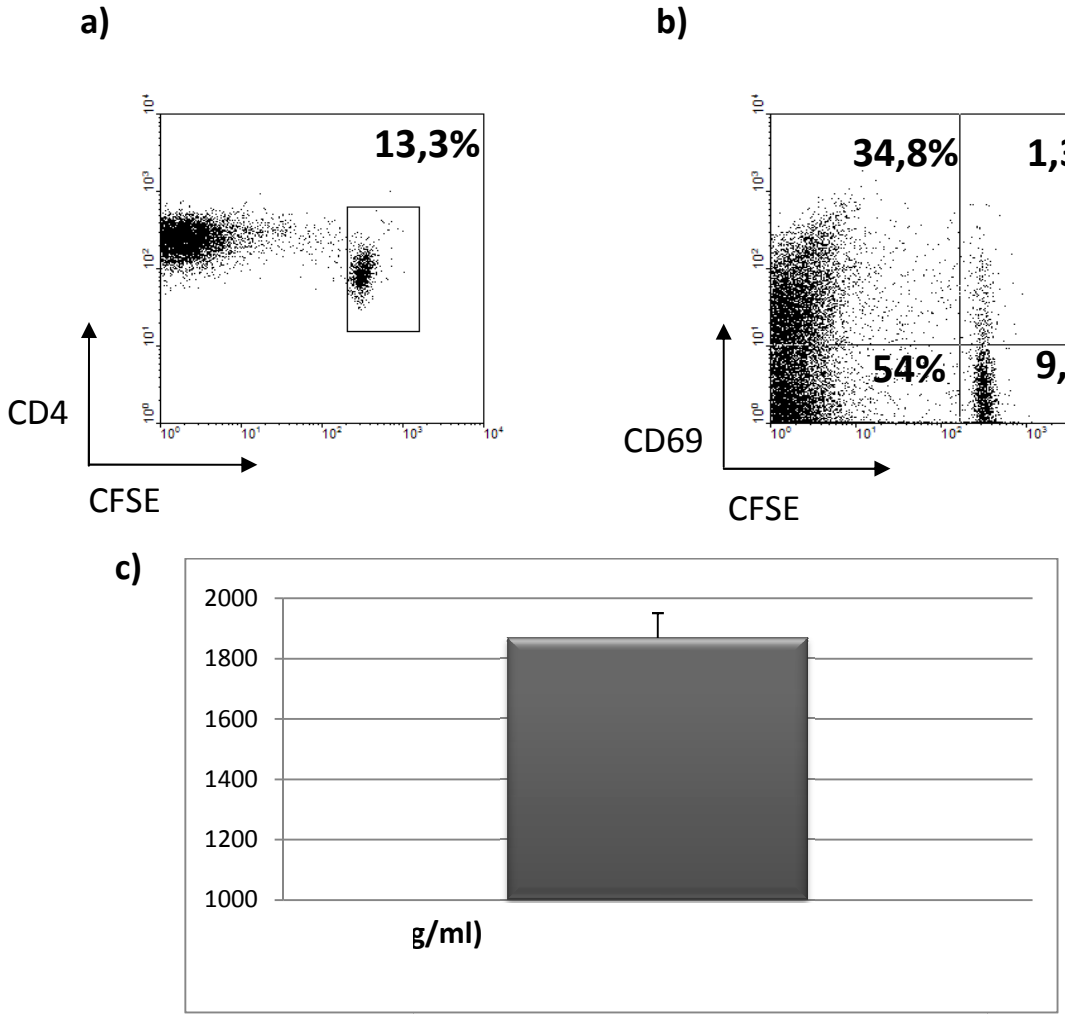


Fig.7 DC-driven proliferation, activation and IFN-γ secretion of CD25- T-cells. Mature moDCs were cocultured with allogeneic CFSE-labeled CD25- T-cells in the presence of soluble human aCD3. On day 7 a) T-cell proliferation was measured based on CFSE dilution b) T-cell activation was assessed by surface expression of CD69 and c) culture supernatants were harvested and examined for production of IFN-γ.

4.4 Abatacept strongly inhibits DC-driven T-cell proliferation and cytokine secretion

Addition of 100 μg/ml of abatacept in the previous MLR conditions resulted in marked reduction in T-cell proliferation (**Fig.8a**), activation (**Fig. 8b**) and IFN-γ secretion (**Fig.8c**) by day 7 of culture, as expected from competition of CTLA-4 with CD28 for B7 ligands. As above, there were no significant differences between experiments with moDCs generated from healhy volunteers and those from patients with RA. In Fig.8 results from a healthy subject are shown.

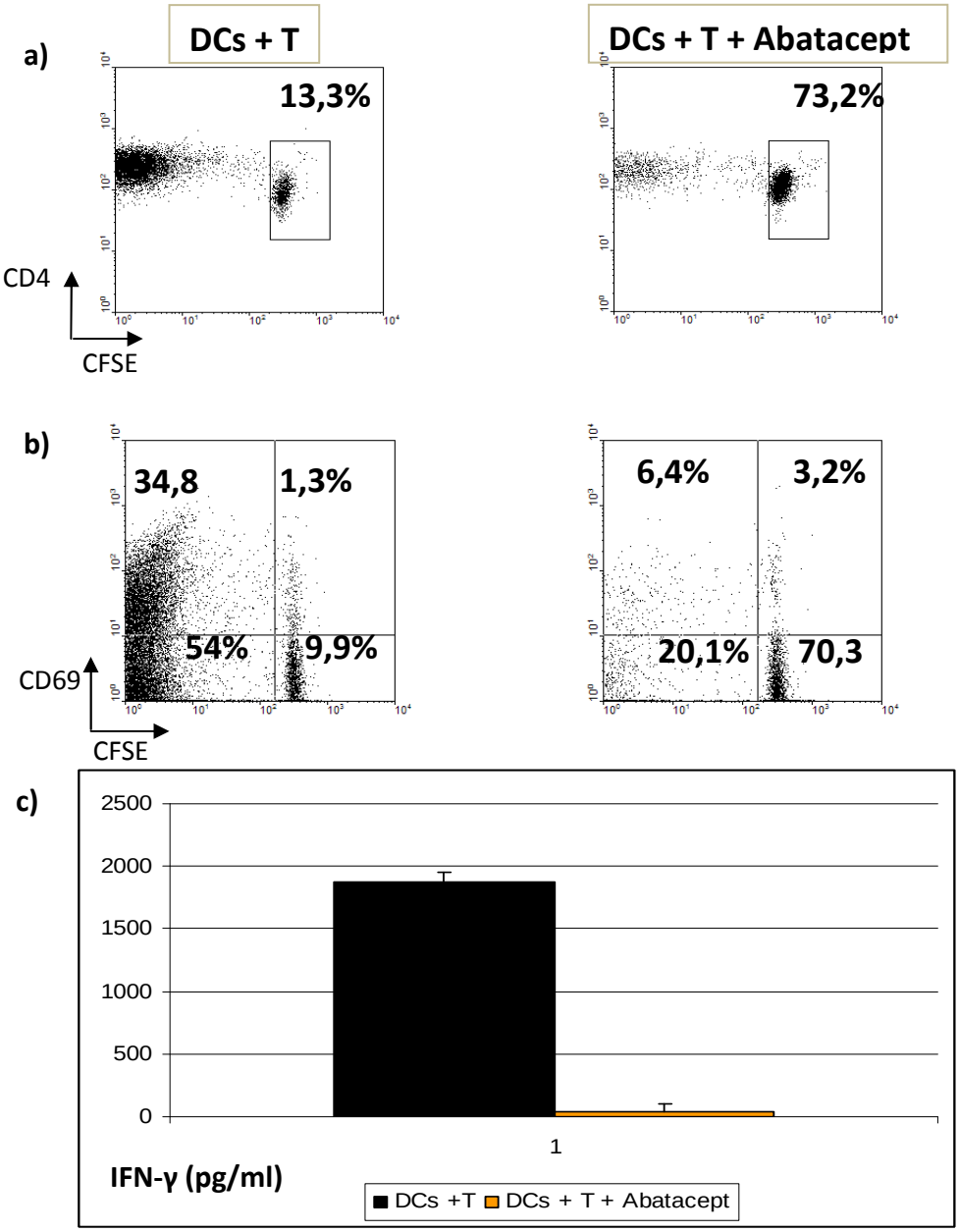


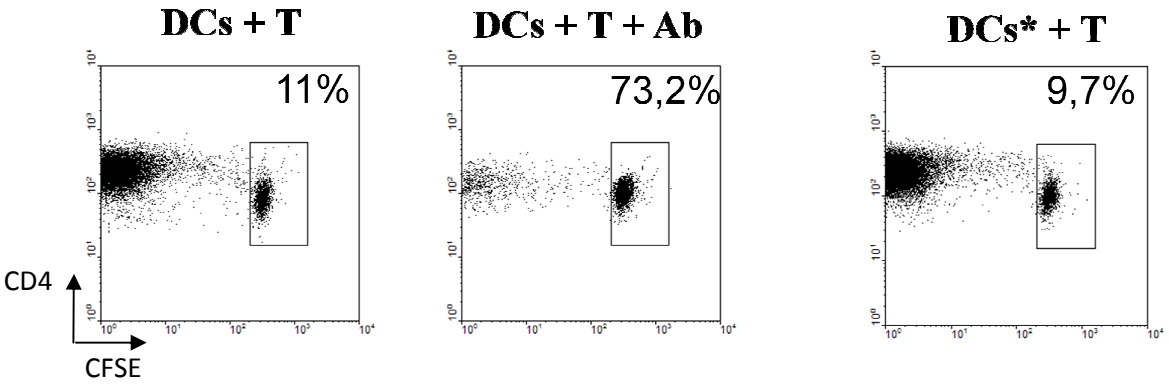
Fig.8 Abatacept blocks DC-driven proliferation, activation and IFN-γ secretion of CD25$^-$ T-cells. Mature moDCs were cocultured with allogeneic CFSE-labeled CD25- T-cells in the presence of soluble human aCD3, with or without abatacept (100 μg/ml). On day 7 a) T-cell proliferation was measured based on CFSE dilution b) T-cell activation was assessed by surface expression of CD69 and c) culture supernatants were harvested and examined for production of IFN-γ.

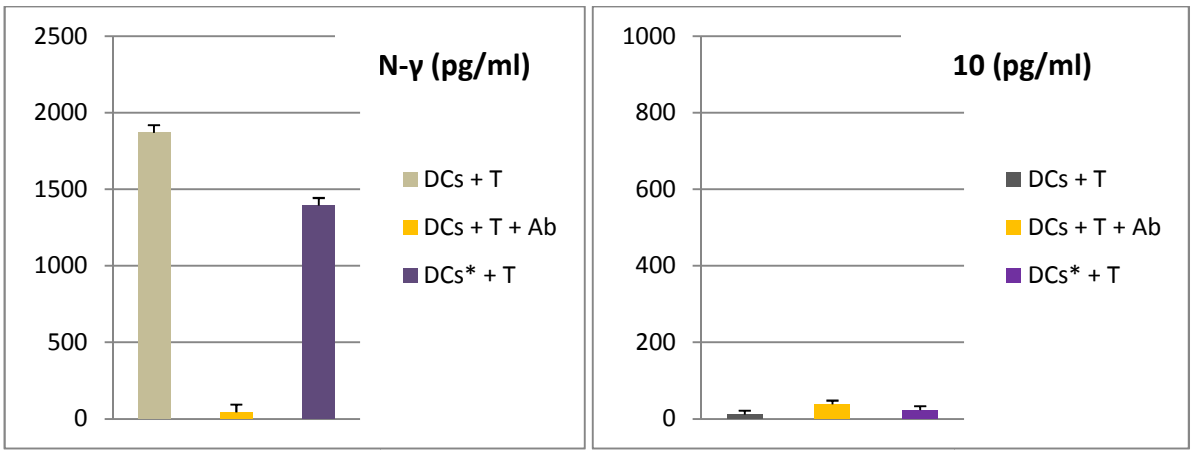
4.5 Abatacept-pretreated DCs of healthy subjects and RA patients fail to suppress T-cell proliferation and cytokine secretion

Considering the main question of this set of experiments, pretreatment of moDCs with 100 µg/ml of abatacept was not able to halt the proliferation, activation and cytokine secretion of responder T-cells, when the latter were cocultured with the pretreated moDCs for 7days. **Fig. 9a and b** depict results from healthy individuals and RA patients, respectively. In some patients, pretreatment led to a minor reduction in proliferation (around 10%, Fig.9b), however this finding was not universal.

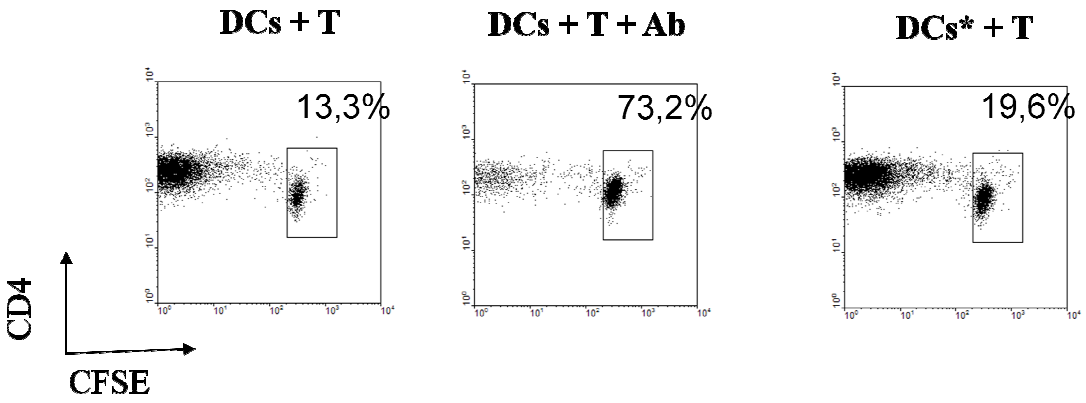
Together with IFN-γ, IL-10 was also mesured in culture supernatants at 7 days. As shown in figures 9a and b, only minor amounts of IL-10 were secreted in all conditions.

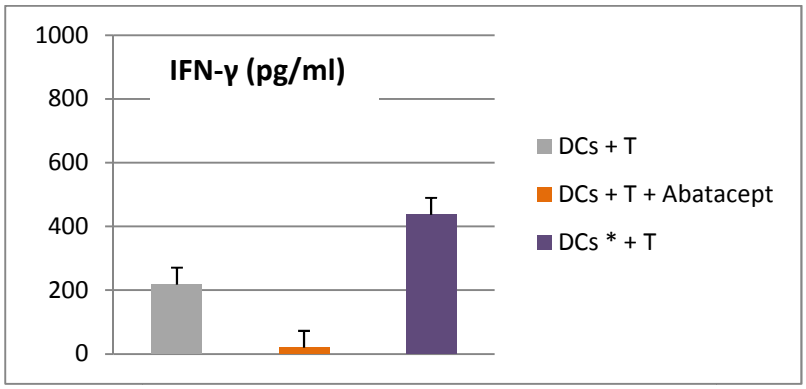
a)

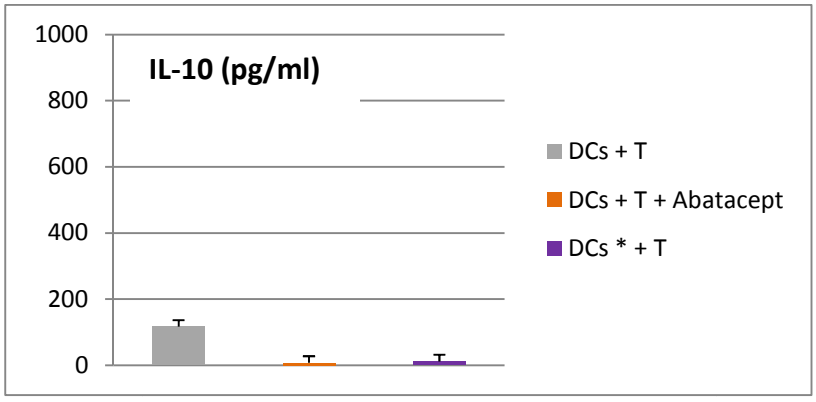




b)







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4.6 Abatacept does not induce IDO production in human DCs

We attempted to determine if abatacept, by binding to CD80/86 on APCs, could trigger IDO activation, which could contribute to the inhibitory effects of abatacept on T-cell proliferation. As shown in **Fig.10**, treatment of moDCs with abatacept for 24 hours (during their maturation with CD40L) did not result in upregulation of IDO mRNA expression compared to no treatment. Instead, in both healthy individuals and patients with RA, moDCs treated with abatacept exhibited a slight reduction in the amount of IDO produced.

There were significant variations between healthy and patients, as well as inbetween patients, considering the magnitude of IDO fold induction relative to the reference gene. However, in all circumstances, treatment with abatacept resulted to less IDO induction compared to no treatment.

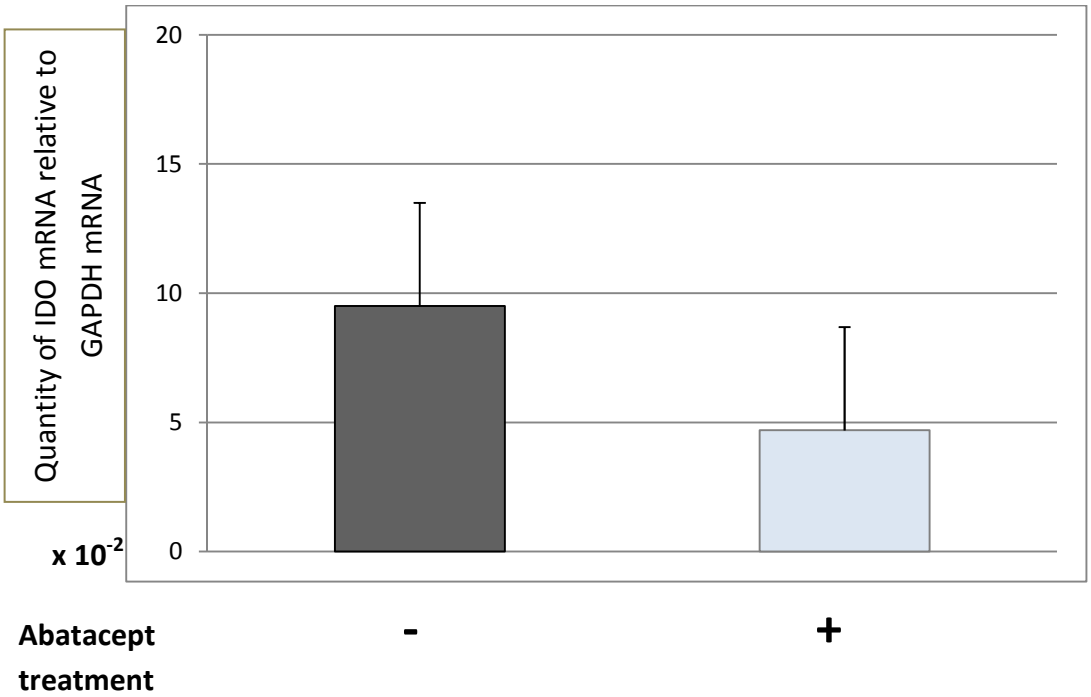
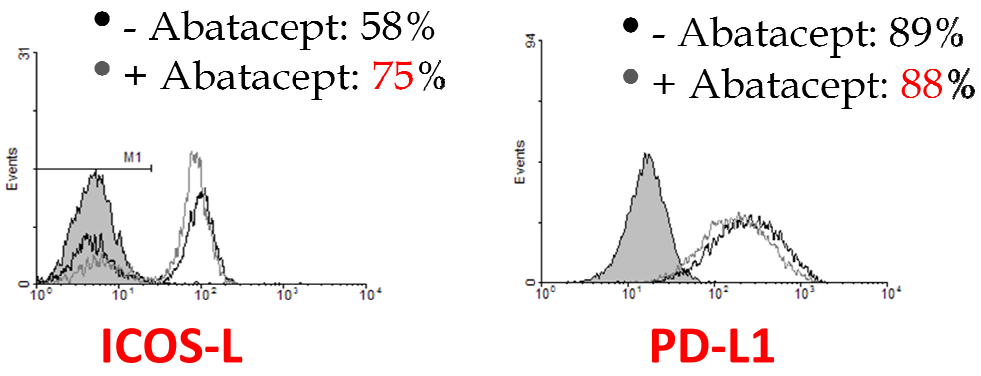


Fig. 10 Abatacept does not induce IDO production in human DCs. IDO mRNA expression on moDCs after abatacept treatment for 24 hours was compared to no treatment. A total of two healthy and 5 patients were tested. In the figure**,** results from two patients are shown, due to significant interindividual differences in IDO fold induction. IDO-specific mRNA levels were quantified by quantitative reverse transcription–polymerase chain reaction and normalized to GADPH mRNA levels. Bars show the mean and SD results.

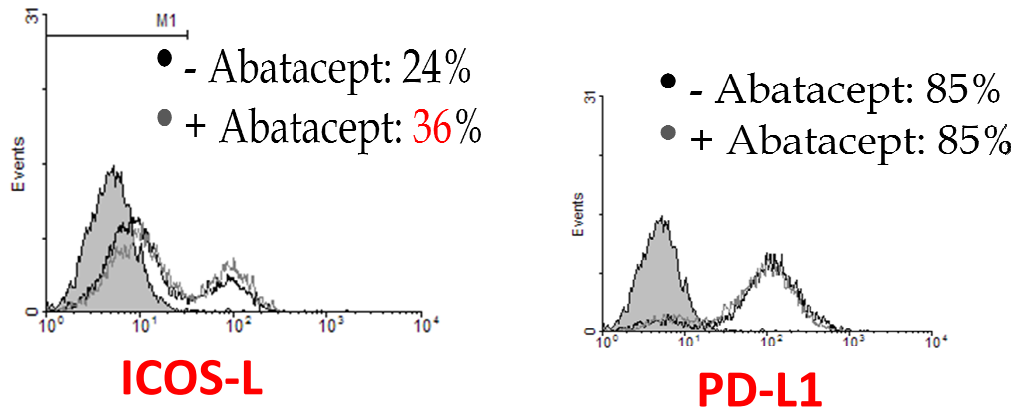
4.7 Effect of abatacept on the expression of surface markers of moDCs in healthy subjects and RA patients

We concomittantly tested whether abatacept has any effect on the expression of surface molecules of human DCs, such as ICOS-L and PD-L1, that could potentially confer tolerogenic potential to these DCs. While PD-L1 remained essentially unaltered following abatacept treatment, expression of ICOS-L was partially upregulated by abatacept in some, but not all, of both healthy and RA patients (**Fig. 11**). In either case however, these modest alterations were not accompanied by functional consequences on DCs, as shown by the inability of abatacept-treated DCs to halt the proliferation of allogeneic T-cells. Expression of oher common DC markers (HLA-DR, CD80 and CD86) were also not significantly influenced by abatacept treatment (data not shown).

A) RA patient



B) Healthy



S-L and PD-L1 in a
3) RA patients were
) and subsequently
ctivation markers).

5. DISCUSSION

Although abatacept was originally developed as a soluble antagonist of CD28 and its main mode of action is considered to be the competition with CD28 for binding to B7 molecules, recent data have questioned this single unifunctional mechanism and proposed additional roles for this T-cell costimulation modulator. Our study attempted to explore potential effect of abatacept on the function of human DCs.

Indeed, the theory of "reverse signaling" has refined the concepts of cell-cell interactions, claiming that cells expressing ligands may undergo significant functional changes upon ligation with their receptor (bidirectional interaction). In this regard, CTLA4-Ig may have an additional role through manipulation of properties of DCs towards a tolerogenic direction. Studies in mice have shown that CTLA4-Ig can upregulate IDO expression in DCs through induction of IFN-γ[78]. Consistent with this finding, addition of 1-methyltryptophan (1-MT), an IDO antagonist, resulted in reversal of the protective effect of CTlA4-Ig *in vivo* in a model of transplantation[78]. However, in contrast to rodent studies, data from human DCs have revealed conflicting results. While Munn et al initially showed that CTLA4-Ig is able to induce IDO in human moDCs[83], a subsequent study from Terness et al reported that DCs generated under similar experimental conditions were unable to express IDO or suppress T-cell responses[80]. More importantly, a recent study using abatacept and not other commercially available forms of CTLA4-Ig showed that abatacept blocks DC-stimulated T-cell proliferation independently of IDO induction[81]. Importantly, all the aforementioned studies testing potential IDO induction by CTLA4-Ig in human DCs utilized DCs generated by monocytes. Overall, with limited studies in humans, the biological significance of IDO induction by CTLA4-Ig remains debatable.

In the present study we sought to determine whether abatacept can indeed promote IDO expression in human DCs and additionally we explored a potential role for this molecule in influencing DC tolerogenicity independently of IDO, namely through expression of surface receptors like ICOS-L or PD-L1. We tested DCs from both healthy subjects and patients with RA already receiving abatacept *in vivo* to assess potential differences between these two populations. Similarly to the published human studies, we chose to utilize monocyte-derived DCs mainly due to

the capability of generating significant numbers of these cells to perform functional experiments, in contrast to either pDCs or mDCs which are both isolated from human peripheral blood in small numbers.

In agreement to the observations of Davis et al, we were unable to show enhancement of IDO expression upon treatment of mature human moDCs with abatacept both in healthy subjects and in RA settings. On the contrary, DCs treated with abatacept expressed less IDO than untreated ones. Importantly, our study together with the study of Davis et al are the only studies that tested IDO induction using abatacept, ie. the form of CTLA4-Ig used *in vivo* in the clinic, and not some other form of commercially available CTLA4-Fc. It has been suggested that the varying structures of CTLA4-Ig molecules used in different studies (mainly differences in the Ig tail leading to varying binding to Fc receptors) may have functional consequences that make it difficult to draw solid conclusions. In any case, the potential role of IDO induction as an additional mode of action of abatacept *in vivo* requires further clarification.

We also tested whether abatacept has any effect in the expression of several molecules on the surface of APCs, which could influence the ability of these cells to drive allogeneic T-cell proliferation. Ito et al recently reported that human pDCs are able to polarize naïve T-cells in the regulatory direction (Tregs) through upregulation of ICOS-L[82]. Similarly, we sought to examine potential alterations in the expression of such molecules caused by abatacept, namely ICOS-L and PD-L1, along with effects in common activation markers of DCs, such as HLA-DR, CD80, CD86. Our results show that treatment with abatacept for 24 hours has minimal, if any, effect in the surface expression of these receptors, as shown in Figure 7. No significant differences were observed between healthy individuals and patients with RA.

The net result of the above observations is that pretreatment of human moDCs with abatacept failed to halt the robust proliferation of responder T-cells driven by these DCs in a 7-day culture. At least in the case of RA patients, a possible explanation, apart from the obvious that abatacept may actually not promote a tolerogenic phenotype in human DCs, could be that our study lacked patients that are true responders to abatacept treatment *in vivo*, as measured by a reduction in their DAS score. Indeed, while we generated DCs from 10 RA patients, none was considered to have inactive disease (as defined by a DAS score less than 2,4), while

only two had significant reductions in their DAS score from baseline during their abatacept treatment. Given the fact that abatacept seems to require a significant period of time (which could reach up to 6 months) in order to act *in vivo* and produce a measurable clinical response in RA patients, it is possible that any potential tolerogenic effects on DCs would be more likely to be seen in DCs from patients that have responded to abatacept treatment or have otherwise been "tolerized". In this regard, it would be interesting to repeat the same experiments in the near future, when more patients receiving abatacept will have responded to therapy by reduction of their DAS.

Another interesting aspect in the exploration of the mechanism of action of abatacept is its potential effect on Treg generation and development. Due to the fact that Tregs are dependent on CD28 for their development, experts on the field are skeptical for a possible negative impact of abatacept treatment on this T-cell subset[22]. However, in a recent clinical study, renal transplant recipients who received belatacept as an immunosuppressant did no exhibit reduced numbers of Tregs compared to patients who received other immunosuppressive regimens[84]. To add more complexity, recent studies in rodents claim that CTLA4-Ig can conversely lead to Treg development, rather than depletion, both *in vitro* and *in vivo*[85-86], in a poorly defined way. Razmara et al reported that administration of CTLA4-Ig converted murine $CD25^-$ T-cells into Foxp3-expressing $CD25^+$ T-cells with suppressive capacity[86]. In a similar manner, we examined whether abatacept may have a similar effect on human naïve $CD25^-$ T-cells *in vitro*. We were unable to show expansion of $Foxp3^+$ T-cells upon treatment of these naïve T-cells with abatacept in the presence of agonist CD3 antibody (data not shown). However, in our experiments we used low doses of abatacept (10 μg/ml) and it would be interesting to examine titrating doses of the drug in order to draw more solid conclusions regarding the abatacept/Treg relationship.

Overall, our study was unable to demonstrate a significant effect of abatacept on the tolerogenic potential of human DCs. However, due to the limited number of RA patients and the different characteristics between them (disease activity, previous treatments etc.), our results are far from conclusive and need to be validated in a larger number of patients.

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