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Delineation of the tolerogenic function of plasmacytoid dendritic cells in Rheumatoid arthritis

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Διερεύνηση της ανοσοκατασταλτικής δράσης των πλασματοκυτταροειδών δενδριτικών κυττάρων στην Ρευματοειδή αρθρίτιδα

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TABLE OF CONTENTS

ABSTRACT

Rheumatoid arthritis (RA) is a chronic immune inflammatory disease characterized by synovial hyperplasia, joint destruction and extra-articular manifestations. RA presents local inflammation in the joints, which subsequently develops into a systemic disorder as a result of the loss of immune tolerance. The inflammation in the joints is the result of a tight interaction between resident cells, such as fibroblast-like synoviocytes (FLS) and cells of the innate and adaptive immune system. Moreover, one of the major contributors in RA pathogenesis are inflammatory cytokines, mostly TNF-α and IL-6, which orchestrate innate and adaptive immune system activation, playing a key role in the tissue damage.

Plasmacytoid dendritic cells (pDCs) are a unique subset of dendritic cells (DCs), specialized in producing large amounts of type I IFNs following their recognition of viruses or self nucleic acids through Toll-like receptor 7 (TLR7) and TLR9. However, pDCs can also secrete other pro-inflammatory cytokines and chemokines, and function as antigen presenting cells (APCs). Interestingly, pDCs can participate in the priming of both immunogenic and tolerogenic immune responses. The role of pDCs in inflammatory arthritis has not been extensively investigated. Data from animal models have shown a rather immunoregulatory role. In addition, a previous study from our laboratory has demonstrated that RA patients in remission have higher number of pDCs in peripheral blood compared to active RA patients, which have a tolerogenic phenotype and induce IL-10 secreting Treg cells in vitro. In this study we investigated the molecular mechanism through which human pDCs contribute to restoration of tolerance in RA.

To address this question, we performed transcriptomic analysis (microarray) of pDCs of RA patients responding to anti-TNF therapy and pDCs derived from healthy donors. The results from microarrays revealed more than 6000 genes differentially regulated between RA and healthy pDCs. Interestingly, IL-6R was significantly upregulated on RA pDCs. Given that IL-6 has a major role in the pathogenesis of RA, we focused on its receptors and IL-6 signaling pathway. We firstly validated the result in a new cohort of RA patients, and we found increased IL-6R as well as pSTAT1 expression on pDCs of RA patients on remission as compared with healthy pDCs. These findings indicate that IL-6/IL-6R pathway is up-regulated and activated. Therefore, and based on the aforementioned data supporting a regulatory role of pDCs in the context of arthritis, we hypothesized that the increased IL-6 signaling pathway may enhance the tolerogenic phenotype of pDCs. In order to study our hypothesis, we performed in vitro experiments on isolated healthy pDCs treated with CpG-A, as a general stimulator of pDCs, and recombinant IL-6 (rIL6). Interestingly, we found that IL-6 signaling did not further induce IFN-a expression nor activation status of pDCs, but significantly decreased TFN-a production by pDCs. Considering that ADAM17 mediates the generation of soluble TNF, we assessed the effect of IL6 treatment on the function of ADAM17 in pDCs. We concluded with a trend towards decreased mature ADAM17 expression on the surface of pDCs upon IL-6 signaling. Moreover, IL-6R is another substrate cleaved by ADAM17. We thus assessed the effect of IL-6 signaling on soluble IL-6R secretion by pDCs. We found that IL-6 induces decreased production of soluble IL-6R in culture supernatants of pDCs, further supporting an inhibitory effect of IL-6 signaling in ADAM17

function. Collectively, we had strong evidence that IL-6 signaling on pDCs mediates impaired ADAM17 activity.

In summary, our findings delineated a novel role of pDCs in the induction of tolerance in RA and leaded us to the hypothesis that increased IL-6 signaling on pDCs inhibits ADAM17 activity, therefore limiting TNFa secretion and this process may enhance the tolerogenic phenotype of pDCs.

ΠΕΡΙΛΗΨΗ

Η Ρευματοειδής Αρθρίτιδα (ΡΑ) είναι μια χρόνια φλεγμονώδης νόσος που χαρακτηρίζεται από υπερπλασία, καταστροφή των αρθρώσεων και εξω-αρθρικές εκδηλώσεις. Η ΡΑ παρουσιάζει τοπική φλεγμονή στις αρθρώσεις και σταδιακά εξελίσσεται σε μια συστηματική αυτοάνοση νόσο, αποτέλεσμα της απώλειας ανοχής του ανοσοποιητικού συστήματος. Η φλεγμονή στις αρθρώσεις είναι αποτέλεσμα μιας στενής αλληλεπίδρασης μεταξύ των κυττάρων που βρίσκονται στις αρθρώσεις και τα κύτταρα του έμφυτου και επίκτητου ανοσοποιητικού συστήματος. Επιπλέον, ένας από τους σημαντικότερους παράγοντες στην παθογένεια της ΡΑ είναι οι φλεγμονώδεις κυτταροκίνες, κυρίως ο TNF-α και η IL-6, οι οποίες συντονίζουν την ενεργοποίηση τόσο του έμφυτου όσο και του επίκτητου ανοσοποιητικού συστήματος, διαδραματίζοντας σημαντικό ρόλο στη βλάβη των ιστών.

Τα πλασματοκυτταροειδή δενδριτικά κύτταρα (pDCs) αποτελούν ένα ξεχωριστό πληθυσμό δενδριτικών κυττάρων (DCs), που ειδικεύονται στην παραγωγή μεγάλων ποσοτήτων IFN τύπου Ι μετά την αναγνώρισή ιών ή νουκλεϊνικών οξέων εαυτού, μέσω των υποδοχέων TLR7 και TLR9. Ωστόσο, τα pDCs μπορούν επίσης να εκκρίνουν άλλες προ-φλεγμονώδεις κυτταροκίνες και χημειοκίνες και να λειτουργούν ως αντιγονοπαρουσιαστικά κύτταρα (APCs). Είναι ενδιαφέρον, ότι τα pDCs μπορούν να επάγουν ανοσολογικές αποκρίσεις αλλά έχουν και ανοσοκατασταλτικό ρόλο. Ο ρόλος των pDCs στη φλεγμονώδη αρθρίτιδα δεν έχει διερευνηθεί εκτενώς. Τα δεδομένα από ζωικά μοντέλα έχουν δείξει ότι τα pDCs διαδραματίζουν ανοσορρυθμιστικό ρόλο. Επιπρόσθετα, προηγούμενη μελέτη από το εργαστήριό μας έδειξε ότι οι ασθενείς με ΡΑ σε ύφεση έχουν υψηλότερο αριθμό pDCs στην περιφέρεια σε σύγκριση με ασθενείς με ενεργή νόσο, και έδειξαν λειτουργικά ότι τα pDCs των ασθενών διαθέτουν ανοκατασταλτικό ρόλο, επάγοντας την διαφοροποίηση των Τ ρυθμιστικών κυττάρων που εκκρίνουν IL-10 in vitro. Ο στόχος της παρούσας ερευνητικής μελέτης ήταν να διερευνήσουμε τον μοριακό μηχανισμό μέσω του οποίου τα ανθρώπινα pDCs συμβάλλουν στην αποκατάσταση της ανοχής στην RA.

Στην παρούσα μελέτη, μέσω μεταγραφικής ανάλυσης (microarray) των pDCs, δείξαμε ότι περισσότερα από 6000 γονίδια ρυθμίζονται διαφορικά στα pDCs από ασθενείς με ΡΑ που ανταποκρίνονται σε anti-TNF θεραπεία και στα pDCs που προέρχονταν από υγιείς δότες. Το πιο ενδιαφέρον ήταν ότι η έκφραση του υποδοχέα της IL-6 (IL-6R) ήταν σημαντικά αυξημένη στα pDCs των ασθενών. Δεδομένου ότι η IL-6 κατέχει σημαντικό ρόλο στην παθογένεια της ΡΑ, επικεντρώσαμε στο σηματοδοτικό μονοπάτι της IL-6. Αρχικά επιβεβαιώσαμε σε μια νέα ομάδα ασθενών τόσο την αυξημένη έκφραση του IL-6R όσο και της pSTAT1 στα pDCs ασθενών με ΡΑ σε ύφεση σε σύγκριση με τους υγιείς, δείχνοντας ότι το μονοπάτι της IL-6 είναι ενεργό. Στη συνέχεια υποθέσαμε ότι η αυξημένη σηματοδότηση της IL-6 ενισχύει την γνωστή όπως προαναφέρθηκε ανοσορρυθμιστική δράση των pDCs. Προκειμένου να μελετηθεί ο μοριακός μηχανισμός της σηματοδότησης της IL-6 στα pDCs, πραγματοποιήσαμε in vitro πειράματα σε απομονωμένα υγιή pDCs που υποβλήθηκαν σε διέγερση με CpG-A, που επάγει την ενεργοποίησή τους και ανασυνδυασμένη IL6 (rIL6). Έτσι, δείξαμε ότι η σηματοδότηση της IL-6 ούτε προκάλεσε διαφορά στην έκφραση της IFN-a στα pDCs και ούτε αύξησε την αντιγονοπαρουσιαστική τους ικανότητα. Αντίθετα μείωσε σημαντικά την παραγωγή TFN-a από τα pDCs. Λαμβάνοντας υπόψη ότι η

μεταλλοπρωτεϊνάση ADAM17 μεσολαβεί στην παραγωγή του διαλυτού TFN-a, αξιολογήσαμε την επίδραση της θεραπείας με IL6 στη δράση της ADAM17 στα pDCs. Το πείραμα αυτό φανέρωσε ότι ενεργοποίηση του μονοπατιού της IL-6/IL-6R οδήγησε σε μια μικρή μείωση στην έκφραση της ADAM17 στην επιφάνεια των pDCs. Επιπλέον, ο IL-6R, που κόβεται από την ADAM17, βρέθηκε σημαντικά μειωμένος σε υπερκείμενα καλλιέργειας των pDCs με rIL6. Συνεπώς, είχαμε ισχυρές ενδείξεις ότι η σηματοδότηση της IL-6 επί των pDCs οδήγησε σε ελλαττωματική δραστικότητα της ADAM17 στα pDCs.

Συνολικά, τα δεδομένα αυτά αποκαλύπτουν ένα καινοτόμο ρόλο των pDCs στην επαγωγή ανοσολογικής ανοχής στην PA και στηρίζουν την υπόθεση ότι η αυξημένη σηματοδότηση της IL-6 στα pDCs αναστέλλει τη δραστικότητα της ADAM17, περιορίζοντας έτσι την έκκριση της TNF-α και αυτή η διαδικασία μπορεί να ενισχύσει την ανοσοκατασταλτική δράση των pDCs.

INTRODUCTION

Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease, affecting 1% of the population worldwide [1]. RA is characterized by autoantibody production (rheumatoid factor [RF] and anti–citrullinated protein antibody [ACPA]) and inflammation of the synovial tissues of the joints, producing hyperplasia and pain. A hallmark of RA is the progressive joint damage that causes bone and cartilage destruction, which are related to functional disability. As the disease develops, chronic inflammatory burden potentially affects a variety of extra-articular organs as a result of the loss of immune tolerance, while specific comorbidities have been associated to RA [2].

RA is a multifactorial disorder and it is established that both genetic and environmental factors may trigger the disruption of tolerance to self-antigens, leading to uncontrolled inflammation and autoimmunity [3]. The HLA system (particularly HLA-DRB1) remains the predominant genetic predisposing factor, implicating T cells, dendritic cells (DCs) and self-peptide binding process in pathogenesis of mainly RF and ACPA positive group [4]. However, more than 100 common variants in non-HLA loci have been implicated in RA susceptibility. In addition, it has been proposed that environmental factors like smoking, hormones, infection and gut microbiota are involved in the induction of the RA in genetically predisposed individuals via activation of type 17 helper T (Th17) cells, leading to the development of joint inflammation [5].

THE PATHOGENESESIS OF RA

The inflammation in the joints is the result of a tight interaction between resident cells, such as fibroblast-like synoviocytes (FLS) and cells of the innate and adaptive immune system (*Figure 1*). The RA synovium is rich in innate immune cells, including macrophages, mast cells, natural killer (NK) cells, neutrophils, myeloid and plasmacytoid DCs [6]. Macrophages are considered to have a effector role in the pathogenesis of RA, by releasing Tumor necrosis factor-alpha (TNF-α), interleukins (IL-1, IL-6, IL-12, IL-15, IL-18, IL-23), reactive oxygen and nitrogen species, production of prostanoids and matrix-degrading enzymes, phagocytosis and antigen presentation [7]. The interplay of cells, as well as the secretion of inflammatory cytokines, like TNF-α and IL-6, lead to the activation of RA FLS. These cells secrete cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMPs), contributing to the

destruction of the cartilage and bone tissues which? migrate from the affected to the healthy tissue, contributing to the spread of arthritis [8].

Besides the innate immune system, the dysregulation of the adaptive immune responses plays a critical role in the development of RA. Infiltration of leukocytes into the synovial joint results in synovitis. RA is mainly considered to be a disease that is mediated by type 1 helper T (Th1) cells. However, several studies have focused on the role of Th17 cells, a CD4+ subset that produces IL-17A, 17F, 21, and 22 and TNF-α [9]. IL-17A, which synergizes with TNF-α to promote activation of fibroblasts and chondrocytes, is currently being targeted in clinical trials [10]. T regulatory (Treg) cells are often detected in tissues from patients with RA, but appear to have limited functional capability [11]. Pathogenic T cells not only help B cells to produce autoantibodies, but also actively mediate tissue destruction by secreting pro-inflammatory cytokines (IL-17, IL-6, and TNF-α) and creating an inflammatory microenvironment that favors macrophage and neutrophil recruitment and osteoclast activation [12].

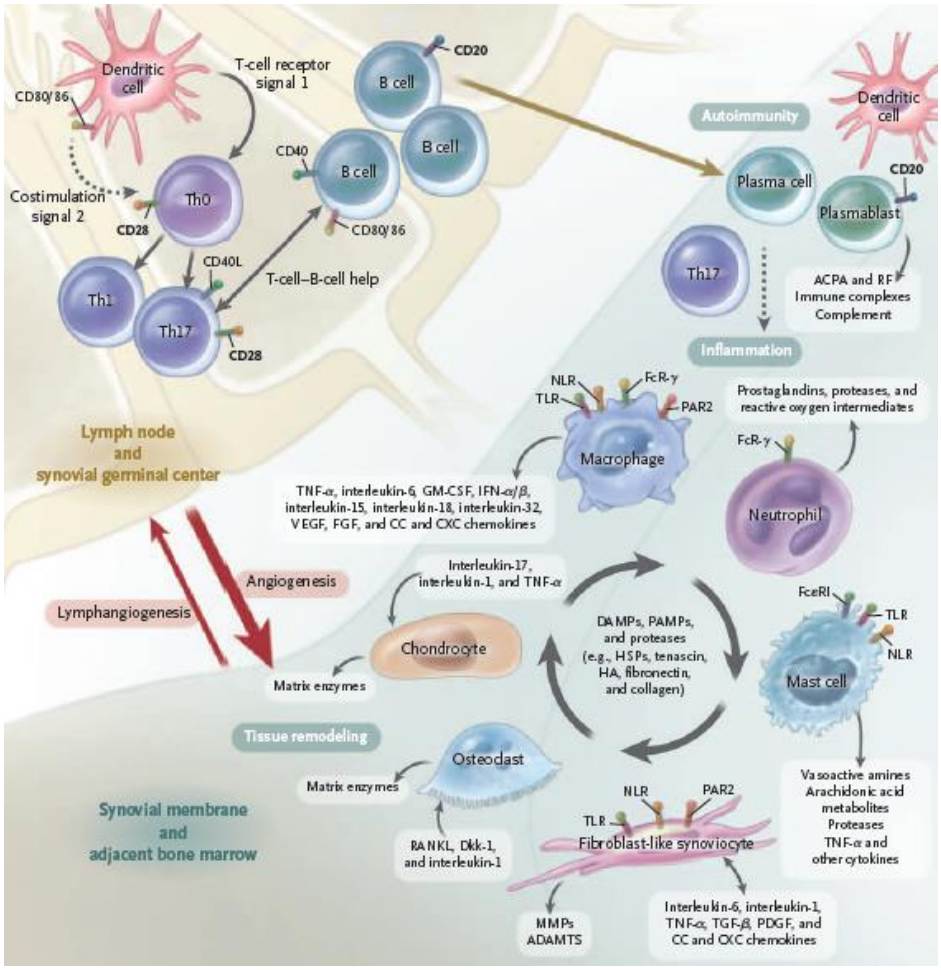


Figure 1: Innate and adaptive immune responses in the RA joints. In the lymph node, DCs interact with T cells and B cells, resulting in the generation of autoimmune response to ACPA. In the synovial membrane and adjacent bone marrow, adaptive and innate immune pathways promote tissue remodeling and damage. The interactions among leukocytes, synovial fibroblasts, chondrocytes, and osteoclasts, together with the molecular products of

damage, drive the chronic phase in the pathogenesis of rheumatoid arthritis. The figure is derived from McInnes et al, 2011 [3].

Soluble mediators, autoantibodies, adhesion molecules and signal transduction pathways control the human immune system and are involved in autoimmune diseases. One of the major contributors in RA pathogenesis are inflammatory cytokines, mainly TNF-α and IL-6. They orchestrate innate and adaptive immune system activation contributing both in disease initiation and perpetuation, while their role in tissue damage has been proven [13]. The remarkable clinical benefit upon blocking TNF-α or IL-6 in patients with RA also confirms their significant role in the pathogenesis of the disease [2].

THE IL-6 SIGNALING IN IMMUNE RESPONSES

IL-6 is secreted by a large number of cell types, including T and B cells, monocytes, fibroblasts and synoviocytes [14]. There are two mechanisms through which IL-6 exerts its biological effects: classical IL-6 receptor (IL-6R) signal transduction via the membrane-bound IL-6R and IL-6 'trans-signaling', a process in which soluble IL-6R binds IL-6 [15]. IL-6 forms a complex by binding to membrane or soluble IL-6R on the cell membrane, which then combines with gp130, which is ubiquitously expressed on the cell membrane, forming a homodimer and giving rise to intracellular signal transduction, including the phosphorylation of tyrosine kinases of the Janus kinase (JAK) family, as well as the recruitment and activation of signal transducers and activators of transcription (STAT)-1 and STAT-3 [16] (*Figure 2*). Interestingly, the expression of IL-6R on the cell surface is restricted only on a few cell types, such as macrophages, neutrophils, some T cell populations and hepatocytes. The soluble IL-6R is released by activated cells mainly via ectodomain shedding and the IL-6 trans-signaling has been associated with pathophysiological situations [17, 18]. Consequently, many cells that lack membrane IL-6R can still respond to IL-6 through trans-signaling, in contrast to other soluble receptors, such as the receptors for IL-1 and TNF-α, which are known to inhibit the effects of their ligands [19].

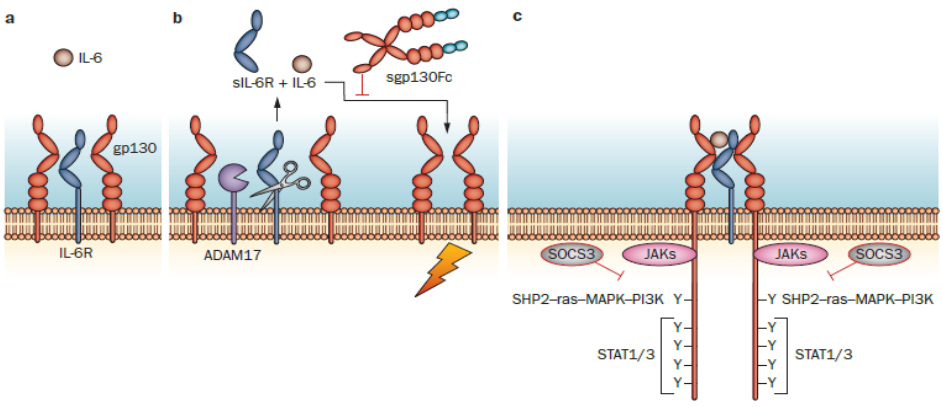


Figure 2: IL-6 signaling via the membrane and soluble receptor. A) In classic signaling, IL‑6 binds to the membrane-bound receptor and this complex associates with the membrane protein gp130, initiating dimerization and intracellular signaling. B) In trans-signaling, membrane IL‑6R is cleaved mainly by metalloprotease ADAM17 in the cell surface, and soluble IL-6R is secreted. The IL‑6/soluble IL-6R complex associates with membrane gp130 on cells that do not express the membrane IL‑6R, to induce dimerization and intracellular signaling. sgp130Fc specifically blocks IL‑6 trans-signaling without affecting IL-6 classic signaling. C) Upon gp130 dimerization, JAKs become activated and phosphorylate gp130, which subsequently stimulates the MAPK and STAT pathways. The STAT proteins are phosphorylated, dimerize, and translocate to the nucleus, where they induce transcription of target genes. Figure source Calabrese et al, 2014 [20].

IL-6 is a pleiotropic cytokine and the classic IL-6 signaling or trans-signaling leads to the activation of distinct pathways. In the classic signaling IL-6 binds to the membrane IL-6R, leading to anti-inflammatory responses. In contrast, signaling via soluble IL-6R often leads to pro-inflammatory responses. This is further supported by in vivo studies showing that the therapeutic targeting of IL-6 trans-signaling blocked the development of arthritis [21].

IL-6 exerts systemic effects on multiple tissues and cells of the immune system. In the context of RA, IL-6 promotes the expansion of fibroblasts and induces neovascularization in the synovial tissue. IL-6 also promotes the infiltration of inflammatory cells, such as macrophages and neutrophils and drives the differentiation of B cells into autoantibody-secreting plasma cells [22, 23]. It also promotes differentiation of T follicular helper (Tfh) cells, which is necessary for T cell-dependent B cell response. IL-6 stimulates osteoblasts and synoviocytes to produce receptor activator of nuclear factor (NF)-kB ligand (RANKL), which activates osteoclasts resulting in joint destruction [24]. In addition, IL-6 promotes the expansion of CD4+ T cells and acts as a checkpoint in the differentiation of naïve T cells towards pro-inflammatory Th17 cells or regulatory Treg cells. The balance between regulatory and effector T cells is crucial to the maintenance of self-tolerance. IL-6 seems to inhibit Treg differentiation, whereas favors the development of Th17 cells [25]. It has been shown that IL-6-deficient mice fail to develop a Th17 response, while Treg cells are dominant in the periphery [26].

Direct evidence of a relationship between IL-6 signaling and chronic joint inflammation in RA arises from the findings that IL-6 and soluble IL-6R concentrations are elevated in serum and synovial fluid of RA patients, and that IL-6 serum levels correlate positively with disease activity [27]. In accordance, it has been reported that IL-6-deficient mice are resistant to the induction of arthritis, and that blocking the soluble IL-6R reduces the incidence of arthritis in mice [28, 29].

THE ROLE OF TNF-A IN RA

TNF-α is produced mostly by macrophages, although it can also be secreted by other immune cell types like, CD4+ T cells, NK cells, neutrophils, mast cells. TNF can signal via two

receptors, TNFR1 and TNFR2. TNFR1 is expressed in most tissues and binds to the membrane and soluble TNF-α, while TNRF2 is found in immune cells and binds to the membrane form of TNF-α.

Several studies have focused on the effector functions of TNF-α. Regarding RA, it has been shown that TNF-α induces the production of other pro-inflammatory cytokines, including IL-1 and IL-6. TNF-α also promotes endothelial cell activation and amplifies the release of chemokines, leading to leukocytes attraction from the periphery into the inflamed tissue. In addition, it has been proven that TNF-α induces angiogenesis and initiates osteoclast and chondrocyte activation, causing destruction of bone and cartilage [30].

In the synovium and serum of RA patients TNF-α is a major cytokine, and it is considered to be a potent inducer of other pro-inflammatory cytokines disturbing the normal physiological balance between pro- and anti-inflammatory mediators. Furthermore, TNF-α receptors have been found to be expressed in human rheumatoid joint tissue [31, 32]. Additionally, inan animal model of RA, it has been reported that administration of a mAb specific for mouse TNF-α after disease onset ameliorated both inflammation and joint damage [33].

THE ADAM17 FUNCTION IN THE IMMUNE SYSTEM

A Disintegrin and Metalloproteinase 17 (ADAM17) is one of the major sheddase involved in a variety of physiological and pathophysiological processes. ADAM17 is ubiquitously expressed and cleaves a broad range of cell surface molecules, the best studied among them being TNF-α, EGFR and IL-6R [34] (*Figure 3*). The key role of ADAM17 implies that its activity has to be tightly regulated. In cells, ADAM17 exists mainly as immature form, which translocate to Golgi apparatus via iRhoms, where its maturation takes place [35]. Most of the mature ADAM17 is located intracellularly, whereas only a small amount is located at the cell surface, where shedding takes place. After cell activation, PKC signaling promotes the translocation of ADAM17 to the cell surface. The rapid increase of membrane ADAM17 is followed by internalization and lysosomal degradation of ADAM17 [36] (*Figure 4*).

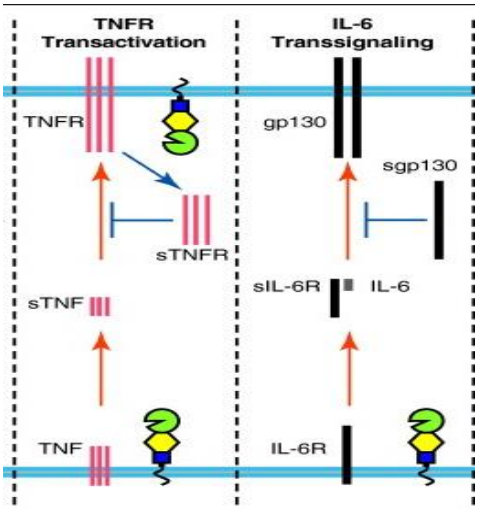


Figure 3: ADAM17 cleaves various transmembrane proteins, including TNF-α, TNFR and IL-6R. ADAM17 induces 'TNFR-transactivation' via soluble TNF-α, TNF-α inhibition via generation of an antagonistic soluble TNFR. In addition, ADAM17 is the master regulator for the generation of the soluble IL-6R.The figure is derived from Scheller et al, 2011 [37].

More than 90% of mature TNF-α in the circulation is derived from the direct action of ADAM17. It has been shown that mice with a specific depletion of the ADAM17 gene in myeloid cells were protected from endotoxin shock lethality and produced reduced amounts of TNF-α [38]. Moreover, it has been found that ADAM17 contributes to the production of TNF-α in synovial tissues from RA patients and in human RA cartilage ADAM17 is overexpressed. [39]. This target has been validated in preclinical trials for the treatment of RA and studies in animal models of arthritis showed that inhibition of ADAM17 leads to amelioration of disease symptoms [40, 41].

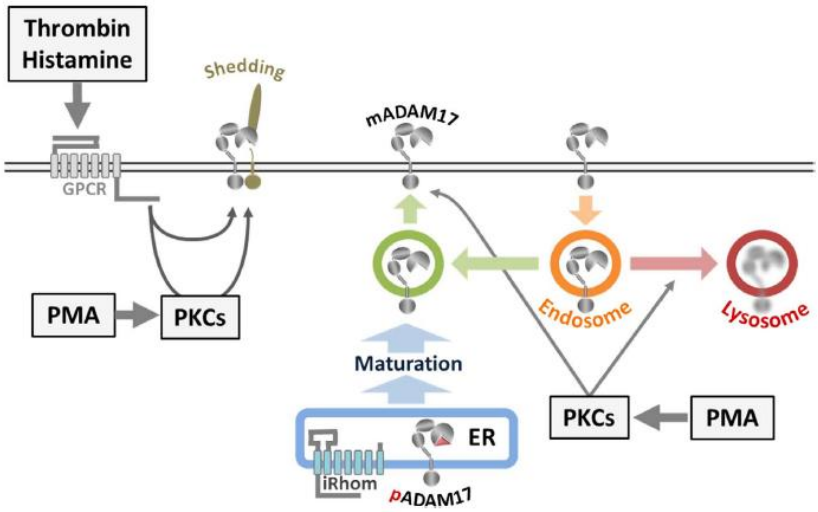


Figure 4: ADAM17 exists as immature proform (pADAM17) and as mature protease (mADAM17) in cells. The maturation of ADAM17 takes place in the Golgi apparatus. The majority of mADAM17 is located intracellularly, and PKC activation influences ADAM17 surface expression. ADAM17-mediated shedding takes place at the cell surface. After shedding process, most of the mADAM17 at the cell surface is internalized and degraded through lysosomes. The figure obtained from Lorenzen et al, 2016. [36].

Plasmacytoid dendritic cells (pDCs) and their multifactorial role

Plasmacytoid dendritic cells (pDCs) are a unique subset of DCs that originate from the bone marrow and display plasma cell morphology. The most commonly used cell surface markers to identify human pDCs are the blood-derived dendritic cell antigen-2 (BDCA-2), BDCA- 4 and immunoglobulin-like transcript 7 (ILT7). They also express CD4 and IL-3R-a (also known as CD123) but not CD11c, which is a common DC marker. They represent a rare cell population (<1% of peripheral blood immune cells) and the distinctive feature of them is that pDCs specialize in producing type I IFNs following the recognition of viruses or self nucleic acids. Indeed, pDCs produce large amount of type I IFNs, which is 200 to 1000 times more than the amount produced by any other blood cell type [42]. By contrast myeloid DCs have the ability to produce high levels of IL-12, but only low levels of type I IFNs.

In humans, pDCs selectively express endosomal Toll-like-receptors (TLR) 7 and TLR9 that sense pathogens and endogenous nucleic acids. Activation of these receptors in pDCs leads to secretion of type I IFNs via myeloid differentiation primary response protein 88(MYD88)-interferon-regulatory factor 7 (IRF7) pathway, as well as the production of pro-inflammatory cytokines and chemokines via the MYD88- nuclear factor-kB(NF-kB) [43].

pDCs play a major role in both innate and adaptive immune responses and several studies strongly support that pDCs possess a multifactorial role [44]. Type I IFNs derived from pDCs apart from antiviral responses, also activate NK cells and, in conjunction with IL-6, influence B cell activation and antibody secretion by plasma cells [45, 46]. However, pDCs can also secrete other pro-inflammatory cytokines and chemokines, including TNF-α, IL-6, IL-12, CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand 3 (CCL3) and CCL4. Moreover, expression of MHC class II and co-stimulatory molecules enables pDCs to present antigens to CD4+ T cells. pDCs have the ability to increase the antigen presenting capacity of myeloid DCs to CD8+ T cells and promote the differentiation of naïve T cells into Th1 cells. Interestingly, pDCs can participate in the priming of both immunogenic and tolerogenic adaptive immune responses. pDC expression of indoleamine 2,3-dioxygenase (IDO) and inducible T cell co-stimulator ligand (ICOSL) and pDC production of transforming growth factor-β (TGFβ) and IL-6 promote Treg cell or Th17 cell commitment, respectively [47, 48]. Thus, the biology of pDCs is multifaceted (*Figure 5*). Human pDCs also continue to be extensively studied in order to understand their potential roles in the pathogenesis of autoimmune diseases, cancer and HIV infection.

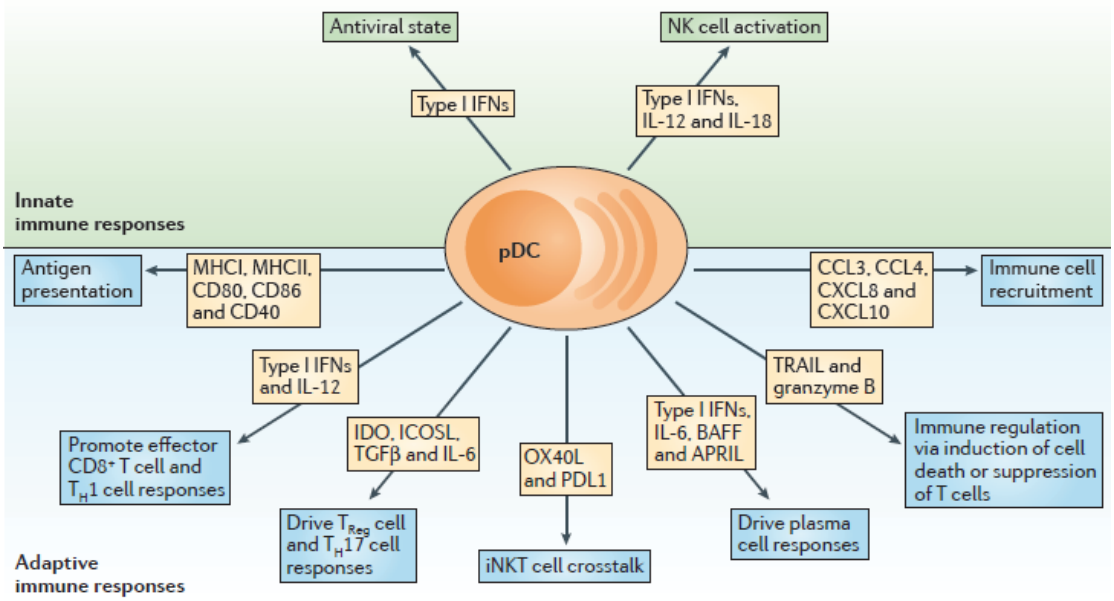


Figure 5: pDCs are specialized cells that produce high amounts of type I IFNs in response to viruses. They also secrete other pro-inflammatory cytokines and chemokines and they are characterized by their antigen presenting capacity to T cells. pDCs contribute to inflammatory responses by promoting NK activation and attraction of immune cells to the sites of infection, enhancing Th1 and Th17 cell responses and influencing B cell activation and antibody secretion. Nevertheless, they also induce immune regulation and tolerance, expressing anti-inflammatory molecules that drive Treg cell responses. The figure is derived from Swiecki et al, 2015 [44].

THE ROLE OF PDCS IN AUTOIMMUNE DISEASES AND RA

Infiltration of pDCs into involved tissues and evidence of interferon responses have been found in a number of autoimmune disorders. pDCs secrete large amounts of type I IFNs, which are inflammatory cytokines of greatest importance in the pathophysiology of many autoimmune and autoinflammatory diseases [44]. As a result, pDCs have been implicated in the pathogenesis of autoimmune diseases characterized mostly by type I IFN signature, such as systemic lupus erythematosus (SLE). Indeed, studies in lupus-prone mice that lack pDCs have shown reduced antibody production against nuclear autoantigens (ANA), impaired expansion and activation of T and B cells, confirming that pDCs are pathogenic in SLE [49, 50]. In addition, pDC depletion or blocking of TLR7 and TLR9 signaling in lupus-prone mice, ameliorate autoimmune skin inflammation [51]. Moreover, in mouse models of psoriasis and type I diabetes (T1D), the blockade of type I IFNs or antibody-mediated depletion of pDCs seems to be protective [52, 53]. On the other hand, many studies have suggested a protective role of pDCs and IFN-I. In experimental autoimmune encephalomyelitis (EAE), IFNAR-deficient in central nervous system (CNS) myeloid cells exacerbates disease development. In addition, pDCs have been shown to inhibit myeloid DC functions and the development of Th17 responses, whereas pDC depletion increases EAE severity [54, 55]. Moreover, pDC function seems to be effective in preventing the development of asthma. In

mouse asthma model, pDCs depletion induces classic features of asthma, whereas the adoptive transfer of pDCs before sensitization prevented disease's initiation [56]. Accordingly, an immune regulatory role of pDCs has been shown in transplantation [57].

The role of pDCs in RA remains controversial. Data from animal models have shown a rather immunoregulatory role. Investigation of the contribution of pDCs to experimental arthritis in various animal models, concludesthat pDC depletion in vivo enhanced the severity of articular pathology and increased T and B cell autoimmune responses against type II collagen [58]. In addition, it has been shown that recruitment and activation of pDCs in arthritic joints of pDC-deficient mice significantly reduce arthritis [59]. Moreover, in the antigen induced arthritis model (AIA) it has been shown the IFN-a secreted by pDCs activates an IDO1/TGF-b–dependent anti-inflammatory program that upon antigenic rechallenge prevents inflammation [60].

Our lab has a special interest in pDCs biology in autoimmune diseases. We have previously demonstrated that RA patients on remission with anti-TNFa agents have higher number of pDCs in peripheral blood compared to active RA patients. In functional studies we found that pDCs from RA patients in remission have a tolerogenic phenotype and induce IL-10 secreting Treg cells in vitro [61].

Aim of the study

It is known that a significant percentage of RA patients under therapy, does not achieve remission. It is of major immunological importance to investigate targeted, cell-specific mechanism contributing to the induction of remission. Elucidating those mechanisms could pave the way of therapeutic strategies to increase the number of patients achieving remission.

We have demonstrated that pDCs are increased in the periphery of RA patients on remission and exert a regulatory effect [61]. In this study we aim to investigate the molecular mechanism through which human pDCs contribute to restoration of tolerance in RA. In order to better characterize those pDCs we will perform transcriptomic analysis (microarray) of pDCs from RA patients in remission and pDCs derived from healthy donors. Then, we will investigate how pathways, with key role in pathogenesis of RA, are regulated on pDCs.

MATERIALS AND METHODS

Human subjects

Peripheral blood samples were obtained from RA patients in remission with anti-TNF therapy, RA patients with active disease activity and healthy individuals. Active disease activity was defined as a Disease activity score (DAS28) higher than 3,2. The samples were recruited from Rheumatology clinic at the University Hospital of Heraklion (Crete, Greece). The study was approved by the Institutional Review Committee, and all subjects gave written informed consent. Healthy samples and buffy coats were recruited from the University Hospital of Heraklion and Venizeleio Hospital (Crete, Greece).

Serum collection

Healthy and RA peripheral blood sera were added in a collection tube without anticoagulants. The tube was centrifuged at 2500 rpm for 15minutes and the serum was collected under sterile conditions.

Reagents

RPMI-1640 (Gibco), fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μg/ml), were all from Gibco, Carlsbad, CA. For culture of pDCs Recombinant IL-6, was purchased from PeproTech EC Ltd. CpG-A (2216) was from Invivogen and TAPI-1 was from Selleck Chemicals. DAPI, Ficol Histopaque 1077 and Ficol Histopaque 1119 were from Sigma-Aldrich. Fluorescent-conjugated monoclonal antibodies to CD303, CD123, IL6R, ADAM17 were all from R&D systems also. Fluorescent-conjugated monoclonal antibodies to pSTAT1 pSTAT3 were from Ebioscience. ADAM17 Ab for immunofluorescence was from Atlas Antibodies. Secondary antibodies anti-rabbit CF488 and anti-goat CF555 were purchased from biotium.

pDCs isolation for microarray analysis

Whole blood samples (20ml) from 5 RA patients in remission with anti-TNF therapy and 5 healthy donors were used for pDCs isolation via magnetic-bead-associated cell sorting with the Miltenyi Biotec (Auburn, CA) human pDC isolation kit for positive selection. pDC purity was ascertained by labeling for CD303 and CD123, and the samples were used, more than 80% pure. The total amount of pDCs isolated was used to maximize the total RNA yield (mini RNA isolation kit) and microarray analysis performed in the German Rheumatism Research Centre Berlin (Germany).

Cell isolation and cultures

Buffy coats were subjected to Ficol-Histopaque 1077 density-gradient centrifugation at 1800 rpm for 30 minutes to obtain peripheral blood mononuclear cells (PBMCs). Cells were washed once at 1500 rpm for 10 min using sterile PBS. Erythrocytes were lysed if needed. The lysis was performed by resuspending the cell lysate in water for injection for 35 sec. Then equal amount of 1,8% w/v NaCl solution was added in order to reduce the osmotic shock. The PBMCs were magnetically sorted using the CD304 (BDCA-4/Neuropilin-1) human MicroBead Kit (Miltenyi Biotec) in order to positive select the pDC population. pDCs were washed and then cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin mix. pDCs were stimulated with CpG-A (0,5μM), rIL6 (1ng/ml) for 4,

13, 18 hours. In addition, in specific experiments pDCs were pre-incubated with TAPI-1 (20 μM) for 30 minutes and then stimulated with CpG-A and rIL6 for 18 hours.

Flow cytometry (FACS)

Cells were stained for extracellular markers for 20 min at 4°C in PBS/5% FBS. Monoclonal antibodies specific for CD303, CD123 were used to identify pDC population. Monoclonal antibodies specific for HLA-DR, CD80, CD86, CD40 were used as maturation-activation markers. Additionally, monoclonal antibodies specific for IL-6R and ADAM17 were used. Intracellular staining with the monoclonal antibodies specific for pSTAT1 pSTAT3 was performed. Apoptosis and cell death was obtained with staining with Annexin V (BD Pharmingen) and 7AAD (Biolegend). Cells were acquired on a FACS Calibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Quantification of Gene Expression by RT-PCR

Total RNA from cultured cells was collected using the TRIZOL extraction protocol. Total RNA was treated with DNAse in order to eliminate any genomic DNA contaminations. Turbo DNAse kit (Ambion) was used according to manufacturer's protocol.

cDNA was prepared from isolated RNA using Perfect Real time cDNA Synthesis Kit (Takara) according to manufacturer's protocol. 50ng of RNA was used as a template for every reaction. RNAse H (2U/reaction) was added in order to clean the resulting cDNA from any RNA and incomplete cDNA products. cDNA was stored at -20 °C. PCR amplification of the resulting cDNA samples was performed using appropriate volumes of KAPA SYBR® FAST Universal 2x qPCR Master Mix and specific for each gene primers at a CFX Connect™, Real-Time System. Total volume of each PCR reaction was 20μl. PCR primers used were as follows:

Primers	Forward	Reverse
GAPDH	5'CATGTTCCAATATGATTCCACC3'	5'GATGGGATTTCCATTGATGAC3'
IFNa	5'TCTGATGCAGCAGGTGGG3'	5'AGGGCTCTCCAGACTTCTGCTCTG3'
IL-6R	5'ACATTCACAACATGGATGG3'	5'AGGACTCCTGGATTCTGTC3'
TNF-α	5'GAGGCCAAGCCCTGGTATG3	5'CGGGCCGATTGATCTCAGC3'
ADAM17	5'GACTCTAGGGTTCTAGCCCAC3'	5'GGAGACTGCAAACGTGAAACAT3'

Confocal microscopy

Confocal analysis was carried out on a Leica TCS SP8. For analysis of TNF and ADAM17 expression pDC were fixed after culture with 4% PFA/PBS and stained with Humira (TNF-blocker) and ADAM17 Ab, washed, stained with DAPI (Invitrogen) as indicated.

Enzyme-linked Immunosorbent Assay (ELISA)

Detection of human TNF-α (eBioscience) and IL-6R (R&D Systems) in culture supernatants harvested at the indicated time, were performed by sandwich ELISA, following the

manufacturer's recommendations. Light absorbance at 450 nm was measured using the ELx800 Biotek.

Statistical Analysis

Statistical analysis was performed with the Prism Software (GraphPad) by nonparametric Mann-Whitney test or paired t test. p values less than 0.05 were considered significant. ns, not significant.

RESULTS

Identification of more than 6000 deregulated genes on pDCs - upregulation of IL-6R on RA patients in remission

To investigate the molecular mechanism through which human pDCs contribute to restoration of tolerance in RA, knowing that pDCs are limited in the periphery of active RA patients, we focused on pDCs isolated from RA patients in remission and we compared them with healthy pDCs. Specifically, to assess the molecular signature, pDCs isolated from 5 RA patients responding to anti-TNF therapy and pDCs derived from 5 healthy donors subjected to microarray analysis. The results from the microarrays revealed the existence of more than 6000 genes to be differentially regulated between pDCs from RA patients in remission as compared to isolated healthy pDCs. Interestingly, among the most prominent genes that were significantly upregulated on RA pDCs was IL-6R (Figure 6).

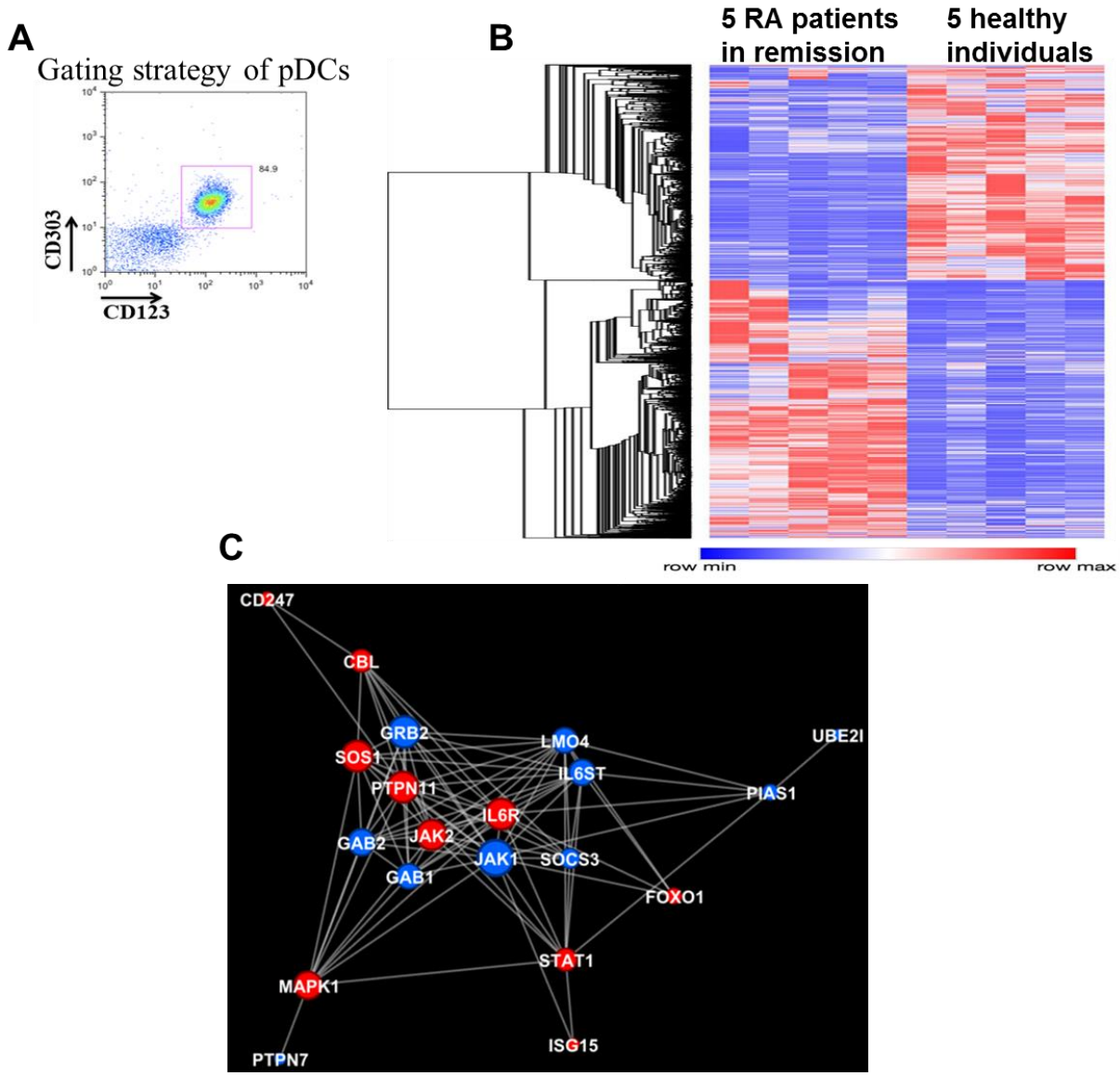


Figure 6: A) pDC gating strategy. pDCs were magnetically isolated from peripheral blood of healthy or RA samples. Then, pDCs were stained for CD303 and CD123 markers. The % of double positive cells represented the isolated pDCs. B)Heat map of genes found to be deregulated on pDCs from RA patients responding to anti-TNF

therapy (left) as compared to pDCs from healthy individuals (right). C) Network of enriched KEGG pathway illustrates molecules of IL-6 signaling to be upregulated (red) or downregulated (blue) on RA pDCs.

Given that IL-6 has a major role in the pathogenesis of RA, we focused on its receptors and IL-6 signaling pathway. Next, we validated these results in a new cohort of RA patients responding to anti-TNF therapy, where total PBMCs were isolated form peripheral blood and stained for pDCs markers (CD303, CD123) and IL-6R. Using flow cytometry, we found an important increase of IL-6R expression on RA pDCs as compared to pDCs derived from healthy donors. However, in the few pDCs found in the periphery of active RA patients, the IL-6R protein levels were reduced compared to pDCs from RA patients in remission (Figure 7A).

It is well established that binding of IL-6 to the cell surface-expressed IL-6R results in the phosphorylation of STATs proteins, which translocate to the nucleus where they induce transcription of target genes. To assess if IL-6 signaling is activated, we examined ex vivo the pSTAT1 and pSTAT3 expression levels gated on pDCs We found significantly increased pSTAT1 expression levels on RA pDCs, whereas no differences were observed on pSTAT3 expression levels (Figure 7B).

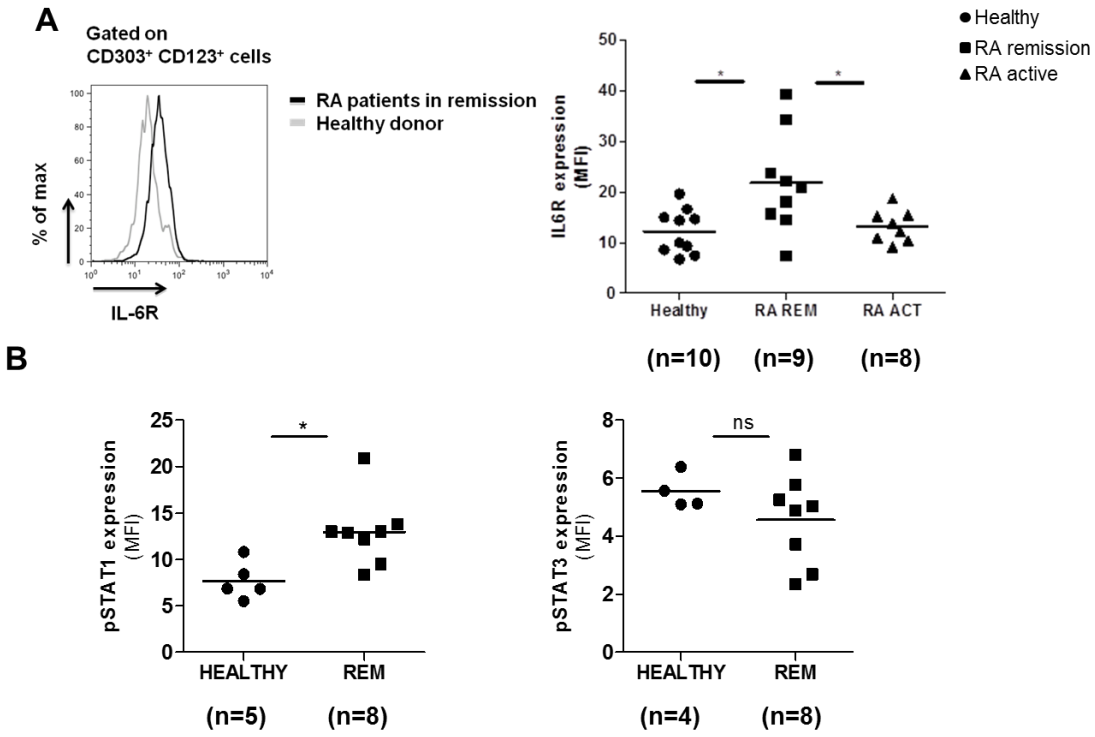


Figure 7: A) Representative figure of IL-6R expression gated on CD303+CD123+ cells (pDCs) analyzed by flow cytometry. Graph of mean fluorescence intensity (MFI) values of IL-6R expression gated on pDCs from healthy and RA samples. B) MFI of pSTAT1 and pSTAT3 expression gated on pDCs from healthy and RA samples. Statistical tests: Mann-Whitney.

These results highlighted that pDCs play a role in the pathogenesis of RA, since a great number of genes found to be differentially regulated between healthy pDCs and RA pDCs in remission state. Focus on molecular pathways that are known to be implicated in the pathogenesis of RA resulted in IL-6 signaling and especially IL-6R and pSTAT1 which were found to be significantly upregulated on pDCs from RA patients in remission. Previous

studies support that classic IL-6 signaling via the membrane bound receptor leads to anti-inflammatory responses. Therefore, we hypothesized that the increased IL-6 signaling pathway enhances the tolerogenic phenotype of pDCs.

IL-6 signaling induced no difference in IFN-a expression and activation status, but decreased TFN-a production by pDCs in vitro

In order to study the molecular mechanism of IL-6 signaling on pDCs we performed in vitro experiments on isolated healthy pDCs. Activation of pDCs was induced by CpG oligodeoxynucleotides (CpG-ODN)-A in the presence or absence of recombinant IL-6 (rIL6). CpG-A binds to TLR9 and gives rise to robust IFN-a production. To understand how IL-6 contributes to the tolerogenic phenotype of pDCs, we focused on the major features of pDCs and one of them is the expression of type I IFNs. Magnetically isolated pDCs cultured with CpG-A in the presence or absence of rIL6 and the assessment of IFN-a mRNA levels in different time points concluded with no detectable differences between these two conditions, a finding that is also supported by the microarray results (Figure 8A). Another feature of pDCs is their antigen presenting capacity. Therefore, we tested with flow cytometry whether there is differential expression of co-stimulatory molecules on pDCs cultured with CpG-A in the presence or absence of rIL6 for 18 hours. To this end, we did not observe significant differences in HLA-DR, CD80, CD86, CD40 molecules expressed by pDCs in the presence of rIL6 (Figure 8B).

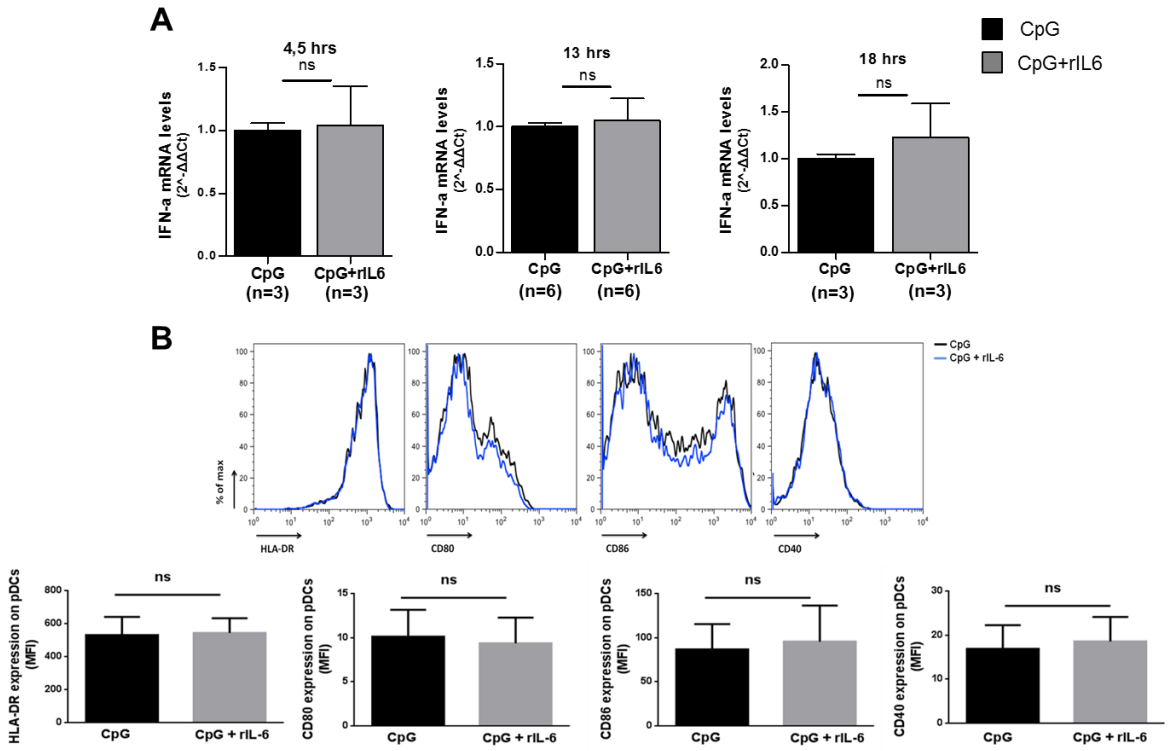


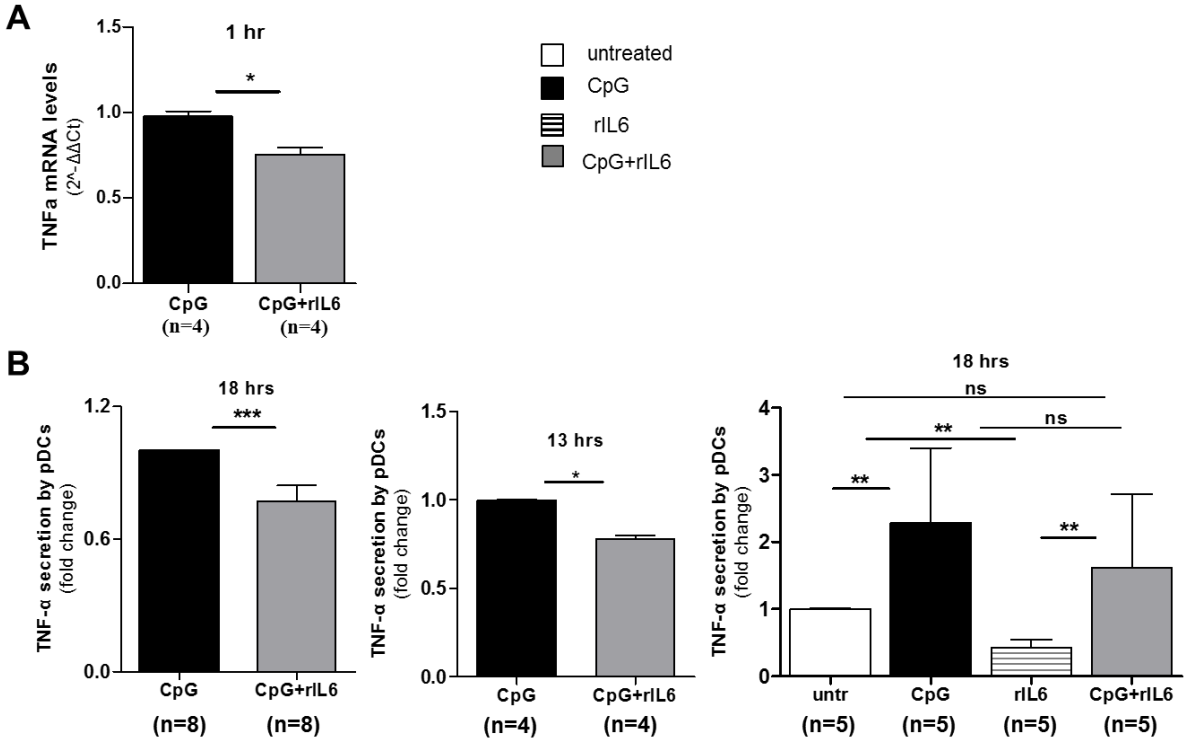
Figure 8: A) fold change of mRNA levels of IFN-a in pDCs treated with CpG (black) and pDCs treated with CpG+rIL6 (grey) for 4,5 , 13 and 18 hours. B)Representative figure of HLA-DR, CD80, D86, CD40 expression on cultured pDCs analyzed by flow cytometry. Graphs of mean fluorescence intensity (MFI) values of surface expression of HLA-DR, CD80, D86, CD40 on cultured pDCs with CpG in the presence or absence of rIL6 for 18 hours.

pDCs are specialized in producing type I IFNs following viruses or self nucleic acids recognition. However, pDCs have been characterized for the secretion of other pro-

inflammatory cytokines, such as TNF-α, IL-6. To elucidate how IL-6 shapes the pDC functions we assessed TNF-α mRNA and protein levels on pDCs treated with CpG-A in the presence or absence of rIL6. In mRNA levels, pDCs treated with rIL6 for 1 hour characterized with reduced transcription of TNF-α gene. Interestingly, we checked cultured supernatants for the secreted TNF-α and we found a vast decrease of TNF-α by pDCs cultured for 13 and 18 hours in the presence of rIL6(Figure 9A,B). This is an important finding knowing that TNF-α is one of the major cytokines that also contributes to RA pathogenesis.

This significant result was also supported by the microarray analysis, demonstrating reduced expression levels of TNF on RA pDCs responding to therapy (Figure 9C). Moreover, to validate this outcome, we sorted pDCs from RA patients responding to anti-TNF therapy and active state of disease and we observed by confocal microscopy that pDCs from RA patients in remission expressed lower TNF levels (Figure 9D). However, this observation has to be repeated in additional RA and healthy samples.

Collectively, these results suggest that IL-6 signaling on pDCs does not affect their antigen presenting capacity and IFN-a production. However, we concluded that IL-6 signaling inhibited the secretion of pro-inflammatory cytokine TNF-α. This is a novel result and we have to investigate both the importance of TNF-α secreted by pDCs in the pathogenesis of RA as well as the mechanism through which IL-6 signaling on pDCs seems to regulate TNF-α secretion.



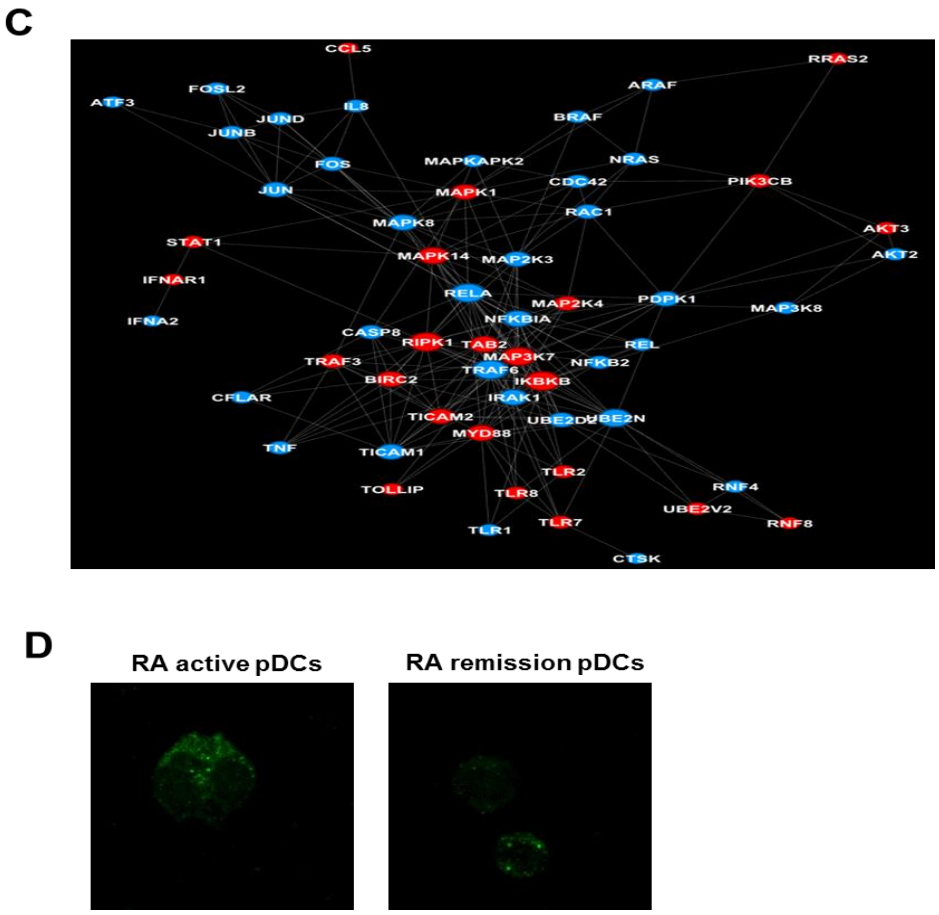


Figure 9: A) fold change of mRNA levels of TNF-α in pDCs treated with CpG-A (black) and pDCs treated with CpG-A+rIL6 (grey) for 1 hour. B) Fold change of protein levels of TNF-α in culture supernatants from pDCs untreated, treated with CpG-A, rIL6, CpG-A+rIL6 for 13 and 18 hours. C) Network of enriched KEGG pathway illustrates molecules of TNF signaling to be upregulated (red) or downregulated (blue) on RA patients responding to anti-TNF therapy as compared to pDCs from healthy individuals. D) Immunofluorescence confocal microscopy for TNF on sorted pDCs from active RA patient and RA patient in remission.

TNF-α secretion on pDCs is ADAM17-depedent

Several studies have demonstrated that the function of ADAM17 contributes to the production of mature TNF-α. However, ADAM17 expression and activity at the surface of pDCs have not been studied. In order to unravel whether TNF levels that we identified in culture supernatants of CpG-A activated pDCs were produced by ADAM17-mediated shedding, we used the ADAM17 inhibitor TAPI-1. Specifically, pDCs were cultured with CpG-A in the presence or absence of TAPI-1 and after 18 hours, soluble TNF-α concentrations in the culture supernatants were measured. Activation of pDCs resulted in vast increase of TNF-α release. However, in the presence of TAPI-1, TNF-α shedding was blocked returning to low concentrations in the culture (Figure 10A). Thus, ADAM17 activity on pDCs contributed to higher soluble TNF-α levels in culture supernatants.

IL-6 signaling seems to inhibit ADAM17 activity on pDCs

Our data suggested that IL-6 signaling on activated pDCs resulted in reduced TNF-α secretion, nevertheless the molecular mechanism that IL-6 pathway controls TNF-α secretion needs investigation. Major producers of TNF-α are macrophages, although it can also be secreted by other immune cell types like, CD4+ T cells, NK cells, neutrophils, mast

cells and pDCs. In addition, we demonstrated that ADAM17 mediates TNF-α cleavage on pDCs, which is in line with previous studies on other cell types.

On the basis of these observations, we sought to investigate whether ADAM17 activity would be altered by IL-6 signaling on pDCs. To get an insight into the fate of ADAM17, its expression at the cell surface of pDCs was analyzed by flow cytometry after activation with CpG-A in the presence or absence of rIL6. We concluded that there was a small decrease of mature ADAM17 expression on the surface of pDCs upon IL-6 signaling (Figure 10B). Considering that we have proved that ADAM17 mediated TNF cleavage on pDCs and IL-6 signaling resulted in reduced expression levels of ADAM17, as well as significantly lower soluble TNF-α levels in culture supernatants, we assumed that IL-6 signaling leads to impaired ADAM17 function.

Interestingly, came out when. pDCs cultured with CpG-A in the presence or absence of rIL6, and soluble IL-6R concentrations were measured by ELISA in the culture supernatants. The cleavage of IL-6R was significantly inhibited, after activation of IL-6 pathway on pDCs (Figure 10C). Thus, we provide an additional evidence that ADAM17 activity is impaired upon IL-6 signaling since we evaluated another substrate, known to be cleaved by ADAM17

Taken together, these results show that ADAM17 function controls the secretion of TNF-α and soluble TNF-α and IL6-R is significantly reduced in culture supernatants of pDCs upon rIL6 treatment. Consequently, we have strong evidence that IL-6 signaling leads to impaired ADAM17 activity on pDCs.

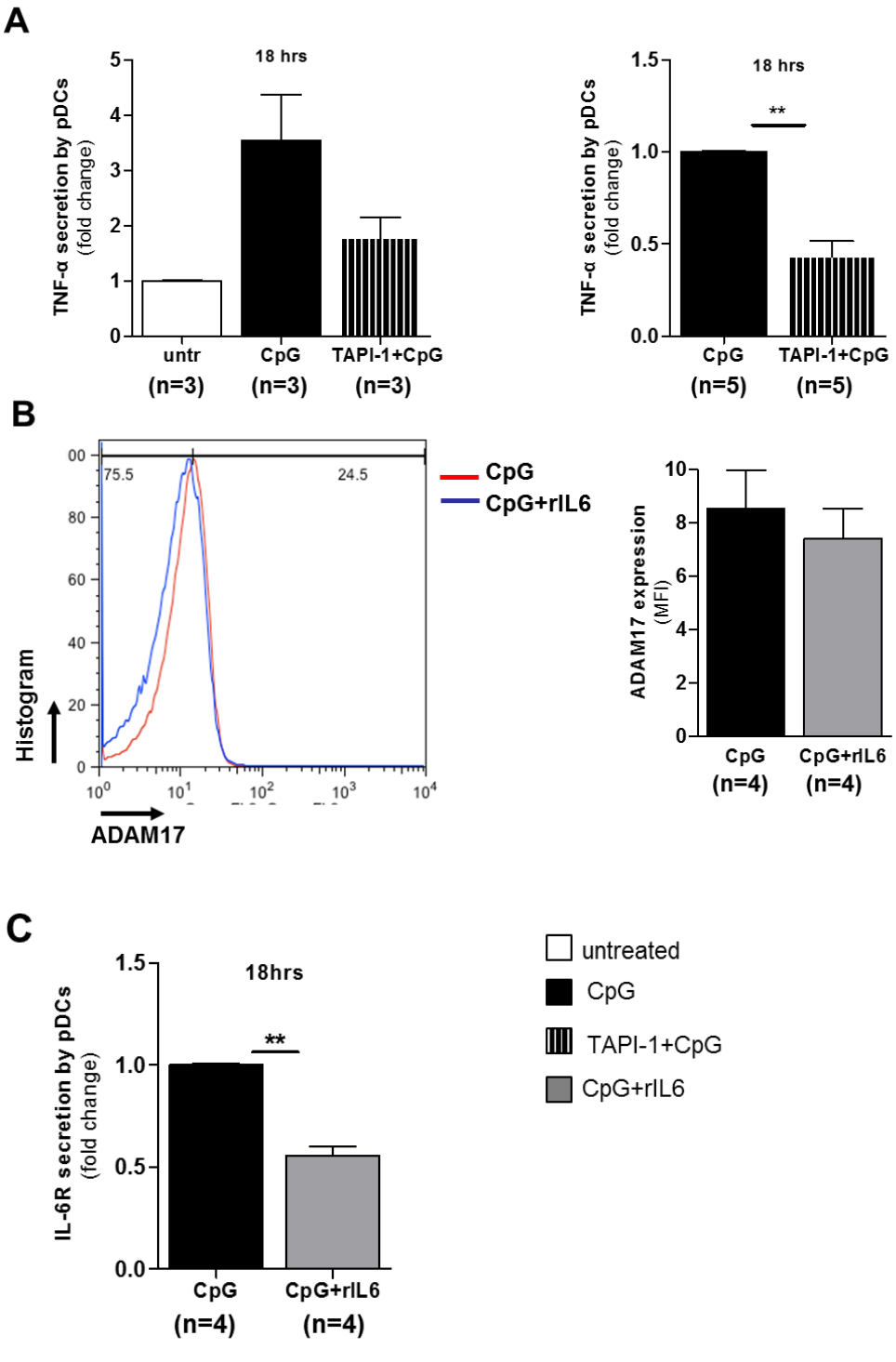


Figure 20: A) Fold change of protein levels of TNF-α in culture supernatants from pDCs untreated, treated with CpG-A and pre-treated with TAPI-1 and then cultured with CpG-A for 18 hours. B) Representative figure of ADAM17 expression on cultured pDCs analyzed by flow cytometry. Graphs of mean fluorescence intensity (MFI) values of surface expression of ADAM17 on cultured pDCs with CpG-A in the presence or absence of rIL6 for 4 hours. C) Fold change of protein levels of soluble IL-6R in culture supernatants from pDCs treated with CpG-A and CpG-A+rIL6 for 18 hours.

Intracellular accumulation of ADAM17 on pDCs from RA patients in remission

Based on microarray analysis, we found that apart from reduced TNF transcriptional levels, pDCs from RA patients in remission exhibited lower ADAM17 expression levels (Figure 11A). Moreover, we sorted pDCs from RA patients responding to anti-TNF therapy and active state of disease and we observed by confocal microscopy increased intracellular accumulation of ADAM17 on pDCs from RA patients in remission, whereas ADAM17 was significant reduced on pDCs from active RA patients (Figure 11B).

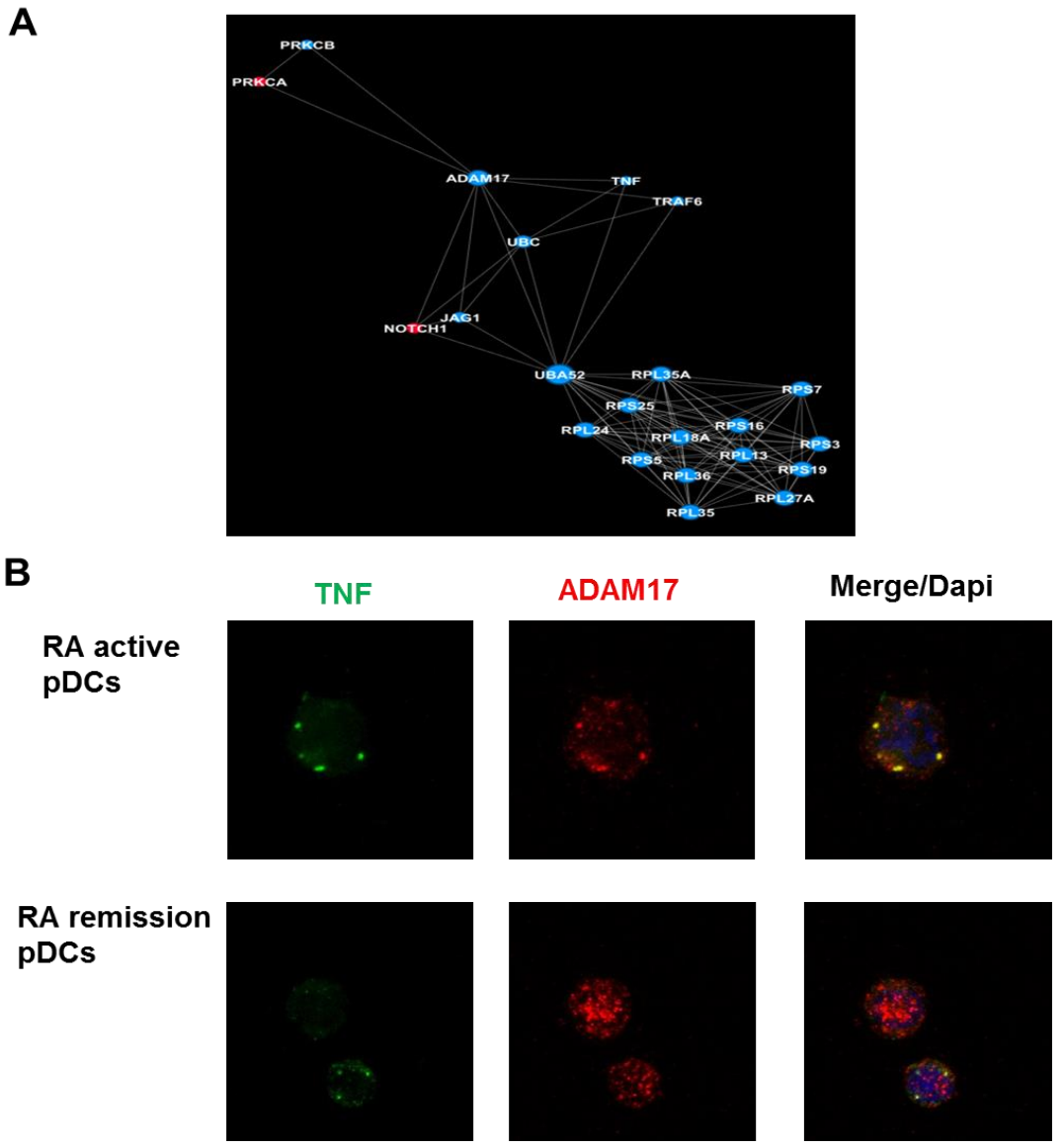


Figure 11: A) Network of enriched KEGG pathway illustrates molecules of ADAM17 signaling to be upregulated (red) or downregulated (blue) on RA patients responding to anti-TNF therapy as compared to pDCs from healthy individuals. B) Immunofluorescence confocal microscopy for TNF and ADAM17 on sorted pDCs from active RA patient and RA patient in remission.

DISCUSSION

pDCs link innate and adaptive immunity and have been characterized for their multifactorial role. It is known that pDCs contribute to inflammatory responses in the development of autoimmune diseases [44]. Nevertheless, there is strong evidence from the literature that pDCs also promote immune regulation and tolerance. Regarding RA, studies in mouse model have shown that pDC depletion enhances the severity of disease [58]. In addition, previous study from our laboratory demonstrates that pDCs from RA patients responding to therapy have a tolerogenic phenotype inducing IL-10 secreting T regulatory cells in vitro [61]. Here, we study the molecular mechanism through which pDCs contribute to restoration of tolerance.

In this study, we performed microarray analysis on pDCs from RA patients responding to anti-TNF therapy and pDCs derived from healthy individuals and surprisingly we identified 6741 genes to be de-regulated, underlining the different signature of pDCs in health and remission state of RA. This important finding combined with previous studies, lead us to the hypothesis that pDCs in RA play a key role in the remission of the disease. However the exact mechanisms of pDCs contribution to the regulation of inflammation is not known.

Interestingly, among the most prominent genes that were significantly upregulated on RA pDCs was IL-6R. Considering the well-studied pleiotropic role of IL-6 and its contribution in pathogenesis of RA, we focused on the role of IL-6 signaling on pDCs in vitro. We concluded that there are no differences in the transcription levels of IFN-a as well as the expression of antigen presenting and co-stimulatory molecules, upon IL-6 on activated pDCs. However, we showed for the first time that classic IL-6 signaling regulated TNF-α secretion by pDCs. It is known that macrophages are the major producers of TNF-α and pDCs are specialized in producing high amounts of type I IFNs, although they can also secrete other pro-inflammatory cytokines, like TNF-α. There is no literature to support the contribution of TNF-α secreted by pDCs in the pathogenesis of RA. Thus, the functional importance of TNF-α produced by pDCs in the immune responses as well as the reduced levels of TNF-α upon IL-6 signaling on pDCs, remains to be investigated. In order to address this question, in on-going experiments we investigate the effect of TNF-α in monocytes attraction and differentiation. Culture supernatants from pDCs treated with CpG-A in the presence of rIL6 (low levels of TNF-α) and in the absence of rIL6 (high levels of TNF-α) for 18 hours, will be used to treat CD14+ cells (monocytes). In order to find how IL-6-mediated reduced TNF-α levels by pDCs drive the immune responses relevant to RA pathogenesis, we will assess the upregulation of maturation markers, such as HLA-DR, CD80, CD86, the mRNA levels of pro- and anti-inflammatory molecules as well as the cytokine secretion.

TNF-α secretion is controlled by ADAM17-mediated shedding [34]. So, we checked if IL-6 signaling could regulate the function of ADAM17. Since ADAM17-mediated shedding takes place at the cell surface, we evaluated the effect of IL-6 signaling in the expression of ADAM17 on the membrane of pDCs. We found a trend for reduced expression of ADAM17 after rIL6 treatment. Additional evidence for the functional importance of ADAM17 was shown when we checked the production of soluble IL-6R in culture supernatants of pDCs, which is another substrate cleaved by ADAM17. We showed that IL-6 signaling limited the

soluble IL-6R secretion by pDCs. Taken together, our data support the hypothesis that IL-6 signaling on pDCs leads to impaired function of ADAM17. It has been shown that TNF-α induces the production of other pro-inflammatory cytokines, including IL-1 and IL-6 [30]. Here, we supported that IL-6 signaling reduced the secretion of TNF-α and we had evidence that this regulation was through impaired ADAM17 activity.

ADAM17 is a crucial part of many signaling pathways and thereby orchestrates many physiological but also pathophysiological processes. In order to keep the balance, ADAM17 activity has to be tight regulated. One important level of regulation is the localization of the mature and therefore active ADAM17. The abundancy of ADAM17 to the cell surface is controlled by the transport to the membrane via PKC signaling and the removal from the cell surface by internalization [36]. Based on our finding, we assumed that IL-6 signaling could regulate the transport of ADAM17 to the cell surface, and in the future we will further investigate the possible role of IL-6 on the PKC expression on pDCs.

Our findings from the in vitro experiments were consistent with microarray analysis on pDCs from RA patients responding to anti-TNF therapy and healthy donors. On pDCs from RA patients in remission IL-6R transcriptional levels were elevated, and the STAT1 levels were increased, indicating that IL-6 signaling was activated. These results were validated in a new cohort of healthy individuals and RA patients, where we found significantly enhanced membrane expression of IL-6R and pSTAT1 levels on RA pDCs in remission. Moreover, microarrays supported reduced transcriptional levels of TNF and ADAM17 on pDCs from RA patients in remission. From the validation of these findings so far, immunofluorescence microscopy on sorted pDCs from RA patients in remission compared to active state of the disease showed a trend for reduced TNF levels on pDCs from RA patients in remission. However, we found intracellular accumulation of ADAM17 on pDCs from RA remission patients, an observation which is not in consistency with the in vitro experiment, where we did not observe high intracellular expression levels of ADAM17 on activated pDCs upon rIL6 treatment. One explanation would be that on RA active pDCs ADAM17 which is expressed can maturate and transport to the surface to cleave proteins, whereas on pDCs from patients in remission, where we found increased membrane IL-6R and pSTAT1, IL-6 signaling regulated the mechanism of ADAM17 translocation to the membrane, leading to intracellular accumulation of ADAM17.

Based on these results we demonstrate a novel role of pDCs in RA through decreased secretion of TNF-α and we propose that increased IL-6 signaling on pDCs inhibits ADAM17 activity, by regulating ADAM17 translocation to the surface therefore limiting TNF-α secretion and this process may enhance the tolerogenic phenotype of pDCs (Figure 12).

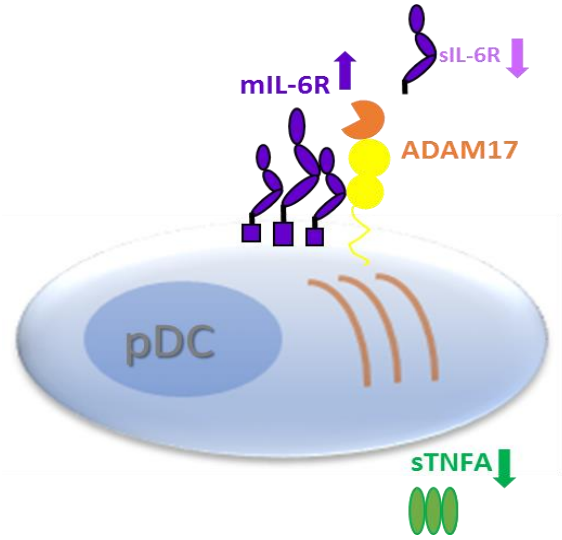


Figure 12: Proposed model for the tolerogenic function of pDCs in RA. Enhanced classic IL-6 signaling on pDCs might contribute to tolerance through limiting ADAM17 activity therefore reducing the levels of TNF-α.

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