Διδακτορική Διατριβή

Ο ρόλος των πλασμακυτταροειδών δενδριτικών κυττάρων στην επαγωγή ανοχής σε ασθενείς με ρευματοειδή αρθρίτιδα

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PhD Thesis

The role of plasmacytoid dendritic cells in the induction of tolerance in rheumatoid arthritis patients

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Dedicated to my parents, Ariadni and Giorgo

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Table of Contents

1. Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial inflammation, which is orchestrated by both innate and adaptive immune responses. Major hallmarks of autoimmunity in this disease include the appearance of serum autoantibodies and the persistent activation of self-reactive $CD4^+$ cells. Although therapeutic strategies targeting cytokines and B or T cells have had a major clinical impact, disease in many patients remains refractory to current biologic interventions, and for patients who show a response to therapy, true remission associated with reestablishment of immune tolerance is rare. This has increased the interest in exploring strategies to reestablish immune tolerance and provide longterm disease suppression.

Dendritic cells (DCs) are professional antigenpresenting cells (APCs) that can induce either immunity or tolerance. Myeloid DCs and plasmacytoid DCs represent the 2 major subsets of DCs, with human plasmacytoid DCs defined as CD45+CD123+CD303+CD11c- cells and myeloid DCs defined as CD45+CD1a+CD11c+CD1c+ cells. Although both subsets exhibit a functional plasticity in directing T cell responses, current evidence supports a predominant role of plasmacytoid DCs in the maintenance of tolerance through the expansion/induction of Treg cells. To date, two major subsets of Treg cells have been described: thymus-derived Foxp3+ Treg cells and adaptive interleukin-10 (IL-10)–producing Treg cells (Tr1 cells) generated in the peripheral blood.

The importance of Treg cells in the maintenance of tolerance is highlighted by the development of spontaneous multiorgan autoimmunity following Treg cell deletion in rodents. In humans, reduced numbers of Treg cells and impaired function of Treg cells have been reported in patients with autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus, and multiple sclerosis. The molecular pathways involved in prompting plasmacytoid DCs to promote Treg cell development are not fully understood. Expression of the indoleamine 2,3-dioxygenase (IDO) enzyme by plasmacytoid DCs is thought to play a significant role in plasmacytoid DC–mediated Treg cell induction.

Whether human DCs exploit similar mechanisms of Treg cell induction that might facilitate reestablishment of tolerance in a disease setting remains to be seen. Cytokine-directed therapy may have an impact on Treg cell function. Thus, Treg cells in patients with active RA are functionally defective. Anti–tumor necrosis factor α

(anti-TNFα) therapy reverses this defect, through conversion of naive T cells into Foxp3+ Treg cells. Whether therapy may also have an impact on DCs, in terms of changing their effects on Treg cells, has not been explored.

Herein, we present findings demonstrating that plasmacytoid DCs isolated from patients with RA whose disease is in remission induce an additional, distinct population of Treg cells, Tr1 regulatory cells, in a manner that is dependent on the presence of IDO. Unlike the previously described anti-TNFα–induced Treg cells, this population of genuine Tr1 cells does not express Foxp3 but, instead, secretes high levels of IL-10. Most notably, these cells suppress the proliferation of autologous naive CD4+ cells, in a dose-dependent manner.

Finally, we studied how IL-21 cytokine effects on the function of pDCs, given that IL-21 is found in high levels at the periphery of autoimmune diseases such as systemic erythematosus lups (SLE). IL-21 is an immunomodulatory cytokine that is implicated in the pathogenesis of autoimmune diseases, by promoting B cells differentiation, or by affecting Th17 and Tregs function. Dendritic cells are potential regulators of immune responses, since mature dendritic cells promote autoimmunity, while immature dendritic cells promote tolerance. Thus, we studied the potential of dendritic cells matured in the presence of IL-21 to affect on the activation and proliferation of naïve T cells.

Our results suggest that dendritic cells matured in the presence of IL-21 significantly promote the proliferation of allogeneic $CD4^{+}CD25^{-}$ T cells, when IL-21 is present during the activation of T cells. This increased proliferation correlates well with high levels of IFN-γ and TNF-α. Together our results highlight the intrinsic ability of pDCs to regulate T cell responses in an autoimmune setting in humans and further support the use of pDCs as a potential therapeutic modality for autoimmune diseases and transplantation.

1. Περίληψη

Η ρευματοειδής αρθρίτιδα (PA) είναι μία χρόνια, φλεγμονώδης αυτοάνοση νόσος, που χαρακτηρίζεται από φλεγμονή των αρθρικών υμένων, η οποία ενορχηστρώνεται τόσο από την φυσική όσο και από την ειδική ανοσία. Βασικά χαρακτηριστικά της αυτοανοσίας στη νόσο αυτή αποτελούν η παρουσία αυτοαντισωμάτων στον ορό και η συνεχής ενεργοποίηση αυτοδραστικών CD4+ Τ κυττάρων. Αν και οι θεραπευτικές μέθοδοι που στοχεύουν κυττοκίνες, Β και Τ κύτταρα έχουν σημαντικό κλινικό αποτέλεσμα, η νόσος σε πολλούς ασθενείς παραμένει ανθεκτική στις τρέχουσες βιολογικές θεραπείες, και ακόμα και σε ασθενείς που ανταποκρίνονται στη θεραπεία, αληθινή ύφεση με αποκατάσταση της ανοσολογικής ανοχής είναι σπάνια. Αυτό έχει αυξήσει το ενδιαφέρον στην μελέτη νέων μεθόδων για την αποκατάσταση της ανοσοανοχής, ώστε να επιτευχθεί μακροχρόνια ύφεση της νόσου.

Τα δενδριτικά κύτταρα (DCs) είναι επαγγελματικά αντιγονοπαρουσιαστικά κύτταρα που μπορούν να επάγουν είτε την ανοσολογική απάντηση είτε την ανοχή. Τα μυελοειδή δενδριτικά κύτταρα (mDCs) και τα πλασμακυτταροειδή δενδριτικά κύτταρα (pDCs) αποτελούν τις δύο κύριες υποκατηγορίες των δενδριτικών κυττάρων. Τα ανθρώπινα πλασμακυτταροειδή δενδριτικά χαρακτηρίζονται ως CD45+CD123+CD303+CD11c- κύτταρα και τα μυελοειδή δενδριτικά ως CD45+CD1a+CD11c+CD1c+ κύτταρα. Αν και τα δύο υποείδη χαρακτηρίζονται από λειτουργική ΄΄πλαστικότητα΄΄ στο να κατευθύνουν τις Τ κυτταρικές απαντήσεις, πρόσφατες μελέτες υποστηρίζουν ότι τα πλασμακυτταροειδή δενδριτικά κύτταρα παίζουν έναν ως επί το πλείστον κυρίαρχο ρόλο στην διατήρηση της ανοσοανοχής, μέσω της διεύρυνσης ή/και της επαγωγής των Τ ρυθμιστικών κυττάρων (Tregs).

Μέχρι σήμερα, δύο κύρια είδη ρυθμιστικών Τ κυττάρων έχουν περιγραφεί: τα Foxp3+ Treg κύτταρα που προέρχονται από τον θύμο, και τα ειδικά Treg κύτταρα που παράγουν IL-10 (Tr1 κύτταρα), τα οποία παράγονται στην περιφέρεια.

Η σημασία των ρυθμιστικών Τ κυττάρων στην διατήρηση της ανοχής επισημαίνεται από την εμφάνιση αυτόματης, πολυοργανικής αυτοανοσίας, μετά από διαγραφή των Tregs σε ποντίκια. Στους ανθρώπους, μειωμένα ποσοστά και μειμένη λειτουργικότητα των Tregs έχουν περιγραφεί σε ασθενείς με αυτοάνοσα νοσήματα, όπως η ρευματοειδής αρθρίτιδα (PA), ο συστηματικός ερυθηματώδης λύκος (ΣΕΛ) και η σκλήρυνση κατά πλάκας. Τα μοριακά σηματοδοτικά μονοπάτια που εμπλέκονται στον τρόπο με τον οποίο τα πλασμακυτταροειδή δενδριτικά κύτταρα επάγουν ρυθμιστικά Τ κύτταρα δεν είναι πλήρως κατανοητά. Η έκφραση του ενζύμου

IDO (2,3 δεοξυγενάση της ινδολεαμίνης) από τα pDCs θεωρείται ότι παίζει σημαντικό ρόλο στην επαγωγή Tregs από τα pDCs.

Εάν τα ανθρώπινα pDCs χρησιμοποιούν παρόμοιους μηχανισμούς επαγωγής ρυθμιστικών κυττάρων που να διευκολύνουν την αποκατάσταση της ανοχής στα πλαίσια ενός νοσήματος παραμένει να αποδειχθεί. Οι θεραπείες που στοχεύουν τις κυττοκίνες πιθανόν να έχουν κάποια επίδραση στην δράση των ρυθμιστικών Τ κυττάρων. Έτσι, τα Tregs σε ασθενείς με ενεργό ρευματοειδή αρθρίτιδα είναι λειτουργικά ελαττωματικά. Η θεραπεία με anti-TNFα παράγοντες αναστρέφει αυτό το έλλειμμα, μέσω μετατροπής άωρων Τ κυττάρων σε Foxp3+ Τ ρυθμιστικά κύτταρα. Εάν η θεραπεία έχει επίπτωση και στα δενδριτικά κύτταρα, τροποποιώντας τις επιδράσεις τους στα ρυθμιστικά Τ κύτταρα, παραμένει να αποδειχθεί. Σε αυτήν την διατριβή, παρουσιάζουμε ευρήματα που συνηγορούν στο γεγονός ότι πλασμακυτταροειδή δενδριτικά κύτταρα που έχουν απομονωθεί από ασθενείς με ρευματοειδή αρθρίτιδα σε ύφεση, επάγουν έναν επιπλέον, διακριτό πληθυσμό ρυθμιστικών κυττάρων, τα Tr1 ρυθμιστικά Τ κύτταρα, και μάλιστα με τρόπο που εξαρτάται από την παρουσία του ενζύμου IDO. Σε αντίθεση με τα έως τώρα περιγραφέντα Tregs που επάγονται από τις anti-TNFα θεραπείες, αυτός ο πληθυσμός των Tr1 ρυθμιστικών κυττάρων δεν εκφράζει τον Foxp3, αλλά εκκρίνει σημαντικά επίπεδα IL-10 κυττοκίνης. Επιπλέον, τα κύτταρα αυτά καταστέλλουν τον πολλαπλασιασμό αυτόλογων άωρων CD4+ Τ κυττάρων, με δοσο-εξαρτώμενο τρόπο.

Τέλος, μελετήσαμε την επίδραση της κυττοκίνης IL-21 στην δράση των pDCs, δεδομένου ότι η IL-21 ανευρίσκεται σε υψηλά επίπεδα σε ασθενείς με αυτοάνοσα νοσήματα, όπως ο συστηματικός ερυθηματώδης λύκος. Η ιντερλευκίνη-21 αποτελεί μία ανοσοτροποποιητική κυττοκίνη που εμπλέκεται στην παθογένεια αυτοάνοσων νοσημάτων, προάγοντας την διαφοροποίηση των Β λεμφοκυττάρων, ή τροποποιώντας την δράση των Th17 και των ρυθμιστικών Τ (Treg) κυττάρων. Τα δενδριτικά κύτταρα (DCs) είναι εν δυνάμει ρυθμιστές των ανοσιακών απαντήσεων, καθώς τα ώριμα κύτταρα προάγουν τις αυτοάνοσες απαντήσεις, ενώ τα άωρα δενδριτικά προάγουν την ανοσοανοχή.

Μελετήθηκε επομένως, η δυναμική των δενδριτικών κυττάρων που έχουν ωριμάσει παρουσία της IL-21 να επιδρούν στην ενεργοποίηση και τον πολλαπλασιασμό των Τ λεμφοκυττάρων. Τα αποτελέσματά μας υποδεικνύουν ότι τα δενδριτικά κύτταρα που ωρίμανσαν παρουσία της IL-21, προάγουν σημαντικά τον πολλαπλασιασμό των αλλογενών $CD4^{+}CD25^{-}$ Τ κυττάρων, όταν η IL-21 είναι παρούσα κατά την διάρκεια

της ενεργοποίησης των Τ κυττάρων. Αυτός ο αυξημένος πολλαπλασιασμός των Τ λεμφοκυττάρων συνάδει με αυξημένη έκκριση IFN-γ και TNF-α. Συνολικά, τα αποτελέσματά μας τονίζουν την ενδογενή ικανότητα των pDCs να ρυθμίζουν τις Τ ανοσιακές απαντήσεις στα πλαίσια ενός αυτοάνοσου νοσήματος στον άνθρωπο, και υποστηρίζουν περαιτέρω την χρήση τους ως θεραπευτικό μέσο για τα αυτοάνοσα νοσήματα και την μεταμόσχευση.

2.Introduction

2.1 Autoimmune diseases

Autoimmune diseases comprise a diverse group of complex clinical disorders that affect approximately 5% of the population in Europe and North America [1-2] . Among the most common autoimmune disorders are rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), type I diabetes mellitus, multiple sclerosis, myasthenia gravis, Crohn's disease, Graves' disease, Hashimoto's thyroiditis.

The autoimmune diseases are characterised by systemic inflammation and symptoms (SLE, rheumatoid arthritis, Sjögren's syndrome) or organ-specific damage (type I diabetes, Graves' disease, Hashimoto's thyroiditis, autoimmune hepatitis).

Autoimmune diseases develop when the body's immune responses are directed against its own tissues. This may happen when self-reactive lymphocytes escape from the immune system's tolerance mechanisms and become activated, resulting to inflammation and tissue destruction. The immune system has evolved mechanisms that protect from invading and self-directed threats. The immunologic unresponsiveness towards self-constituents is called self-tolerance.

Self-tolerance is established and maintained through two fundamental mechanisms: (*a*) elimination of self-reactive cells in the thymus during selection (central tolerance) and (*b*) generation of a variety of peripheral regulatory mechanisms (ignorance, deletion, anergy and suppression by regulatory T cells (Tregs)) to control potentially pathogenic self-reactive cells that escape the thymus (peripheral tolerance). Break-down of the peripheral mechanisms of tolerance could lead to activation of self-reactive cells and the development of autoimmune diseases.

Although the mechanisms by which an autoimmune disorder occurs are not entirely understood, autoimmune responses are thought to result from a combination of genetic and environmental factors.

Genomewide association studies have revealed more than 200 genetic loci associated with one or more autoimmune disorders. In many cases, the precise causal alleles or genes have not been identified. Nevertheless, some loci are associated with the intracellular signaling that drives the activation of T and B cells, signaling by cytokines and cytokine receptors, and pathways that mediate innate immunity and microbial responses.

Considerable evidence also implicates environmental triggers like infection, in the development of autoimmune diseases, such as multiple sclerosis and type 1 diabetes[3].

Mechanisms that could lead from infection to autoimmunity include the release of autoantigens through tissue damage[4], the activation of T cells by superantigens[5] or the induction of inflammatory cytokines by microbial products[6-10]. In addition, 'molecular mimicry', the structural similarity between microbial and self antigens, is proposed to play a key role in inducing autoreactivity[11-15].

2.2 Rheumatoid Arthritis

RA is a complex, debilitating, chronic, systemic autoimmune disease characterised by an immunological, inflammatory and mesenchymal tissue reaction in the synovium accompanied by polyarticular synovitis, ultimately leading to the progressive destruction of articular and periarticular structures. It is one of the most prevalent autoimmune diseases, affecting up to 1% of the adult population in Western countries and causes significant morbidity and disability. RA is usually diagnosed before the age of 60 and is more common in women than men. RA presents with several extra-articular manifestations, including cardiac (pericarditis, atherosclerosis), pulmonary (pleuritis, fibrosis), ocular (episcleritis, scleritis), cutaneous (nodules, vasculitis), neurological (peripheral neuropathy) and haematological (leukopenia, anemia of chronic disease). Rheumatoid arthritis is a systemic disease, characterised by inflammation, which is orchestrated by both innate and adaptive immune responses. Infiltration of cells of the innate and adaptive immune system into the joint space drives the local production of proinflammatory T-helper type 1 and T-helper type 17 cytokines, chemokines, and matrix metalloproteinases by infiltrating monocytes and synovial cells. Proliferation of synovial fibroblasts leads to the formation of pannus tissue, which invades and degrades articular cartilage and subchondral bone. Major hallmarks of autoimmunity in RA include the appearance of serum autoantibodies and the persistent activation of self-reactive $CD4^+$ T cells. In the treatment of RA, several drugs were used, non-steroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs (DMARD) and steroids. When the importance of proinflammatory cytokines in the pathogenesis of RA was established, several biological agents (TNFα antagonists, B cell depleting, CTLA-4 costimulation agent), acting through cytokine inhibition, have been introduced for the treatment of patients with RA.

2.3 Immune response: T cells and DCs

Mature dendritic cells are not only well-recongnized for their role in T cell activation and initiation of antigen-specific immune responses[16-18], but they also induce different types of T cell immune responses depending on the type of original maturation signal. There are two groups of signals that stimulate immature dendritic cells (imDCs) to induce Th1 versus Th2 differentiation[19]. Molecules derived from pathogens, such as lipopolysaccharide (LPS), bacterial CpG DNA and double-stranded viral RNA, as well as T cell signals such as CD40 ligand and IFN-γ, all promote imDCs to produce IL-12 and prime for Th1 responses. By contrast, anti-inflammatory molecules such as IL-10, TGF-β, PGE-2, and corticosteroids, all inhibit imDC maturation and IL-12 production, and induce Th 2 or regulatory T cell responses.

Many dendritic cells reside in and traffic through nonlymphoid peripheral tissues, continuously surveying the environment for invading microorganisms, tissue necrosis and local inflammation[20]. These 'danger' signals induce DC to undergo a maturation process that includes their migration into T cell areas of draining lymph nodes. During infection, DCs in the periphery are triggered by the exposure to microbial agents or inflammatory mediators to increase their expression of major histocompatibility complex (MHC) molecules and costimulatory molecules such as CD80 and CD86. This activates peripheral DCs into a state ready for T cell activation. Exposure to microbial products also modifies DC expression of chemokine receptors and adhesion molecules, causing migration from the periphery to the T cell zone of secondary lymphoid organs[21]. Activated DCs then display pathogen encoded antigens to naïve antigen-specific T cells circulating through secondary lymphoid tissues, which respond and initiate primary T cell immune responses[22-23]. More recently, DCs have been shown to play an important role in regulating peripheral tolerance to self-antigens. Under steady-state conditions, in the absence of microbial stimulation, immature dendritic cells encounter self-antigens (e.g. apoptotic cells), and transport them to secondary lymphoid organs[24-25] A major source of antigens during the steady-state, is apoptotic tissue cells that die during physiologic tissue turnover. Apoptotic cells represent a random source of self-antigens, critical for the maintenance of peripheral tolerance[26]. Cell death by apoptosis is not accompanied by inflammation, and DCs that internalize apoptotic cells do not become activated[27-28]. Antigen-loaded DCs migrate spontaneously to secondary lymph nodes, mature en route, and acquire capacity to stimulate T cells. However, the outcome of T cell stimulation by steady-

state DCs can be apoptosis [29], anergy[30], or the development of regulatory T (Treg) cells[31], depending on the state of maturation of the DCs. Each of these mechanisms results in some form of T cell tolerance[25-26,32]. Thus, unabated DC activation may skew self-antigen presentation from tolerance to autoimmunity.

2.4 Dendritic cells : cDCs and pDCs

Dendritic cells (DCs) are professional antigen-presenting cells, that display an extraordinary capacity to stimulate naive T cells and to initiate primary immune responses. Based on their lineage origin, DCs can be divided in two subsets: conventional DCs (cDCs) that come from myeloid progenitors and plasmacytoid DCs (pDCs) that originate from lymphoid progenitors. This diversity permits the adaptive immune system to mount functionally distinct types of responses.

cDCs, the original DC type identified by Steinman & Cohn[33] as a powerful stimulator of T cell responses, possess dendritic morphology with prominent cytoplasmic veils and protrusions, differentiate and reside in tissues and lymphoid organs in the steady state and turn over rapidly and undergo proliferation in situ. cDCs express a broad range of pattern-recognition receptors; they express high levels of MHC class II and the integrin CD11c (a specific marker in the mouse), but not B220. cDCs have a unique capacity for naive T cell priming. cDCs comprise two main subsets: $CD8^{-}CD11b^{+}$cDCs, also called myeloid, showing high capacity for MHC class II–mediated presentation of exogenous antigen and $CD8^{+}CD103^{+}$cDCs, in tissues, capable of antigen cross-presentation to cytotoxic T cells. Myeloid DCs are found in three compartments: peripheral tissue, secondary lymphoid organ and blood.

The best studied human mDC subsets are those from skin, where three subsets can be identified. The epidermis hosts only Langerhan's cells (LCs) while the dermis displays two mDC subsets, $CD1a^{+}$ DCs and $CD14^{+}$ DCs, as well as macrophages[34-35]. LCs and $CD14^{+}$ DCs differ in their molecular profiles including cytokines they secrete upon activation. While $CD14^{+}$ DCs produce a large set of soluble factors including IL-12, LCs produce only a few cytokines, including IL-15[36]. This distinct cytokine secretion pattern might contribute to the unique biological functions of these DC subsets.

pDCs are a rare cell type constituting only 0.2% to 0.8% of peripheral blood cells in healthy individuals. pDCs are round-shaped cells with a prominent rough endoplasmatic reticulum (ER) resembling that of immunoglobulin-secreting plasma cells (therefore called plasmacytoid)[37]. Human pDCs were first isolated from human blood and tonsils as $CD4^{+}CD123^{+}$ (IL-3 receptor-α chain) cells that lack lineage markers for B cells, T cells, myeloid cells, and natural killer (NK) cells as well as the classical DC-marker CD11c [37-38]. More recently, a number of pDC-specific markers have been identified and have greatly facilitated the isolation of human pDCs. Blood

DC antigen 2 (BDCA-2) is a member of the C-type lectin family of transmembrane glycoproteins that is specific for pDCs[39].However, the use of monoclonal antibody against BDCA-2 for pDC enrichment is limited as BDCA-2 engagement results in inhibition of their functional capability. Other receptors specific for human pDCs include immunoglobulin-like transcript 7 (ILT-7), belonging to the leukocyte immunoglobulin-like receptor gene family[40], and BDCA-4/neuropilin-1, a receptor for members of the semaphorin family[41]. Antibodies to BDCA-4 do not impair pDC function and are now commonly used for enrichment of human pDCs from blood.

In the mouse, pDCs can be identified as $CD11b^{-}CD11c^{low}B220^{+}$ cells[42-44]. Mouse pDCs also react with the GR1 antibody, which recognizes Ly6G on granulocytes and Ly6C expressed by pDCs. Siglec-H (sialic-acid-binding immunoglobulin-like lectin H) and mPDCA-1 (mouse pDC antigen 1) are specific surface markers on mouse pDCs, and antibodies against these markers have been recently developed to deplete pDCs in naive mice[45-46].

A number of findings have suggested that the developmental pathways of pDCs are distinct from cDCs and may be related to the lymphoid lineage rather than the myeloid lineage[47-50]. There is however evidence that pDCs can arise from both common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs)[51]. Furthermore, Flt-3 ligand (Flt-3L) appears to provide the central signal for the development of pDCs through its receptor Flt-3 and transcription factor STAT3 (signal transducer and activator of transcription 3), the same molecules that also drive the development of cDCs[52-55].

However, the recent identification of E2-2, a basic helix-loop-helix transcription factor (E protein) as the essential and specific transcriptional regulator of pDC development has provided strong evidence for a distinct developmental pathway of pDCs[55]. E2-2 directly regulates the synthesis of other transcription factors that are important for pDC development (SpiB) but also regulates expression of specific markers for the pDC lineage (BDCA-2, ILT-7) and genes that control the production of interferon (INF-α regulatory factor 7 [IRF7])[56].

pDCs develop fully in the bone marrow from where they are released into the blood stream. The migration and peripheral distribution of pDCs are significantly different from those described for cDCs. cDCs typically seed peripheral tissues and become resident cells that migrate into T cell-rich areas of lymphoid organs through afferent lymphatics[57-58]. In contrast, pDCs directly migrate from the blood into T cell-rich

areas of lymphoid organs under steady state conditions. This migration occurs through high endothelial venules (HEVs) and appears to be associated with the expression of CD62L and CCR7[59-61]. Under steady state conditions, pDCs are difficult to detect in most peripheral tissues, such as skin, mucosa, and lung[62-63]. However, large numbers of pDCs have been found in peripheral tissues during viral infections, in both mice[64-66] and human[67-68]. Large numbers of pDCs have also been described in tissues of many autoimmune inflammatory conditions: pDCs accumulate in skin lesions of psoriasis[62,69], lupus erythematosus[70-71], in inflamed synovial of rheumatoid arthritis[72-73], in salivary glands of patients with Sjogren's syndrome[74], and in inflamed muscle tissue of dermatomyositis[75]. Furthermore, pDCs are recruited to the brain lesions of patients with multiple sclerosis[76].

Several studies have shown that pDCs efficiently present endogenous antigens, either constitutively expressed[77-78] or derived from viruses infecting the pDCs[79-81]. By contrast, pDCs poorly present exogenous antigens when compared to cDCs, mainly because pDCs are unable to uptake exogenous antigens, by phagocytosis[37] and secondly, because pDCs are unable to accumulate long-lived MHC-II peptide complexes on their surface[79,82]. However, pDCs may internalize certain exogenous antigens, through BDCA-2, Siglec-H, DCIR (dendritic cell immunoreceptor) receptors[83-85].

pDCs are professional type I interferon producing cells. pDCs were found to produce over 95% of type I IFNs by peripheral blood mononuclear cells in response to many viruses. pDCs produce 200 to 1000 times more type I IFNs than any other blood cell type[86]. Human pDCs express almost all the subtypes of type I IFNs, namely IFN-α, IFN-β, IFN-λ, IFN-ω, and IFN-τ[87-88]. Type I IFNs strongly activate immature cDCs to produce IL-12, IL-15, IL-18, and IL-23, cytokines that are not produced by human pDCs themselves[89-90]. Moreover, type I IFNs differentiate monocytes into cDCs, which subsequently induce strong T helper (Th)1-mediated immune responses[89,91-93]. Type I IFNs also increase the ability of mDC to crosspresent antigens to $CD8^+$ T cells[94], promote $CD8^+$ T cell survival and clonal expansion[95], and polarize $CD4^+$ T cells into Th1 cells[96-97]. pDC-derived type I IFNs also stimulate activation of NK cells[98] and differentiation of B cells into antibody-secreting plasma cells[99].

The ability of pDCs to mount a robust type I IFN response is linked to several unique features of pDCs[100]. First, pDCs selectively express TLR7 and TLR9 but not other TLRs. This is in contrast to cDCs, which express TLR1, TLR2, TLR3, TLR4, TLR5,

and TLR8[101-102]. Secondly, pDCs constitutively express high levels of IRF7. This allows the rapid assembly of a multiprotein signal transduction pathway, following TLR7 and TLR9 activation[103]. pDCs also constitutively express high levels of IRF8, which amplifies type I IFN production by pDCs[104]. The third feature that makes pDCs professional IFNs-producing cells is related to their unique ability to retain DNA in early endosomes for extended periods of time, which allows a sustained activation of IRF7 by the MyD88 signal transduction complex with induction of IFNs[105-106].

Upon activation, mature pDCs initiate protective immune responses through the secretion of large amounts of type I IFNs. However, activated pDCs also acquire dendritic morphology and they upregulate expression of major histocompatibility complex (MHC) and T-cell co-stimulatory molecules, which enables them to directly engage and activate naive T cells[37]. Upon activation through TLR7 and TLR9, human pDCs produce IFN-α and TNF-α and differentiate into mature DCs that prime naive $CD4^+$ T cells to produce IFN-γ and IL-10[107]. Human pDCs also mature into DCs in culture with IL-3 or IL-3 plus CD40L[37]. pDCs activated by IL-3 and CD40L do not produce significant amounts of IFN-α but upregulate the costimulatory molecule OX40L, which leads to IL-4- independent priming T cells secreting Th2 cytokines IL-4, IL-5, and IL-10[38,108]. Thus, like classical cDCs, pDCs display functional plasticity with the ability to prime different effector T-cell responses, depending on the type of maturation signals.

Nonactivated, human and mouse pDCs express low to undetectable levels of CD80 and CD86 and are therefore incapable of inducing significant proliferation of naive T cells[37,77,109]. However, nonactivated human pDCs can induce antigen-specific anergy and IL-10 production in $CD4^+$ T-cell clones[110]. Human pDCs activated through both TLR and IL-3-dependent pathways have an intrinsic capacity to prime a population of IL-10-producing T cells with regulatory function[111-114].

pDCs constitutively express high levels of inducible co-stimulator ligand (ICOS-L)[111], which promotes survival, expansion, and IL-10 production of a subset of a $FoxP3^+$ T regulatory cells expressing ICOS[115]. Murine studies have shown that nonactivated pDCs can convert T cell receptor–transgenic T cells into T regulatory cells with the ability to suppress antigen-specific T-cell proliferation mice [65,116-117]. Together these findings suggest a specialized role of nonactivated pDCs in peripheral tolerance, by suppressing inflammatory responses or mediating tolerance by inducing Tregs[63,118-121].

2.5 DCs in autoimmunity

With the recognition of elevated IFN levels as a pathogenesis factor in several autoimmune diseases, the potentially important role of pDCs in autoimmunity has been recognized[122]. The strongest evidence for pDC involvement has been accumulated from the study of two diseases: psoriasis and systemic lupus erythematosus[100,123]. pDCs efficiently sense viral nucleic acids but do not respond to self-nucleic acids released into the extracellular environment by dying host cells. This discrimination appears to be mainly achieved by the intracellular localization of TLR7 and TLR9, which allows recognition of nucleic acids released into endosomal compartments by the endocytosed virus[124]. By contrast, free self-nucleic acids are rapidly degraded in the extracellular environment and do not access TLR7- and TLR9-containing endosomes spontaneously. However, recent evidence suggests that self-nucleic acids are able to enter TLR-containing endosomes when complexed with host-derived factors that are aberrantly expressed in certain autoimmune diseases[125-126].

In psoriasis, early skin lesions are highly infiltrated by activated pDCs, corresponding with decreased numbers of circulating pDCs in the blood[69]. Blocking IFN production by pDCs using anti-BDCA-2 antibody inhibited the development of skin lesions in a xenograft model, providing causal proof of pDC function in the disease[69]. Subsequently, complexes of self-DNA with the antimicrobial peptide LL-37 were identified as the activating stimulus for pDCs[125]. DNA complexed with LL37 is highly resistant to nuclease degradation, enters endosomal compartments of pDCs and triggers high levels of type I IFN production via TLR9 in early endosomes[125]. As a result of LL37 overexpression in psoriatic skin, the continuous formation of DNA–LL37 complexes triggers a sustained pDC activation to produce type I IFNs, which triggers T cell-mediated autoimmunity and disease development[69]. This, and possibly other, homologous proteins promote the aggregation of released cellular DNA and RNA into large complexes that efficiently activate pDCs[125,127]. Although the origin of these complexes and the consequences of pDC activation remain to be elucidated, the major role of pDCs in psoriasis appears well established.

Similarly, lupus patients show a decrease in circulating pDCs and the accumulation of activated, IFN-producing pDCs in affected tissues such as the skin[70]. The hallmark of lupus is the production of antinuclear antibodies, and immune complexes of such antibodies with endogenous nucleic acids were shown to activate pDCs through

TLR7/9[126,128]. These complexes may be delivered into the endosomal compartment of pDCs via Fc receptor FcγRII[126,128-130], and their stimulatory capacity can be augmented by the nuclear DNA-binding protein HMGB1[131], released by dying cells. HMGB1 binds RAGE (receptor for advanced glycation end products), thereby facilitating the association of DNA with TLR9 in early endosomes[131]. As a result, pDCs are continuously activated to produce type I IFNs, leading to an unabated activation and maturation of cDCs that stimulate autoreactive T cells[93]. In addition, self-DNA forms complexes with LL-37 and other antimicrobial peptides released by neutrophils, and the resulting complexes induce IFN secretion in pDCs through TLR9. Notably, TLR-activated pDCs become resistant to glucocorticoids, which could underlie the limited efficacy of these drugs in lupus[132-133].

Moreover, pDC-derived type I IFNs, together with IL-6, promote the differentiation of autoreactive B cells into autoantibody-secreting plasma cells[99]. Recent studies demonstrate that the expression of type I IFN genes and IFN-induced genes represent the most striking molecular signatures of SLE peripheral blood cells and correlates with disease activity[134]. Thus, type I IFNs may represent the most important effector molecules in SLE pathology.

2.6 Tregs

Specific immunologic tolerance to self antigens requires the immune system to discriminate between self and non-self. Elimination of self-reactive T cells in the thymus during T cell development leads to central tolerance. Peripheral tolerance is designed both to control responses to foreign antigens encountered in the periphery and to maintain tolerance to self antigens. While peripheral tolerance is mediated by a variety of mechanisms, a specific subset of T cells known as regulatory T cells play a critical role.

Tregs are characterised as $CD4^+CD25^+$ T cells, both in human and mice. CD25, the interleukin-2 receptor α-chain (IL-2Rα), is also expressed by activated T cells, but the $CD4^+CD25^{high}$ cells among the $CD4^+CD25^+$ subset are considered to be Treg cells. An important marker of the suppressive capacity of Tregs is the expression of the X chromosome encoded forkhead winged-helix transcription factor Foxp3 (forkhead box p3)[135]. Foxp3 is uniquely expressed by Treg cells and is critically important in the development of these cells. Humans with loss of function mutations of Foxp3 develop a severe, multiorgan, autoimmune and inflammatory disorder, called dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX syndrome)[136-137]. Spontaneous Foxp3 mutation in mice results to an analogous phenotype, called scurfy[138-140].

Different Tregs subsets are now subdivided based on expression of cell surface markers, production of cytokines, and mechanisms of action. Two subsets of Treg cells have been identified, the $CD4^+CD25^+$ natural Treg (nTreg) cells, which mediate central tolerance, and induced Treg (iTreg) cells, which mediate peripheral tolerance.

Natural occurring Treg cells are a T population with immunosuppressive properties that constitutes 5–10% of the total peripheral $CD4^+$ T cells[141-142]. Besides the expression of CD25, they constitutively express other several activation markers, such as the glucocorticoid-induced tumor-necrosis factor (TNF) receptor-related protein (GITR), OX40 (CD134), L-selectin (CD62 ligand (CD62L)), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152). However, it should be noted that none of these markers exclusively identifies Tregs as they can also be expressed to various degrees on activated T cell subsets and various antigen-presenting cells (APCs). However, Foxp3 is a more exclusive intracellular marker for the identification of Tregs[135-136].

nTreg cells develop in the thymus, have a repertoire similar to that of conventional T cells but more skewed toward recognition of autoantigens, undergo clonal expansion upon antigen exposure, and yet maintain their suppressive phenotype. Since the exact mechanism of the development of nTreg cells is not completely understood, it is possible that some thymocytes receive a strong signal via their T cell receptor (TCR) and CD28, escape negative selection, and differentiate into nTreg cells. The nTreg cells then migrate to the periphery and suppress autoreactive T cells, thus suppressing autoimmunity. Stimulation through the CD28 molecule is also required for the survival and proliferation of nTreg cells in the periphery.

Furthermore, several studies reported the existence of various subsets of induced Treg cells, generated in the periphery after antigen recognition by naïve $CD4^+CD25^-$ T cells[143]. The suppressive function of these induced Tregs is mediated by the production of suppressive cytokines (IL-10 and transforming growth factor-β (TGF-β)). Therefore, the current classification of induced Tregs is based on expression of different suppressive cytokines. $CD4^+$ regulatory T cells of type 1 (Tr1) express high levels of IL-10 and moderate levels of IL-5, IFN-γ, and TGF-β, and they are negative for IL-2 and IL-4[144-145]. T helper 3 (Th3) regulatory T cells express high levels of TGF-β[146-147]. These cells acquire FoxP3 and CD25 expression and have suppressive functions.

Apart from the $CD4^+$ naturally occurring and induced Tregs in controlling proper function of the immune system, $CD8^+$ T suppressor cells have also been described. $CD8^+$ T suppressor cells are derived from an oligoclonal T cell population, and they lack CD28 and express FOXP3, GITR, CTLA-4, OX-40, and CD62L at the same level as compared to $CD4^+CD25^+$ Tregs[148-149]. In addition, $CD8^+$ T suppressor cells, that are able to inhibit T cell proliferation, can be induced by xenogenic APCs or by peptide-pulsed autologous APCs[150-151].

Treg cells are anergic and require IL-2 or T cell receptor ligation for their proliferation and suppressive function. Their effector function is not restricted by histocompatibility antigens, and they can suppress both $CD4^+$and $CD8^+$ T cells in an antigen-nonspecific manner[143]. Treg cells can selectively suppress various aspects of T effector function, such as proliferation, cytokine production, chemokine receptor expression, or cytolytic function (figure a). The precise mechanisms underlying suppression mediated by Treg cells are controversial. It is possible that Treg cells suppress immunologic responses in multiple ways, which may involve contact

dependent processes, with direct contact between Treg cells and T effector cells, or may involve Treg interactions with antigen-presenting cells (APCs), which then suppress T effector cells. Other mechanisms include local secretion of inhibitory cytokines and local competition for growth factors[152].

Both *in vitro* and *in vivo* analyses suggest that Treg cells can suppress the proliferation and/ or cytokine production of effector T cells (Teff). Additionally, Treg cells prevent $CD8^+$ cells from differentiating into cytolytic effector cells *in vivo* without affecting their proliferation or interferon-γ (IFN-γ) production[153].

Because the suppression of Teff cell proliferation by Tregs cells was observed *in vitro* in an APC-free system, it was suggested that Treg cells suppress through direct contact with Teff cells[154]. Possible mechanisms include delivery of large amounts of cytoplamic cAMP to Teff cells by contact through gap junctions[155], or direct killing of Teff cells through the release of granzyme B and perforin[156-159].

Because natural Treg cells constitutively express CD25, the high-affinity receptor for IL-2, it has long been suspected that Treg cells can suppress by consuming IL-2 produced by Teff cells, thereby preventing their proliferation and differentiation. Teff cells undergo apoptosis after being exposed to Treg cells[160].

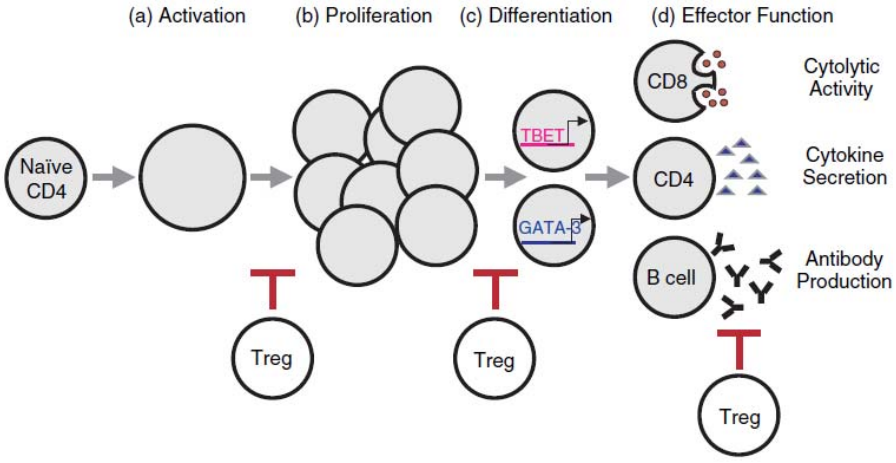

Figure a: Regulatory T cells (Tregs) inhibit multiple stages of target cell activity. (a) Tregs appear to be unable to inhibit the early activation events (up-regulation of CD25 and CD69) of the first 6–10 hr of target $CD4^+$ T-cell activation. (b) Tregs suppress proliferation of multiple immune cell types possibly via attenuation of interleukin-2 production. (c) Suppression of $CD4^+$ T-cell differentiation by limiting the duration of T-cell receptor signalling or inhibiting the induction of the lineage specific transcription factors GATA3 and

Tbet. (d) Treg suppression of effector T-cell cytokine production (interferon-c and interleukin-4 by T helper type 1 and type 2 cells, respectively); inhibition of lytic granule release by CD8 effectors; inhibition of B-cell antibody production, directly or via blockade of CD4 help[161].

Moreover, Treg cells directly interact with antigen-presenting dendritic cells. The interaction of Treg cells with DCs profoundly affects the ability of Teff cells to subsequently engage and become activated by the same DCs. Treg cells either abrogate the antigen-presenting activity of the DC or promote the secretion of suppressive factors by the target DC population. It has been shown that Treg cells can stimulate APCs to upregulate the activity of indoleamine 2,3-dioxygenase (IDO), a potent immunosuppressive enzyme associated with pregnancy and tumor immune evasion[162-163].

Furthermore, blocking CTLA-4 *in vivo* abrogates Treg cell–mediated suppression in mouse models of inflammatory bowl disease (IBD), autoimmune gastritis and transplantation. In the setting of IBD, CTLA-4-deficient Treg cells are able to prevent disease. However, unlike the protection afforded by wild-type Treg cells, disease protection mediated by CTLA-4-deficient Treg cells is dependent on IL-10[164].

2.7 Tregs in autoimmunity

Reduced functional activity of Tregs results in an increased susceptibility to autoimmune disease. Patients with multiple sclerosis (MS)[165], polyglandular syndrome of type II[166], active rheumatoid arthritis[167], type-I diabetes[168], psoriasis[169], and myasthenia gravis[170] show a significant decrease in the suppressive function of $CD4^+CD25^+$ Tregs as compared with cells from healthy donors. Because the percentage of $CD4^+CD25^+$ Tregs in peripheral blood of these patients is unaltered as compared with healthy controls, it has been suggested that it is mainly defective Treg function, rather than its number, that contributes to disease development in these disease conditions. In addition, in some autoimmune diseases, reduced levels of $CD4^+CD25^+$ Tregs have been observed in the peripheral blood of patients[171-172]. However, in these cases, the recruitment or migration of Tregs from the blood to the inflammatory site may be responsible for the decreased number of Tregs in peripheral blood. Indeed, studies on patients with RA or juvenile idiopathic arthritis (JIA) demonstrated that at the site of inflammation (i.e., in the synovial fluid) the percentage of $CD4^+CD25^+$ Tregs was significantly increased as compared with the percentage in peripheral blood[173-175]. The reasons for the increased frequencies of Tregs in inflamed synovia are not known. In addition to preferential homing to synovia from peripheral blood, it is possible that Treg population expands within the synovia. However, the persistence of inflammation in the rheumatoid joints despite the increased number of Tregs indicates that these cells are ineffective in controlling the inflammatory response. One possible explanation is that Tregs in the joint are defective in mediating their suppressive, anti-inflammatory activity.

IPEX is thus far the clearest example that an abnormality in naturally arising Tregs is a primary cause of autoimmune disease, IBD, and allergy in humans. Although IPEX is a rare disease, its clinical picture and its underlying causative phenotype as a deficiency or malfunction of natural Tregs hold several important implications for the role of Tregs in maintaining immunologic self-tolerance in humans and also for the etiology and the pathogenetic mechanism of human autoimmune diseases.

2.8 IL-21 in autoimmunity

Interleukin-21 (IL-21) is a potent immunomodulatory cytokine that has been implicated in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE). IL-21 is a type I cytokine, clearly related to the γ_c family of cytokines, and more similar to IL-2, IL-4, IL-15. IL-21-receptor (IL-21R) is a type I cytokine receptor, most related to the IL-2 receptor β-chain (27% similar)[176].

IL-21R was observed initially to be expressed by spleen and thymus, consistent with expression in lymphohaematopoietic lineages[176-178]. IL-21R expression has been detected on $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, DCs, macrophages [176,179-183] and also on fibroblasts, keratinocytes and intestinal epithelial cells[184-188], suggesting that IL- 21 has a broad range of actions. IL-21R expression is constitutive on B and NK cells. Expression on B cells is the highest, even on resting cells, with constitutive expression in a number of cell lines[176-177]. Absent to low-level IL-21R expression was observed on resting T cells, but it was significantly increased following stimulation by mitogens, T cell receptor (TCR) activation, or following transformation with human T cell lymphotropic virus, type I (HTLV-I) [176-177,179-180].

IL-21 was first observed to be produced by activated $CD4^+$ T cells[176,179] and by innate immune natural killer T (NKT) cells[185] and the more recently identified Th17 lineage[189-191]. IL-21 has pleiotropic effects on both innate and adaptive immune responses. Depending on the cellular context, nature of costimulation, and the ambient cytokine milieu, IL-21 can induce B cell activation, differentiation and death[176,179-180,192-193], the differentiation and activation of NK cells[179], is involved in NK T cell proliferation, cytokine release, and effector function[185,194], can costimulate T-cell proliferation, enhances $CD8^+$ T-cell function[194-196], and promotes proliferation and differentiation of cells of the macrophage and granulocyte lineages[197-198]. Conversely, IL-21 also has direct inhibitory effects on the antigen-presenting function of dendritic cells[181] and can be pro-apoptotic for B cells[180,192,199] and NK cells. IL-21 is also a critical regulator of Th17 development[190-191,200]. Moreover, IL-21 has been shown to have strong antitumor action via its effects on both NK and $CD8^+$ T cells and also has been identified as a key component in the development of autoimmune disease. Although, IL-21 is a pleiotropic cytokine with actions on many cells, one of its essential non-redundant activities is to regulate B-cell responsiveness in both health and disease.

First insight in the disease-promoting effect of IL-21 was suggested by the observation that transgenic mice expressing very high levels of IL-21 did not survive[199]. Further, in several mouse models of SLE, IL-21 has either been indirectly or directly shown to be a contributing factor to disease. Elevated levels of IL-21 were observed also in NOD mice[201], which develop an autoimmune form of diabetes, and in the BXSB-*Yaa* mouse model[199]. In the BXSB-*Yaa* disease, affected animals develop very severe lupus and die at approximately 6 months of age. Elevated levels of IL-21 were also noted in another autoimmune mouse model, the *Sanroque* mutant, which has defective expression/function of the protein Roquin, which negatively regulates the production of a population of follicular T helper cells that produce high levels of IL-21[202]. These cells produce high levels of IL-21, which is correlated with elevated antinuclear antibodies, as well as with the development of lymphadenopathy and glomerulonephritis. The MRL/lpr mouse model of SLE is also associated with elevated production of IL-21 by $CD4^+$ T cells[203]. Interestingly, treatment of these animals with an IL-21R-Fc fusion protein resulted in disease amelioration, indicative of a pathophysiological role of IL-21 in this disease.. The same agent also exhibited a partial effect in a mouse model of collagen induced arthritis[204].

In human lupus, it has been reported that peripheral blood B cells have reduced IL-21R expression, which was associated with nephritis and higher titers of autoantibody[205]. However, the lower levels of IL-21R on SLE B cells may reflect expansions of altered B-cell subpopulations in these patients[206-207]. Corresponding to the augmented IL-21 expression in mice, a cohort of SLE patients have highly elevated IL-21 plasma levels[208]. The increased IL-21 levels correlated with increases of some cytokines but not others. Specifically, increases of IL-21 correlated with increased IL-1, TNF, IL-6, IL-20, IL-22, IL-15, and IFNg, but not with IL-10, IFNa, IFNb, or IL-12. Furthermore, there were no correlations of increases in IL-21 with SLE disease activity index (SLEDAI) scores, anti-DNA titers, or complement levels[208]. Finally, there is a genetic association of IL-21 polymorphisms with SLE in humans[209], consistent with a role of IL-21 in the pathophysiology of at least some patients with this disease.

The role of IL21 in RA has not been extensively studied, but a few reports in both mouse and human suggest that IL21 may be involved in disease progression. Recently, a pathogenic role of IL21 in two animal models of arthritis was reported[204]. In those studies, blockade of IL21 with an IL21R-Fc fusion protein reduced and

importantly, reversed clinical disease activity in both collagen-induced arthritis and adjuvant-induced arthritis[204]. In human RA, alterations of the IL21R have been described. IL21R has been shown to be expressed by synovial macrophages and fibroblasts from biopsies of patients with RA, but not from patients with osteoarthritis[188]. However, IL21 was not detected in these biopsies. In addition, a significantly higher percentage of IL21R-positive inflammatory cells was found in the blood and synovial fluid from patients with RA[210]. Furthermore, blood and synovial fluid T cells from patients with RA secreted higher levels of tumour necrosis factor and IFNγ after stimulation with anti-CD3 and IL21 than T cells from patients with osteoarthritis or healthy subjects, suggesting that IL21 may play a role in upregulating secretion of proinflammatory cytokines that may contribute to worsening of disease[210].

In a recent study, it was shown that, under lymphopenic conditions, the remaining T cells undergo extensive compensatory expansion, in order to reconstitute the immune system and high levels of IL-21 result to the development of secondary autoimmunity[211]. It is thus proposed that IL-21 may promote autoimmunity by inducing proinflammatory Th17 cells[212], by promoting B cell differentiation and antibody production[213] or by reducing the suppressor effect of Tregs[214-215]. Another possible explanation is that IL-21 could modulate dendritic cell function. Actually, the precise role of IL-21 in DCs maturation and function has not been addressed. There is a study showing that the presence of IL-21 during the differentiation of monocytes towards DCs in vitro leads to altered function of DCs with reduced MHC-II, CD80, CD86 expression and low stimulatory capacity for T cell activation[181]. Inducible-costimulator ligand (ICOS-L) is expressed on dendritic cells and is implicated in both negative and positive co-stimulation of T cells. Interaction of ICOS/ICOS-L can either augment or inhibit T cell mediated immune responses depending on the in vivo setting. Disrupting ICOS/ICOS-L signaling suppressed intragraft T cell activation and cytokine expression and prolonged allograft survival[216-217].

3. Objectives

Even though both subtypes of dendritic cells exhibit a functional plasticity in directing T cell responses, recent evidence supports a predominant role of pDCs in the maintenance of tolerance, through the induction or/and expansion of Treg cells. To this end, the objectives of our study were:

a. to examine whether pDCs from RA patients in remission could induce and/or expand regulatory T cells

b. to investigate whether pDCs-induced Tregs can potently suppress effector cells in vitro

c. to delineate the mechanism through which pDCs instruct naïve T cells towards Tregs

Since IL-21 is shown to be implicated in the development of autoimmunity, in both human disease and mice models, while DCs are capable of differentially instructing effector T cells, the objective of our study was:

d. to address the role of IL-21 in DC maturation and to assess the potential of IL-21-treated DCs to influence T cell immune responses

4. Materials and Methods

Patient population. Patients (n=35) with RA, fulfilling the revised classification criteria of the American College of Rheumatology for RA, were included in this study. Disease activity was assessed by the Disease Activity Score (DAS 28). Disease remission is defined as a DAS below 2.4. 20 patients with active and 15 inactive RA patients in remission were evaluated, after either anti-TNFα therapy (infliximab administered at a dose of 3 mg/kg i.v. at weeks 0, 2, 4, 8 and at every 8 wks) or methotrexate (10 to 15 mg/wk orally). 10 healthy individuals were used as controls. All subjects gave written informed consent prior to study enrolment.

	Active RA	Inactive RA†
Sex, no. female/no. male	11/4	4/8
Age, years	66.2 ± 8.18	52.5 ± 20.58
RA duration, years	12.6 ± 11.74	9.41 ± 8.54
DAS28	5.06 ± 1.02	1.91 ± 0.48
Therapy, no. of patients		
Anti-TNFα	15	8
MTX	0	4

Table 1. Characteristics of the patients with rheumatoid arthritis (RA)*

* Except where indicated otherwise, values are the mean ± SD. Patients with missing data are excluded. DAS28 = Disease Activity Score in 28 joints; anti-TNFα = anti–tumor necrosis factor α; MTX = methotrexate.

† Defined as those achieving disease remission from 6 months up to 2.5 years after the start of therapy for RA.

Media and Reagents. The following fluorescent-conjugated antibodies were used: CD45-PC5 (clone J33), CD69-PE (clone TP1.55.3) and CD4-PC5 (clone 13B8.2) all from Beckman-Coulter (Miami, FL). CD303-FITC (BDCA-2, clone AC144), CD123-PE (IL3-R, clone AC145), CD1c-PE (BDCA-1, clone AD5-8E7) from Miltenyi Biotec (Bergisch Gladbach, Germany). CD11c-FITC (clone 3.9), PD-L1-PE (clone MIH1), ICOS-L-PE (clone MIH12), CD80-PE (B7-1, clone 2D10), CD86-PE (B7-2, clone IT2.2), HLA-DR-PE (clone LN3) and Foxp3-PE (clone 3G3) from eBioscience (San Diego, CA). CD25-FITC (clone M-A251), CD62L-FITC (clone

Dreg-56) from BD Biosciences Pharmingen (San Diego, CA). For CFSE staining of $CD4^+$ T cells, the CellTrace CFSE cell proliferation kit was used (Invitrogen, Eugene, Oregon, USA).

All cell cultures were performed in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100μg/ml) and 2mM L-Glutamine (all from Gibco, Carlsbad, CA). Recombinant human IL-3 (rhIL-3) (R&D, UK & Europe), recombinant human IL-4 (rh-IL-4) and rhGM-CSF (from Peprotech, EC London) were used in DCs cultures as indicated. For DCs activation anti-human CD40L (Peprotech) and LPS was used (500ng/ml) were used. For T cells stimulation, we used 5μg/ml plate bound anti-human purified CD3 (aCD3, OKT-3 clone; eBioscience) and 1μg/ml soluble anti-human purified CD28 (aCD28, clone CD28.3; eBioscience). In some experiments, soluble aCD3 (50ng/ml) was used. The IDO inhibitor 1-MT (1-methyl-DL-tryptophan, Sigma-Aldrich, Germany) was dissolved in DMSO and used at 10 μM concentration in *in vitro* cultures. Recombinant human IL-21 (rh-IL-21) (Peprotech) was used during DCs differentiation and activation (30ng/ml).

Cell isolation and culture. Peripheral blood mononuclear cells (PBMCs) from RA patients or healthy volunteers were isolated by Ficoll-Histopaque (Sigma-Aldrich,) density-gradient centrifugation of heparinized venous blood aspirates, immediately after peripheral blood draw.

In order to generate moDCs, PBMCs were magnetically separated using the CD14 isolation kit (Miltenyi Biotec), following the manufacturer's instructions. Cells were cultured for seven days in the presence of IL-4 (50ng/ml), GM-CSF (50ng/ml). Replacement with fresh media took place on days 3 and 5. The generated moDCs were activated for 24 hours with LPS.

Synovial fluid samples were obtained from active RA patients and mononuclear cells were isolated, upon treatment with hyalouronidase solution [1mg hyalouronidase (Sigma-Aldrich) per ml of hyalouronidase buffer (1M phosphate buffer, pH=7, 5M NaCl, 7,5% BSA)] for 10 minutes at RT, followed by Ficoll-Histopaque density gradient centrifugation. Leucocytes were collected and were used for further analysis.

Flow cytometry analysis. PBMCs were magnetically separated using anti-CD3 microbeads (Miltenyi Biotec), following the manufacturer's instructions. The phenotypic characterization of the DC subsets was performed on the $CD3^-$ cell

fraction, based on the surface expression of CD45, CD303, CD123, CD11c and CD1c molecules. Alternatively, DC subsets were stained with the negative co-stimulatory molecules ICOS-L and PD-L1. The $CD3^+$ cell population was extracellularly stained with CD4, CD25, CD62L, followed by intracellular Foxp3 staining, according to the manufacturer's protocol. For the IL-21 experiments, PBMCs were magnetically separated using the anti-CD14 microbeads (Miltenyi Biotec). Mature moDCs were stained for surface molecules, CD80, CD86, CD40, HLA-DR, PD-L1 and ICOS-L, CD4 and CD69. IgG isotype matched controls were used as negative controls. All stainings were conducted in PBS/5% FCS for 20 min at 4°C. Flow cytometry was performed with an Epics Elite model flow cytometer (Coulter, Miami FL) and data were analysed with WinMDI software.

For the IL-21 experiments, flow cytometry was performed with a Caliber model flow cytometer (BD Biosciences Pharmingen, San Diego, CA) and data were analysed with FlowJo software.

Mixed leukocyte reaction (MLR). mDCs and pDCs populations were isolated from PBMCs of inactive RA patients using the BDCA-1 (CD1c) and BDCA-4 (CD304) magnetic microbeads dendritic cell isolation kits respectively, according to the manufacturer's instructions (Miltenyi Biotec). The purity of the isolated populations ranged from 75% to 95%. To maintain viability, mDCs and pDCs were cultured in flat-bottom 96 well-plate, with 2ng/ml GM-CSF and 1ng/ml IL-3 respectively. After one to two days of culture, DCs were activated for 24h in the presence of 1μg/ml anti-CD40L. $CD4^+CD25^-$ T cells (responders) were isolated from heparinised cord blood using CD4 and CD25 magnetic microbeads (Miltenyi Biotec), and were subsequently labelled with 2μM CFSE, in labelling buffer (PBS, 0.1% BSA) for 10 min in 37°C. MLRs were set up as follows: 10^4 mDCs or pDCs were co-cultured with 2 x 10^4 allogeneic CFSE-labelled $CD25^-$ T cells in round-bottom 96-well plates, for 5-7 days, in the presence or absence of soluble aCD3. Proliferation of T cells was determined based on CFSE dilution by flow cytometry. Culture supernatants were collected in order to be assessed for the presence of cytokines. In other experiments, MLR was performed in the presence of the specific antagonist of the IDO enzyme, 1-MT (1mM), in order to examine the role of IDO in T cell activation/proliferation.

For the IL-21 experiments, DCs were generated from PBMCs of healthy volunteers as mentioned before. MLRs were set up as follows: 10^4 moDCs were co-cultured

with 2 x 10^4 allogeneic CFSE-labelled $CD25^-$ T cells in round-bottom 96-well plates, for 5-7 days, in the presence of soluble aCD3 (10ng/ml). In half conditions soluble IL-21 was added (30ng/ml). Proliferation of T cells was determined based on CFSE dilution by flow cytometry. Culture supernatants were collected in order to be assessed for the presence of cytokines.

Cytokine detection. DC-primed $CD4^+$ T cells were collected and washed after 5 days of primary culture and were re-stimulated (10^6 cells/ml) with 5 μg/ml of plate-bound aCD3 and 1 μg/ml of soluble aCD28 for 24h. The levels of IL-10, IFN-γ and TNF-α in culture supernatants were measured by using the respective human cytokine ELISA kits (eBioscience). The detection limits were 15,65pg/ml for IFN-γ, 16,25pg/ml for IL-10 and 5,81pg/ml for TNF-α.

IDO expression by Real Time-PCR. 2 x 10^5 pDCs isolated from either inactive RA patients or healthy controls were resuspended in TRI reagent (Sigma) and total RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA was then reverse-transcribed into cDNA using ThermoScript Reverse Transcriptase (Invitrogen). cDNA was stored at -20° C until further use.
Expression of IDO enzyme by pDCs was determined by real time-PCR using gene-specific primers for the human gene IDO: 5'-CATGCTGCTCAGTTCCTCCAG-3' (forward), 5'-CAGAGCTTTCACACAGGCGTC-3' (reverse). PCR was run with 5 μl undiluted cDNA template in 20μl reactions in duplicate on a 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. Amplification of GAPDH using gene-specific primers 5'-CATGTTCCAATATGATTCCACC-3' (forward) and 5'-GATGGGATTTCCATTGATGAC-3' (reverse) was used as reference gene. 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.

IL-21R expression by PCR. 2 x 10^5 moDCs generated from healthy controls were resuspended in TRI reagent (Sigma) and total RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA was then reverse-transcribed into cDNA using ThermoScript Reverse Transcriptase (Invitrogen). cDNA was stored at -20° C until further use.
Expression of IL-21R by moDCs was determined by PCR using gene-specific primers for the human gene IL-21R : 5'-CTTACCTGGCAAGACCAGTATGA -3' (forward), 5'-GTAGAAGGCAGGGTCTTCGTAAT-3' (reverse).

Suppression assay. For the suppression experiments, DC-primed T cells (Donor A) were collected and cultured with 2 x 10^4 naïve autologous $CD4^+CD25^-$ T cells (Donor A) in the presence of 10^4 irradiated (40Gy) allogeneic CD3-depleted monocytes as APCs (Donor B), in round-bottomed 96-well plates. Titration of DC-primed T cells was performed, resulting in naïve T: DC-primed T ratios of l:1, 1:0,5 and 1:0,1. After 4 days of culture, 1μCi [^{3}H]thymidine (Amersham Biosciences) was added to each well for the last 18h of culture. The cells were harvested and incorporated radioactivity was measured using a Beckman beta counter.

Statistical analysis. The non-parametric Mann-Whitney *U* test was used for statistical analysis. Results were expressed as mean ± SEM and *p*-values (two-tailed) <0.05 were considered as statistically significant.

5. Results

5.1 Induction of Interleukin-10–Producing Treg Cells by Plasmacytoid Dendritic Cells in Patients With Rheumatoid Arthritis Responding to Therapy

5.1.1 Increased frequencies of Foxp3$^+$ Treg cells in patients with inactive RA

We first sought to confirm whether Treg cells had been restored in the peripheral blood of patients with RA who had been successfully treated and achieved remission of their RA, as reported previously[167,218]. As shown in Figure 1A, the percentages of Foxp3$^+$CD4$^+$ T cells in patients with inactive RA were increased 2–3-fold compared with those in patients with active RA. Of interest, although the CD4$^+$Foxp3$^+$ T cells from all 3 groups were also CD25high (results not shown), they exhibited a distinct pattern of expression for the CD62L molecule. Thus, in patients with inactive RA, almost 34% of the CD4$^+$Foxp3$^+$ cells did not express CD62L, whereas in healthy controls and patients with active RA, the majority of the CD4$^+$Foxp3$^+$ cells (>85%) were CD62L$^+$ (Figure 1B). Taken together, these findings support the notion of the de novo generation of Treg cells, rather than the expansion of these cells from preexisting Treg cells, in the peripheral blood of patients with therapy induced remission of RA.

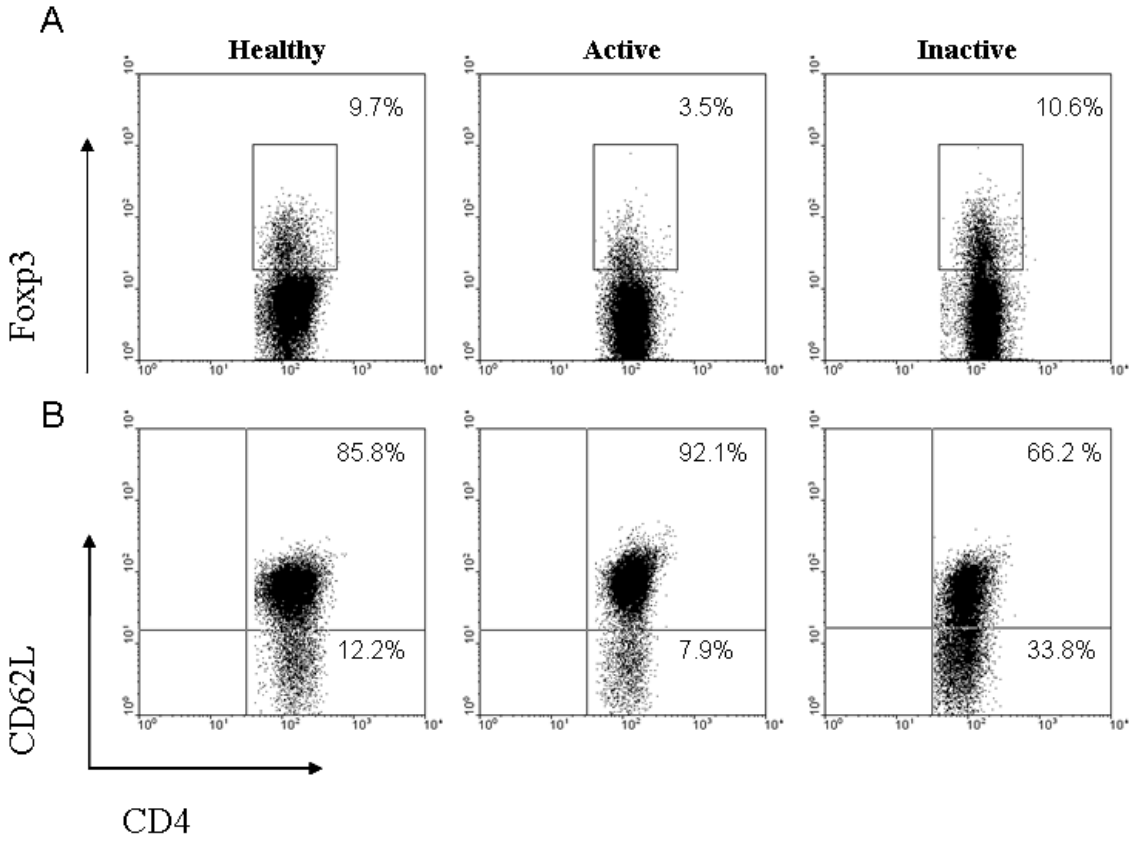

Figure 1. Increase in the levels of $Foxp3^{+}CD4^{+}$ T cells in the peripheral blood of patients with rheumatoid arthritis (RA) responding to therapy. T cells were isolated from peripheral blood mononuclear cells obtained from healthy controls, patients with active RA, and patients with inactive RA, and assessed by fluorescence-activated cell sorting (FACS) analysis. **A,** Intracellular Foxp3 expression was determined by staining $CD3^{+}$ T cells with fluorescence-conjugated antibodies against CD4 and Foxp3. Values are the percentages of gated $CD4^{+}Foxp3^{+}$ T cells. **B,** The gated $CD4^{+}Foxp3^{+}$ T cell population was also assessed by FACS analysis for the expression of the CD62L molecule. Representative results are shown for 10 healthy controls, 20 patients with active RA, and 15 patients with inactive RA.

5.1.2 Restoration of the levels of plasmacytoid and myeloid DCs in the peripheral blood of patients with RA achieving remission

Since DCs have been linked to the induction of tolerance through the generation of Treg cells (21), we reasoned that DCs may be involved in the generation and/or expansion of Treg cells in patients with RA whose disease is in therapy-induced remission. To this end, CD3-depleted peripheral blood leukocytes were stained for the CD303 antigen (BDCA-2) and the CD1c antigen (BDCA-1), which are expressed on the surface of plasmacytoid DCs and the surface of myeloid DCs, respectively. The results were then analyzed by flow cytometry. As shown in Figure 2A, expression of myeloid DCs ($CD45^+CD1c^+CD11c^+CD303^-$) was severely curtailed in the peripheral blood of patients with active RA, in contrast to that in patients with inactive RA, in whom the myeloid DC levels were similar to those in healthy controls. The decrease in myeloid DC numbers in patients with active RA was statistically significant compared with that in either healthy controls or patients with inactive RA (Figure 2B). Similar to the findings regarding myeloid DCs, $CD303^+$ cells were virtually absent (<0.1%) in the peripheral blood of patients with active RA, whereas the levels of $CD303^+$ cells in patients who had achieved remission were restored (~0.5%) and were comparable with the levels in healthy controls (~0.7%) (Figure 2C). We also determined the relative frequencies of $CD45^+CD303^+CD1c^-CD11c^-CD3^-$ plasmacytoid DCs in the peripheral blood of patients with active RA and patients with inactive RA and found that the numbers of plasmacytoid DCs ($CD303^+$ cells) in patients with active RA (mean 250 per 3 x 105 cells) were significantly decreased as compared with those in either patients with inactive RA (mean 1,700 per 3 x 10^5 cells; $P < 0.005$) or healthy individuals (mean 2,100 per 3 x 105 cells; $P < 0.05$) (Figure 2D). To further confirm that CD303 is specifically expressed on plasmacytoid DCs, we performed costaining for CD123 (IL-3 receptor α), which is commonly used as a marker for plasmacytoid DCs. As shown in Figure 2E, the entire population of $CD303^+$ cells (>96%) also stained positive for CD123. The decrease in DCs in the peripheral blood of patients with active RA prompted us to explore whether some of these cells may be accumulating at the site of inflammation within the joint. An increased accumulation of myeloid DCs (~15%) in the SF of patients with active RA was observed. In contrast, we failed to detect $CD303^+$ plasmacytoid DCs in the SF of the same RA patients (results not shown).

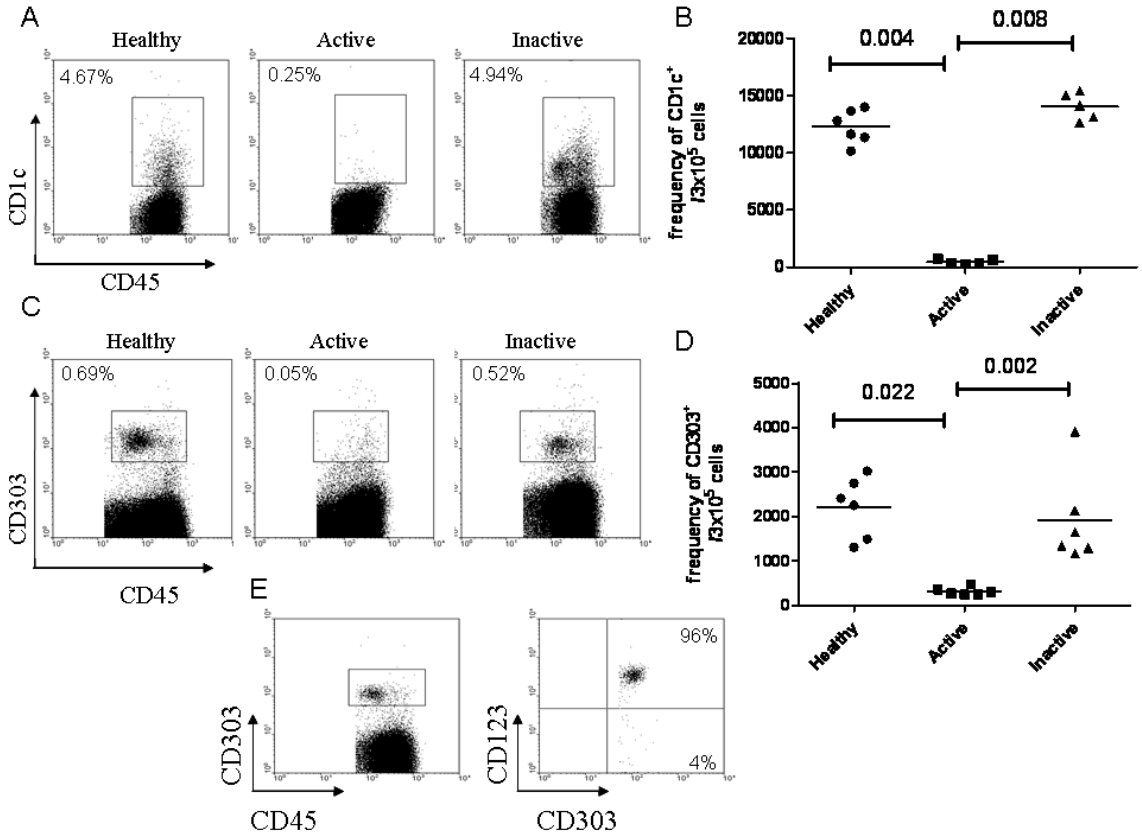

Figure 2. Restoration of the levels of myeloid and plasmacytoid dendritic cells (DCs) in the peripheral blood of patients with rheumatoid arthritis (RA) in remission. CD3-depleted peripheral blood mononuclear cells from healthy controls (n = 10), patients with active RA (n = 20), and patients with inactive RA (n = 15) were stained with myeloid DC (CD1c)– and plasmacytoid DC (CD303)–specific antibodies, and expression of these molecules in the peripheral blood was assessed by flow cytometry. A and B, The proportion of $CD45^+CD1c^+CD11c^+CD303^-$ myeloid DCs (A) and the relative frequencies of $CD1c^+$ myeloid DCs per 3 x 10^5 events (B) were determined. C and D, The fraction of $CD45^+CD303^+CD1c^-CD11c^-$ plasmacytoid DCs (C) and the frequencies of $CD303^+$ plasmacytoid DCs per 3 x 10^5 events (D) were determined. Each symbol in B and D represents the results from an independent experiment, and the horizontal lines indicate the mean. *P* values (bars) were calculated by Mann-Whitney U test. E, The $CD45^+CD303^+$ fraction of cells from patients with inactive RA (left) was also assessed to determine the percentage staining positive (upper right) or negative (lower right) for CD123 (interleukin-3 receptor α). Results in E are representative of 1 of 5 independent experiments.

5.1.3 Induction of IL-10–secreting T cells from naïve $CD4^+$ T cells by mature plasmacytoid DCs in patients with RA responding to therapy

We next sought to determine whether plasmacytoid DCs isolated from RA patients showing a response to therapy have the ability to induce T cells with a regulatory phenotype. To this end, myeloid DCs and plasmacytoid DCs were isolated from the peripheral blood of patients with inactive RA and cultured in the presence of GM-CSF and IL-3, respectively, and then matured with CD40L for 24 hours. The maturation status of the DC subsets was confirmed on the basis of the expression of HLA–DR, CD80, CD86, CD40, and the chemokine receptor CCR7 (results not shown). To detect the ability of CD40L-matured myeloid and plasmacytoid DCs to activate and expand naive T cells, we cultured CFSE-labeled naive $CD4^+CD25^-$ T cells, which were isolated from cord blood, in the presence of CD40L-matured plasmacytoid DCs and myeloid DCs, and monitored cell proliferation based on dilution of the CFSE staining after 6 days. Naive T cells cocultured with mature plasmacytoid DCs were hyporesponsive, as demonstrated by their delayed proliferation, in contrast to that seen in cocultures with mature myeloid DCs, in which a robust expansion of naive T cells occurred (Figure 3A). Specifically, more than 90% of the myeloid DC–primed T cells progressed to the third division, whereas only 40% of the T cells cultured with plasmacytoid DCs entered the third division (gate M3 in Figure 3A). This was further confirmed on the basis of the increased expression of the early activation marker CD69 on $CD4^+$ T cells. Thus, the majority (>90%) of the $CFSE^-CD4^+$ T cells cultured with myeloid DCs were $CD69^-$ after 6 days of culture, whereas almost 35% of the plasmacytoid DC–primed T cells were in the early activation stage ($CFSE^+CD69^+$) (Figure 3B). We next assessed the ability of DC-primed T cells to secrete cytokines upon in vitro restimulation with anti-human CD3/anti-human CD28. T cells cultured in the presence of myeloid DCs produced significant amounts of IFNγ (250–500 pg/ml), whereas T cells cultured with plasmacytoid DCs produced lower amounts of IFNγ (80–100 pg/ml) (Figure 3C). Most notably, plasmacytoid DC–primed T cells secreted large amounts of IL-10 (800–1,200 pg/ml), in contrast to myeloid DC–primed T cells, in which IL-10 was not detectable (Figure 3C). Collectively, these results show that T cells cultured in the presence of plasmacytoid DCs from patients with RA in remission proliferate poorly and secrete significant amounts of IL-10 in response to polyclonal stimulation, indicating that they have acquired features of Treg cells.

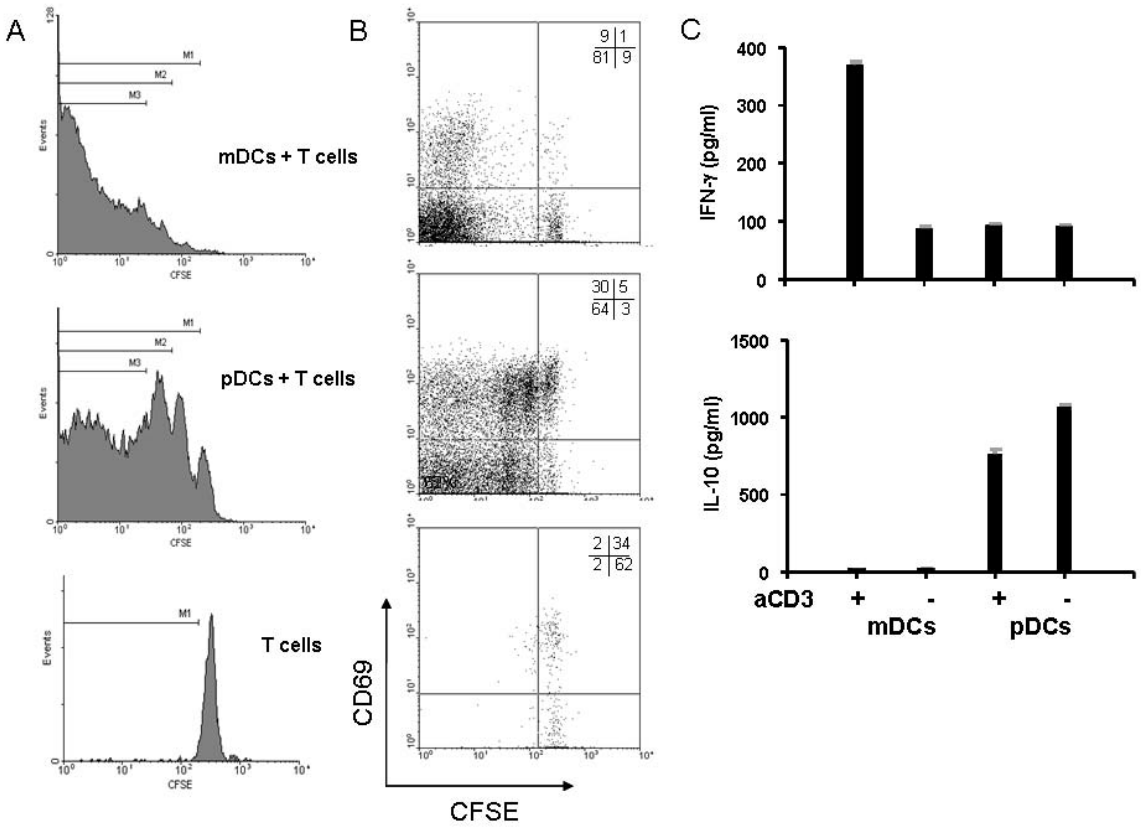

Figure 3. Induction of interleukin-10 (IL-10)–secreting T cells by mature plasmacytoid dendritic cells (pDCs) isolated from patients with rheumatoid arthritis (RA) in remission. Myeloid DCs (mDCs) and pDCs isolated from the peripheral blood mononuclear cells of patients with inactive RA were cultured with 2 ng/ml granulocyte–macrophage colony-stimulating factor and 1 ng/ml IL-3, respectively, and matured upon culture with 1 μg/ml anti-CD40L for 24 hours. Allogeneic naive 5,6-carboxyfluorescein succinimidyl ester (CFSE)–labeled $CD4^+CD25^-$ T cells were isolated from cord blood and were cultured with either mature mDCs or mature pDCs in a DC-to–T cell ratio of 1:2. As the control, CFSE-labeled naive T cells were used. The proliferation was optimized using soluble human purified anti-CD3 (50 ng/ml). **A,** Histograms show the dilution of CFSE staining in $CD4^+CD25^-$ T cells after 6 days of coculture with DC subsets. Gates depict individual cell cycles. **B,** The surface expression of the CD69 molecule was assessed on T cells cultured with mDCs or pDCs. The values indicate quadrant percentages. **C,** DC-primed T cells were collected after 6 days of coculture and restimulated with 5 μg/ml of plate-bound anti-CD3 (aCD3) and 1 μg/ml of soluble anti-CD28 monoclonal antibodies. Culture supernatants were collected 24 hours later and assessed by cytokine enzyme-linked immunosorbent assay for the presence of interferon-γ (IFNγ) and IL-10. The detection limit of the assay was 15.65 pg/ml for IFNγ and 16.25 pg/ml for IL-10. Bars show the mean and SEM representative results from 1 of at least 3 independent experiments.

5.1.4 Suppression of naive CD4_ T cell proliferation by mature plasmacytoid DC–induced IL-10–producing Treg cells in patients with RA in remission

To determine whether IL-10–producing plasmacytoid DC–primed T cells are Treg cells, plasmacytoid DC–primed T cells were cultured with CFSE-labeled autologous naive $CD4^+CD25^-$ T cells in the presence of irradiated allogeneic CD3-depleted monocytes as APCs; myeloid DC–primed T cells were used as the control. As shown in Figure 4A, plasmacytoid DC–primed T cells inhibited the proliferation of the autologous naive T cells, in a dose-dependent manner. In contrast, the addition of myeloid DC–primed T cells into the culture resulted in an increased proliferation of the autologous naive T cells, when compared with that in cultures with naive T cells alone (Figure 4B). These results indicate that mature plasmacytoid DCs in patients with RA in remission have an intrinsic ability to induce the generation of Treg cells that display a potent suppressive activity on autologous T cells in vitro.

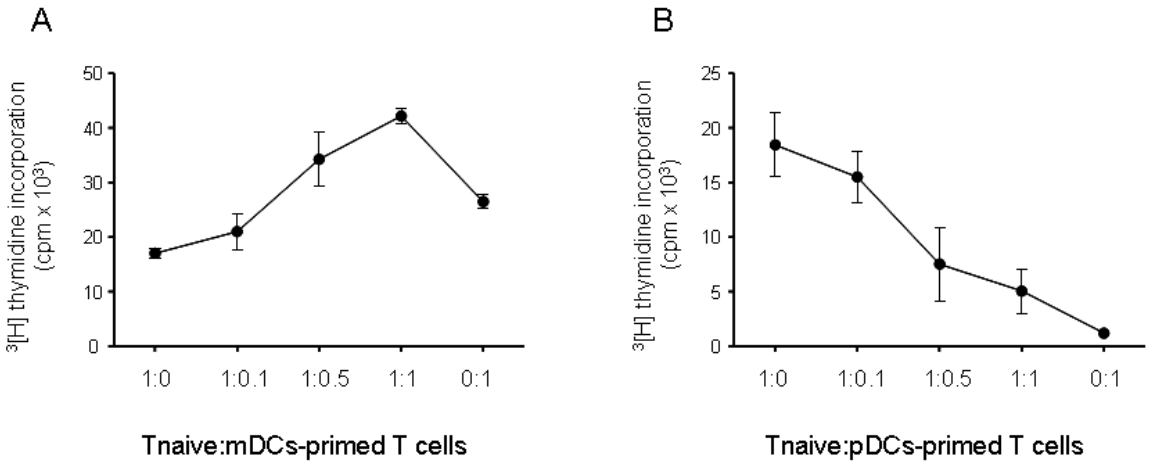

Figure 4. Suppression of autologous T cell proliferation by plasmacytoid dendritic cell (pDC)–primed T cells in vitro. Naïve $CD4^+CD25^-$ T cells from the peripheral blood of a single donor were cocultured with either pDCs **(A)** or myeloid DCs (mDCs) **(B)** from patients with rheumatoid arthritis in remission, and then matured with CD40L. After 7 days of culture, $CD4^+$ T cells were separated from DCs and used for functional assays, in which 2 x 10^4 freshly purified autologous $CD4^+CD25^-$ T cells from the same donor were cultured with 10^4 irradiated antigen-presenting cells from a second donor, and the DC-primed T cells from the first donor were added at graded doses. T cell proliferation was assessed in a 4-day mixed leukocyte reaction assay, with 3H-thymidine added during the last 18 hours of culture. Bars show the mean ± SEM representative results from 1 of 4 independent experiments.

5.1.5 Increased expression of IDO mRNA, but not ICOS-L and PDL-1 molecules, by mature plasmacytoid DCs in patients with inactive RA

Several lines of evidence indicate an important role of negative costimulatory molecules, such as ICOS-L and PDL-1[219-220] as well as the expression of the enzyme IDO[221] by DCs in the induction of Treg cells. As shown in Figure 5A, plasmacytoid DCs from patients with inactive RA and healthy controls expressed comparable levels of either ICOS-L or PDL-1 upon maturation, suggesting that the expression of these molecules most likely does not account for the polarization of T cells by plasmacytoid DCs toward Treg cells in these patients. In contrast, plasmacytoid DCs from patients with RA in remission displayed a 4–8-fold increase in IDO mRNA levels as compared with that in healthy individuals, independent of the maturation status of the plasmacytoid DCs (Figure 5B).

5.1.6 Induction of IL-10–producing Treg cells by plasmacytoid DCs in an IDO-dependent manner

To examine whether the expression of IDO by mature plasmacytoid DCs in patients with RA in remission is required for the differentiation of naive T cells into IL-10–producing Treg cells, we set up cocultures of naive T cells and DCs in the presence or absence of the IDO specific inhibitor 1-MT. As shown in Figure 5C, mature myeloid DCs induced a robust proliferation of naïve $CD4^+CD25^-$ T cells, irrespective of the addition of 1-MT in vitro. In cocultures with plasmacytoid DCs, naive T cells proliferated poorly when cultured with plasmacytoid DCs alone (Figures 3A and 5C), whereas this phenotype was altered in the presence of the IDO inhibitor, since a strong proliferation of naive T cells occurred after inhibition of IDO (Figure 5C). The reversal in plasmacytoid DC–induced T cell proliferation in the presence of the IDO inhibitor was further confirmed by measuring the expression of CD69 by proliferating T cells. In the absence of 1-MT inhibition, only 40% of the $CD4^+$ T cells divided ($CFSE^-CD4^+$) and down-regulated the CD69 molecule (Figure 5D). In contrast, with the addition of the IDO inhibitor, more than 90% of the cells entered cell division, with the majority of cells (~86%) being $CFSE^-CD4^+CD69^-$; only 6% of the cells were activated ($CFSE^+CD4^+CD69^+$) but not dividing (Figure 5D). Furthermore, T cells primed with myeloid DCs with or without the addition of 1-MT did not produce significant amounts of IL-10 (<50 pg/ml) (Figure 5E). In contrast, plasmacytoid DC–primed T cells secreted large amounts of IL-10 upon restimulation (1,400–1,750 pg/

ml), and the addition of the IDO inhibitor during plasmacytoid DC–T cell cocultures effectively abolished the secretion of IL-10 by T cells (180–250 pg/ml) (Figure 5E). In contrast, blocking of the ICOS pathway or blocking of the secretion of cytokines by DCs using neutralizing antibodies did not affect the ability of plasmacytoid DCs to polarize naive T cells toward Treg cells (detailed results available from the corresponding author upon request). Taken together, these findings suggest that plasmacytoid DCs from RA patients whose disease has responded to therapy have the capacity to polarize naive T cells toward Treg cells that secrete high levels of IL-10 in an IDO-dependent manner.

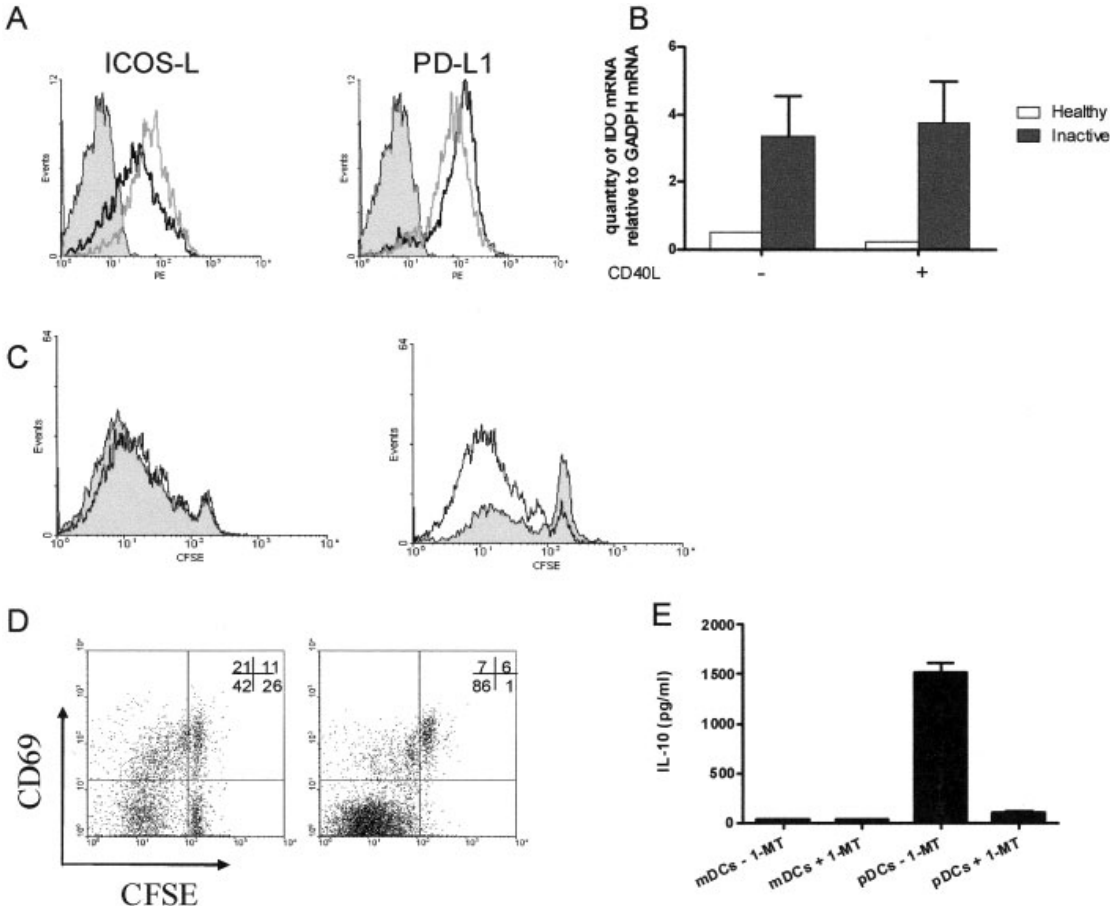

Figure 5. Induction of interleukin-10 (IL-10)–producing Treg cells by plasmacytoid dendritic cells (pDCs) in an indoleamine 2,3-dioxygenase (IDO)–dependent manner. **A**, Surface expression of inducible costimulator ligand (ICOS-L) and programmed

death ligand 1 (PDL-1) on mature pDCs from patients with inactive rheumatoid arthritis (RA) (solid line) and healthy controls (shaded line). Results obtained with isotype-matched control antibody are also shown (filled histogram). **B**, IDO mRNA expression on pDCs from the same patients with inactive RA and healthy controls as in A. 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 with or without maturation by CD40L. **C**, Expression levels of myeloid DCs (mDCs) (left) and pDCs (right) from patients with inactive RA in cocultures with allogeneic 5,6-carboxyfluorescein succinimidyl ester (CFSE)–labeled $CD4^+CD25^-$ naive T cells, in the presence (open histogram) or absence (filled histogram) of the IDO-specific antagonist 1-methyl-DL-tryptophan (1-MT). CFSE dilution of the depicted $CD4^+$ gated cells is shown after 5 days of culture. **D**, Surface expression of CD69 on $CFSE^+CD4^+CD25^-$ T cells cultured with pDCs, with (right) or without (left) 1-MT. The values indicate quadrant percentages. **E**, Secretion of IL-10 in DC primed T cells in the presence or absence of 1-MT. IL-10 levels were measured by enzyme-linked immunosorbent assay in the supernatants collected 24 hours after restimulation. Bars show the mean and SEM representative results of 1 of at least 3 independent experiments.

5.1.7 Increased levels of IL-10–producing T cells in the peripheral blood of patients with inactive RA

To determine whether disease remission is associated with an increase in IL-10–producing T cells in the peripheral blood, we examined the secretion of IL-10 upon polyclonal stimulation of $CD4^+CD25^-$ T cells isolated from patients with inactive RA, patients with active RA, and healthy controls. Although both T cells from patients with active RA and those from healthy individuals showed increased levels of IL-10 upon stimulation, the levels of IL-10 secreted from stimulated T cells from patients with RA in remission were 3–4-fold higher (details available from the corresponding author upon request). Collectively, these results confirm that patients with RA in remission display increased numbers of IL-10–producing T cells, a proportion of which might have been induced upon interaction with the IDOexpressing plasmacytoid DCs.

5.2 IL-21 conditioned-DCs promote T cell reactivity through downregulation of ICOS-L

5.2.1 IL-21R is expressed in human mature and immature dendritic cells, generated with IL-4 and GM-CSF

IL-21 receptor (IL-21R) is expressed on $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, macrophages, keratinocytes and DCs. However, it is not known whether IL-21R is expressed on human mature and immature dendritic cells. For this reason, PBMCs were isolated from healthy volunteers, and after CD14 enrichment and culture with IL-4 and GM-CSF, moDCs were generated. Generated monocyte-derived dendritic cells (moDCs) were submitted to PCR to test the expression of IL-21R. IL-21R is expressed on both mature and immature moDCs, generated with IL-4 and GM-CSF.

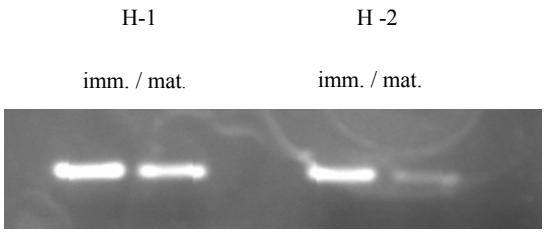

Figure 6: Expression of IL-21R in human myeloid-derived dendritic cells by PCR. Monocyte derived dendritic cells (moDCs) were generated from peripheral blood mononuclear cells (PBMCs) of healthy volunteers, using 50ng/ml of IL-4 and GM-CSF. moDCs were collected on day 7 and half of them were activated with LPS in order to generate mature moDCs. Mature and immature moDCs were submitted to PCR for the expression of IL-21R. moDCs were generated from PBMCs of 8 healthy volunteers.

5.2.2 IL-21-conditioned DCs promote the proliferation of naïve T cells in the presence of soluble IL-21

We next sought to determine whether dendritic cells generated within an environment of high levels of IL-21, such as the periphery of SLE patients, acquire a new function. To this end, we set up co-cultures of moDCs, differentiated in the presence of IL-21, with naïve CFSE labeled $CD4^{+}CD25^{-}$ T cells, isolated from cord blood, in the presence or absence of IL-21. We demonstrate that moDCs matured in the presence of IL-21, significantly increased the proliferation of allogeneic $CD4^{+}CD25^{-}$ T cells, as compared to non-IL-21-treated moDCs. The immunogenicity of IL-21-treated moDCs was further increased when IL-21 was present during T cell activation.

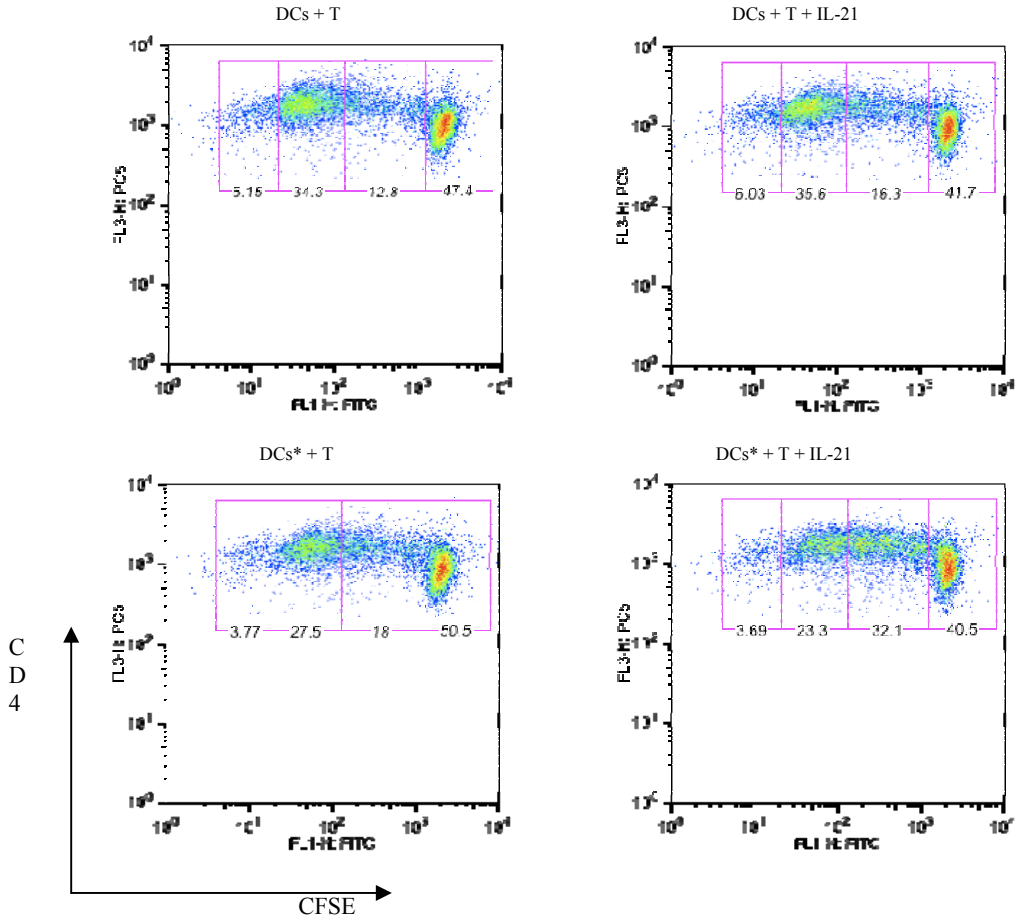

Figure 7: IL-21-conditioned DCs promote the proliferation of naïve T cells in the presence of soluble IL-21. Monocyte derived dendritic cells (moDCs), were generated from peripheral blood mononuclear cells (PBMCs) from healthy donors, cultured with

50 ng/ml of GM-CSF and IL-4, and matured upon culture with LPS for 24 hours. Allogeneic naïve CFSE–labeled $CD4^+CD25^-$ T cells were isolated from cord blood and were cultured with either mature moDCs or mature moDCs generated in the presence of soluble IL-21, in a moDC-to–T cell ratio of 1:2. The proliferation was optimized using soluble human purified anti-CD3 (50 ng/ml). As the control, CFSE-labeled naive T cells were used. **A,** Histograms show the dilution of CFSE staining in $CD4^+CD25^-$ T cells after 5-7 days of coculture with moDCs. Gates depict individual cell cycles. moDCs* are moDCs generated in the presence of IL-21

5.2.3 Increased levels of IFN-γ and TNF-α in culture supernatants in the presence of IL-21

To better establish the results from the proliferation assays showing that IL-21 treated moDCs promote the proliferation of naïve $CD4^+CD25^-$ T cells, we measured the cytokines secreted during these assays. In correlation with the proliferation assays, increased levels of IFN-γ and TNF-α were measured in cultures of naïve $CD4^+CD25^-$ T cells and moDCs in the presence of soluble IL-21, supporting the immunogenic character IL-21 treated moDCs.

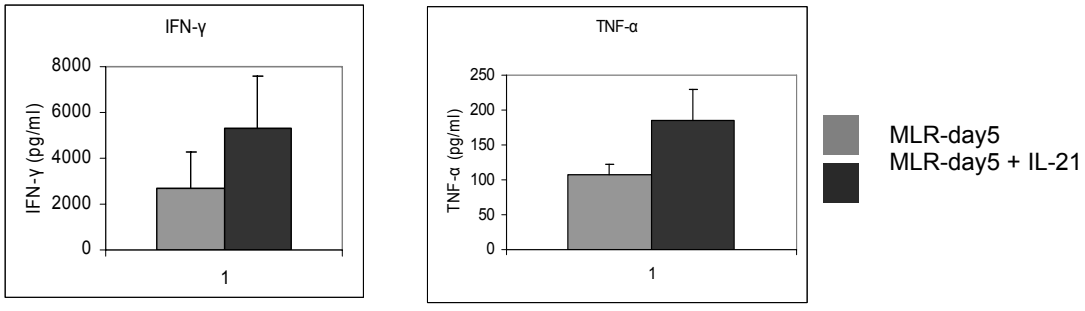

Figure 8: Increased levels of IFN-γ and TNF-α in culture supernatants in the presence of IL-21. Culture supernatants, from the MLRs of moDCs with naïve $CD4^+CD25^-$ T cells in the presence or absence of IL-21, were collected on day 5 and assessed by cytokine enzyme-linked immunosorbent assay for the presence of interferon-γ (IFNγ) and TNF-α. The detection limit of the assay was 15.65 pg/ml for IFNγ and 5,81pg/ml for TNF-α.

5.2.4 IL-21 conditioned-DCs promote T cell proliferation through the downregulation of ICOS-L

Several co-stimulatory molecules on the surface of dendritic cells are implicated in the tolerogenic or immunogenic character that naive T cells acquire after their encounter with dendritic cells. To examine the phenotype that dendritic cells acquire upon IL-21 treatment, we next assessed the levels of several co-stimulatory molecules on the surface of dendritic cells generated and activated in the presence of IL-21. The levels of CD-80, CD-86, CD-40, HLA-DR, ICOS-L and PD-L1 were measured in mature moDCs compared to mature moDCs generated and matured in the presence of IL-21. IL-21 treatment leads to the downregulation of ICOS-L on the surface of dendritic cells, while CD-80, CD-86, CD-40, HLA-DR (results not shown) and PD-L1 are not altered.

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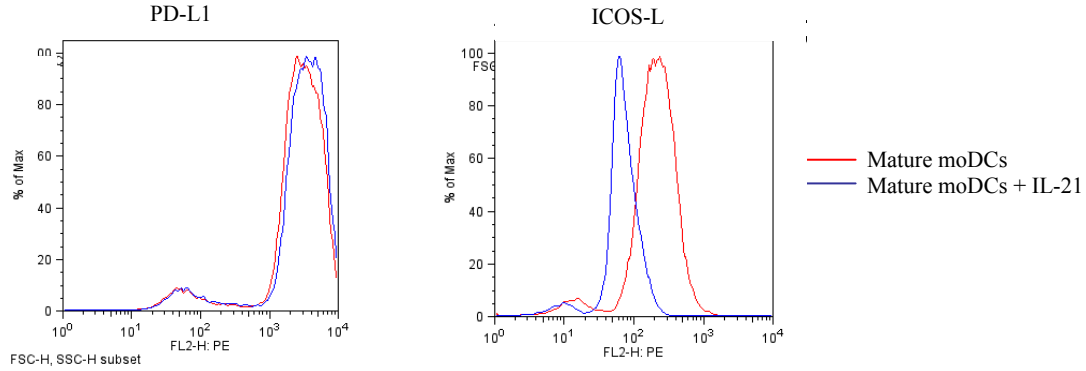

Figure 9: IL-21 treatment results to downregulation of ICOS-L on DCs. The surface expression of the ICOS-L molecule was assessed on T cells cultured with moDCs and moDCs generated and activated in the presence of IL-21. Histograms represent the surface expression of PD-L1 and ICOS-L, on moDCs generated in the presence (blue line) or absence (red line) of IL-21. PD-L1 expression is not altered by IL-21 treatment, whereas ICOS-L is downregulated upon IL-21 treatment.

5.2.5 moDCs matured in the presence of SLE patients serum promote the proliferation of naïve T cells

It is reported that the serum of SLE patients is rich in IL-21[208]. In order to correlate our findings of IL-21 promoting DC immunogenicity with the phenotype that dendritic cells acquire in the microenvironment of the periphery of lupus patients, we next repeated the MLR experiments in the presence of serum obtained from active SLE patients. Proliferation of naïve $CD4^{+}CD25^{-}$ T cells co-cultured with serum-treated moDCs, was promoted in the presence of SLE serum (Figure 9A). Moreover, the addition of SLE serum downregulates ICOS-L on moDCs (Figure 9B). Serum from active SLE patients contains ~600pg/ml of IL-21, assessed by cytokine enzyme-linked immunosorbent assay for the presence of ICOS-L.

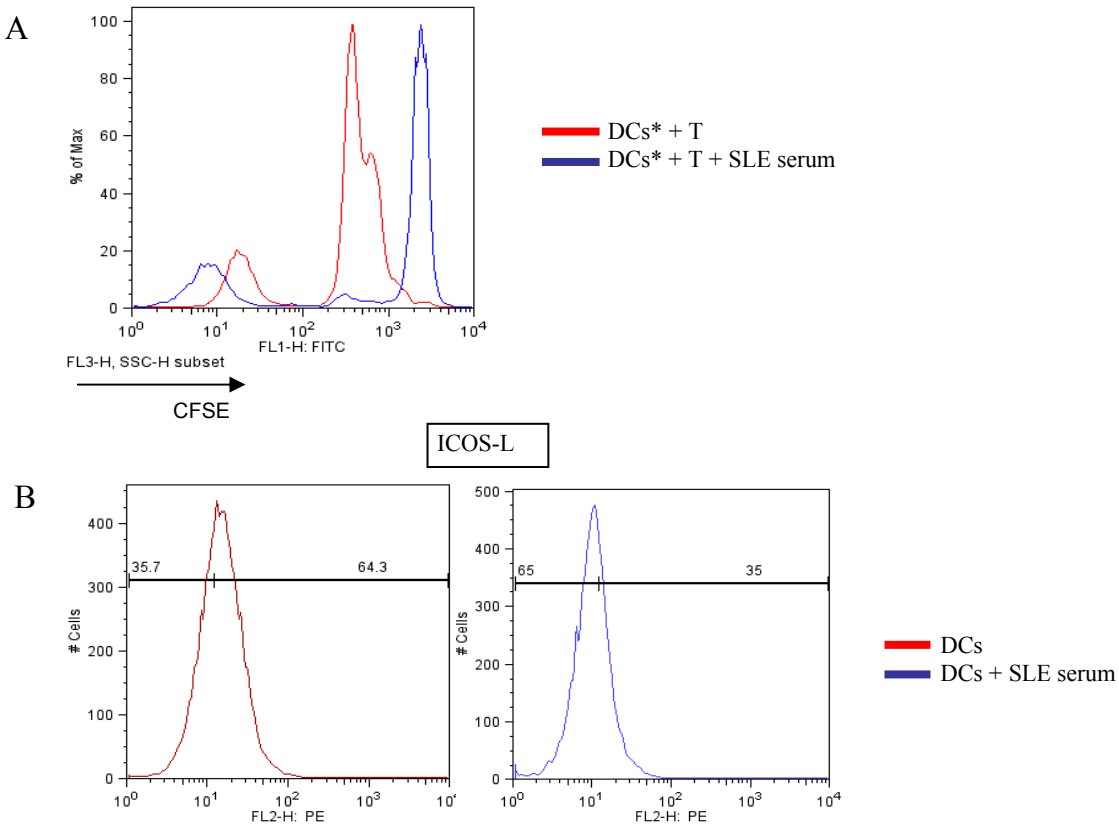

Figure 10: moDCs matured in the presence of SLE patients serum promote the proliferation of naïve T cells. A. Proliferation of T cells cultured with SLE serum-treated DCs, B. ICOS-L expression on SLE serum-treated DCs.

6. Discussion

6.1 Induction of Interleukin-10–Producing Treg Cells by Plasmacytoid Dendritic Cells in Patients With Rheumatoid Arthritis Responding to Therapy

In this study, we have shown that the remission of RA is associated with the restoration of plasmacytoid DC levels in the peripheral blood, and we have provided evidence of an important role for this DC subset in the maintenance of tolerance through the induction of IL-10–secreting Tr1 regulatory cells. Amelioration of disease activity in RA patients resulted in the reestablishment of the levels of both plasmacytoid DCs and myeloid DCs in the circulation. Because plasmacytoid DCs migrate to the sites of inflammation, we sought to determine whether the lack of plasmacytoid DCs in the peripheral blood of patients with active RA may be due to the recruitment of plasmacytoid DCs at the synovium. Although we were unable to detect $CD303^+$ plasmacytoid DCs in the SF of patients with inactive RA, plasmacytoid DCs may still be present at the synovial membrane[222]. There is ample evidence for the functional plasticity of plasmacytoid DCs in directing T cell responses, which mainly depends on their maturation status[223-224]. Thus, virus-infected plasmacytoid DCs elicit a potent Th1 response[225], whereas IL-3–treated plasmacytoid DCs prime Th2 cells in an OX40 ligand– dependent mechanism[108]. In addition, plasmacytoid DCs induce Treg cell–mediated differentiation of naïve T cells, which depends on the activation stimulus used[111,113,226]. Our findings are consistent with those in previous studies in that our results also support the notion that amelioration of an autoimmune disease in humans may be associated with plasmacytoid DC–induced Treg cell development. Based on these observations, we speculate that in patients with active RA, plasmacytoid DCs are exposed to a cytokine milieu and adapt to a tolerogenic phenotype. During disease remission, tolerogenic plasmacytoid DCs are restored in the peripheral blood, where they are able to polarize naive T cells toward IL-10–secreting Treg cells. Although it is not clear whether remission of RA is directly mediated through plasmacytoid DC–primed Treg cells, our results demonstrate that IL-10–secreting Treg cells can potently suppress the activation of naive T cells in vitro, implying a possible role in the homeostatic control of inflammation in vivo. Both naturally occurring Treg cells and Tr1 cells have a crucial role in the induction and maintenance of tolerance to self and foreign antigens[227]. Our results indicate that T cells primed by mature plasmacytoid DCs from patients with

RA in remission have the cardinal features of Tr1 regulatory cells[144], since they secrete high levels of IL-10 and low levels of IFNγ, and they proliferate poorly following polyclonal T cell receptor–mediated stimulation. Most notably, they suppress the activation and proliferation of autologous naive $CD4^+$ T cells, in a dose-dependent manner. Previous studies have convincingly shown that treatment of RA patients with infliximab led to the induction of $Foxp3^+CD25^{high}CD62L^-$ Treg cells that were distinct from natural Treg cells and Tr1 cells[218]. Although we confirmed the presence of $Foxp3^+CD4^+CD62L^-$ T cells in patients with inactive RA (Figure 1A), we were unable to detect Foxp3 expression by plasmacytoid DC–primed IL-10–producing Tr1 cells (results not shown). These results suggest that restoration of tolerance during an autoimmune disease may be mediated by distinct types of Treg cells with specialized functions. Support for this hypothesis has been provided in mouse models, in which distinct types of Treg cells could act in concert to suppress autoimmune phenomena[228]. Secretion of high levels of IL-10 by plasmacytoid DC–primed T cells could have a beneficial role during induction and/or maintenance of remission in these patients. Therefore, the plasmacytoid DC–mediated secretion of IL-10 by Tr1 cells in patients with RA in remission may exert pleiotropic effects toward maintenance of immune tolerance. Secretion of IL-10 by plasmacytoid DC–primed Treg cells might also increase the regulatory activity of other Treg cell subsets[229] or restore the suppressive capacity of Foxp3+ Treg cells that were previously defective. In addition, IL-10 can indirectly inhibit immune responses by abolishing the antigen-presenting ability of APCs, either through down-regulation of the expression of the major histocompatibility complex and costimulatory molecules or through inhibition of cytokine secretion by these cells[230]. Consistent with our findings, an increase in serum IL-10 levels has been demonstrated in patients who have exhibited a prolonged clinical response upon anti-TNF treatment[231-232]. Our results clearly demonstrate that IDO expression by mature plasmacytoid DCs isolated from patients with RA in remission was required for the induction of IL-10–producing Treg cells. Blockage of IDO activity by the inhibitor 1-MT completely reversed the T cell polarization. This is in line with mouse and human studies demonstrating the crucial role of IDO in Treg cell induction[226,233-239]. This reversal was IDO-specific, since blockage of the ICOS pathway or neutralization of cytokines such as TNFα and IL-10 did not affect the ability of plasmacytoid DCs to polarize naïve T cells toward IL-10–producing Treg cells. Recent study findings have suggested that the ICOS pathway is essential for the

induction of anergic T cells with a regulatory phenotype in immature myeloid DCs[240], and therefore it might not be required for the induction of Tr1 cells. Although the exact molecular mechanism that links Treg cell induction with IDO-expressing plasmacytoid DCs is still unresolved, it has been proposed that interactions between Treg cells and plasmacytoid DCs might initiate a so-called reverse-signaling mechanism that leads to the activation of NF-κB signaling and up-regulation of IDO[221,241]. Subsequently, the combined effects of IDO expression and tryptophan depletion may induce Treg cells from naive T cells[242]. What drives the up-regulation of IDO on plasmacytoid DCs in patients with RA who have experienced disease remission remains to be determined. IDO is strongly upregulated on plasmacytoid DCs that have been exposed to IFNγ, a cytokine present during the course of RA[243]. We postulate that plasmacytoid DCs exposed to the cytokine milieu in RA increase the expression of IDO and, upon contact with naive T cells, mediate the conversion of these cells toward Treg cells. In summary, we have shown that plasmacytoid DCs isolated ex vivo from patients with RA in remission display increased IDO expression and prime naïve $CD4^+$ T cells to differentiate in vitro into IL-10–producing Tr1 regulatory cells that potently suppress the proliferation of autologous naive $CD4^+$ T cells. Amelioration of the disease was correlated with the reappearance of circulating plasmacytoid DCs in RA patients, raising the possibility that IDO-expressing plasmacytoid DC–induced Treg cells might be involved, at least in part, in the restoration of immune homeostasis. It is tempting to speculate that plasmacytoid DCs exposed to an autoimmune environment have been conditioned to express a tolerogenic phenotype that induces the de novo generation of Treg cells with the ability to control ongoing immune responses. A better understanding of the pathways involved in the plasmacytoid DC–Treg cell crosstalk will facilitate the exploration of the potential use of cell-based therapies for the induction of tolerance in transplantation and autoimmunity.

6.2 IL-21 conditioned-DCs promote T cell reactivity through downregulation of ICOS-L

We demonstrate that monocyte-derived dendritic cells (moDCs), matured in the presence of IL-21, significantly increased the proliferation of allogeneic $CD4^{+}CD25^{-}$ T cells, as compared to non-IL-21-treated moDCs. The immunogenicity of IL-21-treated moDCs was further increased when IL-21 was present during T cell activation. This enhanced T cell proliferation correlated well with increased secretion of IFN-g and TNF-a in culture supernatants. Compared to untreated moDCs, IL-21 conditioned moDCs exhibited a significant and specific downregulation of the expression of inducible co-stimulator ligand (ICOS-L); in contrast, expression of HLA-DR, CD80, CD86, CD40 and PD-L1 was not altered. Inducible-costimulator ligand (ICOS-L) is expressed on dendritic cells and is implicated in both negative and positive co-stimulation of T cells. Interaction of ICOS/ICOS-L can either augment or inhibit T cell mediated immune responses depending on the in vivo setting. Disrupting ICOS/ICOS-L signaling suppressed intragraft T cell activation and cytokine expression and prolonged allograft survival[216-217]**.** Finally, maturation of moDCs in the presence of serum obtained from active SLE patients, that is previously reported to be rich in IL-21, resulted in enhanced immunogenicity of moDCs and reduced ICOS-L expression on their surface. Together these results suggest that modulation of DCs by IL-21 may represent an important mechanism to explain the increased immunogenicity of DCs in the autoimmune environment. However, the specific mechanism underlying this effect remains to be delineated.

7. Future Directions

Collectively, our data provide the first demonstration that human pDCs, isolated from patients with an autoimmune disease in remission, can mediate the induction of Treg cells. Our results further highlight the intrinsic ability of pDCs to regulate T cell responses in an autoimmune setting in humans. However, several questions that revealed remain to be answered, and the following experiments could be performed to answer them.

In order to better characterize the phenotype of tolerogenic pDCs and the mechanism they use to instruct naïve T cells into regulatory T cells, we will determine genes that are differentially expressed by pDCs in RA patients and healthy controls, using microRNA expression analysis. pDCs will be isolated from healthy and active RA patients with cell sorting, and isolation of RNA and DNA microarray analysis will follow. Results will be confirmed by RT-PCR. Validation of the identified genes on their role in Treg induction will be performed in RA animal models such as collagen induced arthritis.

Specifically experiments in animal models of rheumatoid arthritis will further help delineate the molecular mechanism used in the induction of Tregs by pDCs. Mouse models of RA will be used (Antigen induced Arthritis, or Collagen induced Arthritis models), and pDCs isolated from these mice will be transferred into syngeneic mice, in order to examine whether they can induce Tregs. Secondly, we will study how pDC depletion in vivo will lead to autoimmunity. To deplete the pDC population, mice will be injected with the pDC-depleting antibody (120G8 depleting mAb), and the disease should deteriorate.

Studying how IL-21 promotes T cell response, a remaining question to be answered is how IL-21 modulates DCs function. To this end, IL-21 treated DCs will be cultured with naïve T cells, and we will assess the polarization of T cells into Th1, Th17 and Treg cells. Naïve T cells $CD4^{+}CD25^{-}$ will be cultured with cytokine mixtures that favor the induction of the respective T cell subsets, IL-12 and IL-2 for Th1, TGF-β and IL-6 for Th17 and TGF-β for Treg; in all conditions aCD3 should be present plus IL-21 treated or not DCs. The T cells that will be induced from IL-21-treated DCs will be adoptively transferred during CIA and we will monitor their role in perpetuating or suppressing disease. To this end, we will monitor clinical score (paw

swelling, immunohistochemistry of the joints to determine cell infiltration, re-stimulation of draining lymph nodes to assess immune responses).

Additionally, culture supernatants will be collected and assessed by immunosorbent assay for the presence of IL-12, IFN-γ and IL-10. Accordingly, the T cell polarization can be characterized using intracellular staining of $CD4^+$ T cells for IL-12, IFN-γ or IL-10. Moreover, in mouse models of RA, using an IL-21 neutralizing antibody, we can assess the disease activity by histology, and also characterize the DCs subsets and profile, by measuring the cytokine secreted and the expression of co-stimulatory molecules that are up-regulated. Finally, we will assess the populations of T cells in the lymph nodes, spleen and synovia, and characterize the different populations of Th17, Th1 and Treg cells.

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