T CELLS IN CHRONIC INFLAMMATORY ENVIRONMENTS: THERE'S MORE THAN MEETS THE EYE

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T CELLEN IN CHRONISCHE INFLAMMATOIRE MILIEU:
ER IS MEER DAN ONTMOET HET OOG

(met een samenvatting in het Nederlands)

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Chapter 1

GENERAL INTRODUCTION

Chronic inflammation is a systemic or local pathogenic condition occurring when the agent(s) triggering the immune reaction has not been removed and the inflammatory process may develop into a long term chronic phase. Several actions are put in place to eradicate the causal agent at this stage, including the recruitment of different players of the innate and adaptive immunity. On one hand, the attempt is to promote eradication of the agent causing the immune reaction, and on the other hand, to end the immune response and minimize inflammation-dependent tissue damage. T cells are cells of the adaptive immunity typically involved in all these processes and the (im)balance of their actions in this context contributes to determine the outcome of chronic inflammatory diseases. Little is known about the relative contribution of different T cell subsets (e.g. CD4 and CD8 T cells, and Treg) in human chronic inflammatory environments and about their interplay with other cell subsets, such as the innate immunity compartment. New knowledge in this field will lead to a better understanding of the pathology of chronic inflammatory diseases as well as the identification of specific targets to be investigated for therapeutic use.

T CELL SUBSETS

The majority of human peripheral blood T cells express α and β chains of the T Cell Receptors (TCR) and are called αβ T cells. DNA recombination of variable regions of the α and β TCR genes are responsible for the TCR diversity, which is required for T cell reactivity to the myriad of potential antigens (1). αβ T cells can be divided into the major subsets of CD4 and CD8 T cells. **CD4 T cells** shape and regulate themselves and other immune cells through cytokine production or by cell–cell contact and are defined as T-helper (Th) cells. **CD8 T cells**, on the other hand, are programmed to kill target cells and therefore are called cytotoxic T cells (2).

Both CD4 and CD8 T cells continuously recirculate through the body from the peripheral blood to secondary lymphoid organs searching for specific antigens. **Naïve T cells**, characterized by the expression of CD45RA, CCR7, and CD62L, once encountered the antigen presented by dendritic cells via HLA class II molecules in draining lymph nodes, become specialized **effectors** and express specific trafficking and tissue-homing molecules which allow exit lymph nodes, enter the blood and migrate in target peripheral tissues (3). Differentiation of T cells implies cell specialization including changes in their migratory capacities, modification to their lifespan, and secretion of specific cytokines. Examples of major Th subsets based on their cytokine production are **TH1 cells**, mainly producing IFN-γ and TNF-α, **TH2 cells** secreting IL-4, IL-5 and IL13, **TH17 cells** producing IL-17 (4). In parallel, **memory T cells** are generated, and these have the capacity to enter lymph nodes and recirculate into blood and tissue (5).

Besides Th subsets, specific cell populations endowed with abilities to suppress the immune response are generated, the so called **regulatory T cells (Treg)**. Treg are inducers of immunological tolerance, which is a state of immune unresponsiveness

specific to a particular antigen or set of antigens induced by previous exposure to that antigen(s) (6).

Among regulatory T cells, **natural occuring T cells (nTregs)** and **IL-10-producing type 1 regulatory T cells (Tr1)** have been widely studied in humans and mice (7). Thymus-derived **nTreg** are characterized by the expression of the Forkhead box P3 (FOXP3) transcription factor and use several different mechanisms to inhibit the activity of the effector cells, such as production of immunosuppressive cytokines, expression of inhibitory surface molecules, release of cytotoxic enzymes, competition for IL-2, and degradation of extracellular ATP (7). **Tr1 cells**, instead, arise in the periphery after encountering the antigen in the presence of a tolerogenic environment, i.e. IL-10. Tr1 cells need to be activated through their TCR in an Ag-dependent manner to exert their suppressive functions and they are suppressive selectively towards the antigen(s) encountered during T cell priming (8). They can be identified in the periphery by the surface markers $CD4^{+}CD45RA^{-}LAG3^{+}CD49b^{+}$ (9) and have a unique cytokine production profile (i.e., $IL\text{-}10^{++}IL\text{-}4^{-}TGF\beta^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}$) (reviewed in (10)). Tr1 mechanism of immune suppression is not only mediated by cytokine production, but also by cell contact (11), metabolic disruption (12) and cytolysis (13). The relative importance of each mechanism in vivo remains to be defined since most of the studies regarding human Tr1 cell activity have been done in vitro.

In recent years, studies using parabiosis and intravascular staining have revealed that T cells that remain after the clearance of a viral infection adapt to the tissue and adopt a specific differentiation program that enables residency, rather than continuous recirculation (14). These are called **tissue-resident memory T cells (TRM)** and may play a vital role in the protection of tissues from pathogens.

HUMAN CHRONIC INFLAMMATORY DISEASES

In this thesis, we investigate T cell functional specialization in human chronic inflammatory diseases. In particular, we studied two human diseases characterized by a chronic inflammatory state, such as Juvenile Idiopathic Arthritis (JIA) and Chronic Kidney Disease (CKD). To define the dynamic and differentiation of T cell subsets at the target site of inflammation we used the exudate collected from the synovium of JIA patients (i.e. the synovial fluid). To elucidate whether T cell exposed to an inflammatory milieu can be used as cell therapy, the peripheral blood of patients with chronic kidney failure (CKD) on waiting list for living-donor kidney transplantation was used to generate donor-specific Tr1 cells.

Juvenile Idiopathic Arthritis

JIA is one of the most common autoimmune diseases in children, affecting 16 to 150 children per 100.000 (15). JIA, similarly to RA, is characterized by chronic inflammation of the joints due to infiltration of the synovium by different subsets

of immune cells (16). JIA can be divided into several subclasses of disease, including the **oligoarticular** and **polyarticular JIA** (16), which have been investigated in this thesis. Oligoarticular JIA patients have < 5 affected joints at onset, better prognosis than patients with the polyarticular form and, in some cases, the disease is self-limiting. In case the number of joints involved is > 5 after the first 6 months, the diseases is classified as extended-oligoarticular JIA. Polyarticular JIA involves 5 or more joints with a symmetric pattern, affecting both large and smaller joints. This subtype of JIA usually displays a rather progressive course of disease and it is more similar to the adult form of the disease (i.e. RA).

Over the past decade, dramatic improvements in the treatment of JIA have been made (17). As a first line therapy, non-steroidal anti-inflammatory drugs (NSAIDs) are administered; however, they don't modify the underlying disease process which is instead affected by the so called disease modifying anti-rheumatic drugs (DMARDs), such as Methotrexate (MTX), which target the disease more effectively and limit damage and long-term morbidity (18). Corticosteroids are often administered locally due to their multiple side effects, including growth retardation. In the last decade, specific therapies targeting checkpoints of the immune response, such as TNF-α, have been tested on JIA patients (i.e. biological) (19). Infliximab (chimeric mouse/human monoclonal antibody), adalimumab (fully human monoclonal antibody) and etanercept (fusion protein produced by recombinant DNA) are monoclonal antibodies against TNF-α, which are used in the treatment of autoimmune arthritis. Although many patients respond well to treatment, no permanent remission is achieved and often patients relapse after discontinuation of therapy (20). This is associated with considerable side effects due to general immune suppression, possibly including malignancies and infections (19). Therefore, pursuit of new strategies to treat arthritis as well as other autoimmune diseases is required.

Chronic kidney disease (CKD)

Chronic kidney disease is a general term for heterogeneous disorders affecting the structure and function of the kidney. The definition of CKD is based on the presence of kidney damage (i.e. albuminuria) or decreased kidney function (i.e, glomerular filtration rate [GFR] <60 mL/min per 1.73 m.) for 3 months or more (21). End-stage kidney disease occurs when the GFR is < 15 mL/min per 1.73 m^2, which is regarded as the most serious outcome of CKD. Worldwide, an estimated 200 million people have chronic kidney disease (22) and diabetes is the leading cause of kidney failure (23). Hypertension, cardiovascular diseases, anaemia and hyperparathyroidism are the most common secondary disorders to kidney failure (23). The only treatments available for end-stage renal disease are dialysis and transplantation. Importantly, the survival of patients in long-term dialysis is substantially lower compared to kidney transplant recipients (24). However, transplanted patients require lifelong treatment with immunosuppressive drugs to protect the transplanted organ, which have a myriad of side effects.

T CELLS IN AUTOIMMUNE ARTHRITIS

CD4 T cells in rheumatoid inflammation

CD4 T cells are essential in initiating, controlling, and driving specific immune responses in inflamed sites such as the synovium of patients with autoimmune arthritis. Association of RA and JIA with particular MHC class II alleles, such as HLA-DR4, implies a central role for CD4 T cells (25, 26). This is supported by the evidence that adoptive transfer of CD4 T cells from mice with arthritis to healthy recipient induce inflammatory arthritis (27) and that T cell directed therapies have conferred clinical benefit in RA (28).

Whereas the antigen(s) recognized by autoreactive CD4 T cells is still unknown in RA and JIA, much progress has been made in defining the phenotype and function of those pathogenic CD4 T cells. CD4 T cells present at the site of inflammation of autoimmune arthritis show a predominant **TH1** phenotype, characterized by the secretion of pro-inflammatory cytokines such as IL-2, IFN-γ and TNF (29). However, it is still unexplored which players contribute to the induction of this phenotype at the target site of inflammation. IL-12 and IL-27 are possible candidates, given their well-known ability to skew T cell differentiation towards the TH1 phenotype (30, 31).

Besides TH1 cells, **TH17** cell frequency is elevated in the joints of children with arthritis, particularly those with a more severe clinical course known as extended oligoarticular JIA (32), as well as the synovium of patients with a particular form of arthritis, psoriatric arthritis (PsA) (33).

To conclude, CD4 T cells orchestrate the local inflammation and cellular infiltration, after which a large number of subsequent inflammatory events occur.

CD8 T cells in rheumatoid inflammation

The well-described function of CD8 T cells is protection against viral infections and tumors. CD8 T cells perform this function by killing target cells that express HLA class I molecules and the antigenic peptide. Therefore it is expected that CD8 T cells have a great potential to cause tissue damage also in the autoimmune setting. HLA class I polymorphisms are associated with a higher risk to develop specific forms of autoimmune arthritis, including RA and Ankylosing Spondylitis (34, 35). Activated CD8 T cells can produce high levels of pro-inflammatory cytokines such as IFN-γ and TNF (36), which contribute to target cell destruction and sustain the inflammatory milieu present in autoimmune diseases.

Despite their potentially crucial contribution to tissue damage and local inflammation, investigation of CD8 T cells in autoimmune arthritis has been for a long time neglected and therefore the precise role played by CD8 T cells as effectors and regulators of rheumatoid inflammation remains to be clarified. An extensive review on current knowledge of CD8 T cells in autoimmune inflammation is described in Chapter 2

T CELLS IN CHRONIC KIDNEY DISEASE

Given the high chance to develop infectious diseases, patients affected by end-stage CKD are considered immunocompromised (37). CKD patients present impaired cell-

mediated and humoral immunity (B-cell, T-cell, monocytes) and are characterized by a lower seroconversion rate after vaccination as compared with healthy subjects (38), presence of activated pro-inflammatory monocytes (39, 40), reduced frequency of immunogenic circulating dendritic cells (DC) (41) and elevated levels of plasmatic pro-inflammatory molecules (42, 43). Additionally, CKD patients show (i) lymphopenia (44), (ii) hyporesponsiveness to T-cell priming (25), (iii) impaired FoxP3-Treg suppressive function (45).

Overall, the immune system of patients with end-stage renal failure is over-activated but functionally compromised, explaining how dysfunctional immune responses might lead to increased susceptibility to infections.

TR-1 CELL BASED IMMUNOSUPPRESSION

Animal models have demonstrated that immunological tolerance towards self- or allo-antigens can be achieved by transferring purified preparations of regulatory immune cells from a donor that is tolerant to the same antigens (46, 47). Administration or in vivo expansion of FoxP3$^+$ Treg and Tr1 cells has proved to be an effective strategy to control allograft rejection, autoimmune diseases and prevent Graft versus Host Disease (GvHD) (48-50). These studies provide proof-of-concept data to support the clinical translation of this approach. Our group at Ospedale San Raffaele in Milan conducted the HSR-ALT-TEN trial, testing cell-based immunosuppression with recipient-derived Tr1 cell-enriched lymphocytes following aploidentical haematopoietic stem cell transplantation in patients with haematological cancers. The aim of the study was to prevent GvHD. No safety concerns related to the cell therapy have been registered, and the cell infusions sustained immune reconstitution, with no occurrence of disease relapse and reduced severity of acute GvHD (51). In the context of autoimmune diseases, a phase-I/II study in patients with severe Crohn's Disease has been completed (52) showing that cell therapy with antigen-specific Tr1 cells is safe. The ONE Study (www.onestudy.org) is a cooperative international multicenter clinical trial aiming to develop and test the safety of different immunoregulatory cell products in living donor kidney transplant recipients. Our group participates in this study by testing the tolerogenic ability of the donor-specific Tr1 cell–enriched medicinal product.

However, defining the human regulatory T cell source, the most effective expansion strategy for this specific patient population as well as whether the cell product is endowed with the expected characteristics (such as anergy and suppression), presents many challenges and require extensive effort prior to the start of the clinical testing.

EMERING CONCEPTS AND OUTSTANDING QUESTIONS IN T CELL IMMUNOLOGY

Elucidation of T cell differentiation and specialization is crucial to understand mechanisms and identify checkpoints of inflammatory diseases. The traditional picture where naïve T cells upon encountering the antigen become effectors and, once cleared the antigen,

differentiate into long-lived memory cells that recirculate in blood and tissues is a static view of developmental biology, which is currently being revisited. Cell differentiation is a dynamic process depending on the antigen as well as the environment.

By taking a closer look at the tissues, populations of **tissue resident memory (TRM) cells** are locally generated, which are stably residing in the tissue and acquire a molecular program that contributes to the maintenance of these cells in peripheral tissue (53). This cell subset has been identified in many sites throughout a mouse and human, including the skin, liver, lung, brain, sensory ganglia, thymus, kidney, gut, salivary gland, reproductive tract, and even the spleen and blood vessels (14) and displays a transcriptional program that is distinct from their circulating counterparts (54). These specialized TRM are likely to exhibit an important role in the preservation of human tissue integrity and function during homeostasis, infection, and non-infectious perturbations. However, in the setting of human chronic inflammatory diseases, it is still unexplored how their program is specifically triggered and whether chronic antigen stimulation and inflammation may play a role in their local development.

T cell features and their role in local responses or relapses of chronic inflammatory diseases are still largely unknown. In chronic viral infections and malignant tumours, peripheral T cells have been described to display upregulated expression of the inhibitory receptor PD-1 (programmed cell death protein 1) and have a reduced ability to secrete effector cytokines (55, 56). These features have so far been considered to mark terminally differentiated **'exhausted' T cells**. However, several recent clinical and experimental observations indicate that phenotypically exhausted T cells can still mediate a crucial level of pathogen or tumour control. The role of PD-1-expressing CD8 T cells at the target site of human chronic inflammatory diseases is yet to be explored, leaving still uncertain whether these cells are antigen-specific effectors or harmless bystander in this contex (52).

The inflammatory environment might also interfere with both regulatory and effector T cell function. The prevalence of pro-inflammatory cytokines or activated antigen presenting cells could reduce Treg function or impair the ability of effector T cells to be suppressed. At the site of inflammation of autoimmune arthritis, Treg express elevated levels of FOXP3 and are described to induce potent suppression of cell proliferation and cytokine production (57). Effector CD4 T cells instead, are resistant to suppression and responsiveness to suppression (58, 59) can be successfully restored in vitro by anti-TNF (60). CD8 T cells can also become resistant to suppression (56) but it remains to be explored whether this **resistance is cell intrinsic**, independent of the presence of CD4 T cells and antigen-presenting cells and whether it can be self-sustained.

A myriad of cytokines modulate T cell differentiation. But how the environment regulates T cell differentiation and function in inflammatory conditions is still unclear. For instance, is yet to be explored **what is driving the TH1 phenotype** appearing dominant at sites of chronic inflammation such as in RA and JIA (29, 36). In this context cytokines such as IL-12 and IL-27 (31) might be potentially interesting target to be modulated in chronic inflammatory diseases.

SCOPE AND OUTLINE OF THE THESIS

In this thesis we study T cell functional specialization in human environments characterized by a state of chronic inflammation. The aim of the thesis is to dissect how a pro-inflammatory milieu influences T cell development and dynamics, eventually identifying mechanisms of immune imbalance to be modulated in chronic inflammatory diseases. In Chapter 2 we review recent literature on CD8 T cells in patients with autoimmune arthritis and discuss whether targeting this cell subset can be beneficial for the treatment of these diseases. In Chapter 3 the intrinsic resistance of CD8 T cells at the target site of inflammation of autoimmune arthritis is investigated and the mechanisms underlying the impaired local suppression are identified. In Chapter 4 we study the features of PD-1-expressing CD8 T cells at different sites of chronic inflammation. The effect of IL-27 in the skewing CD4 T cell in autoimmune arthritis is investigated in Chapter 5. Clinical application of cell-based immunotherapy is discussed in Chapter 6, and in particular, we describe an optimized protocol to obtain a Tr1-enriched cell product for infusion in patients with CKD undergoing living-donor kidney transplantation. Finally, in Chapter 7, we discuss the importance of Translational Medicine and what is missing in the current educational system to develop Translational Medicine Professionals (TMPs). In Chapter 8 the findings and opinions described in this thesis are discussed in the context of a need for a shift in the focus of investigation (from blood to tissues), new therapeutic targets and personalized medicine led by professionals to develop products with clear clinical impact to restore immune balance in chronic inflammation.

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Chapter 2

CD8 T CELLS IN HUMAN AUTOIMMUNE ARTHRITIS: THE UNUSUAL SUSPECTS

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ABSTRACT

CD8+ T cells are key players in the body's defence against viral infections and cancer. To date, data on the role of CD8+ T cells in autoimmune diseases have been scarce, especially when compared with the wealth of research on CD4+ T cells. However, growing evidence suggests that CD8+ T-cell homeostasis is impaired in human autoimmune diseases. The contribution of CD8+ T cells to autoimmune arthritis is indicated by the close association of MHC class I polymorphisms with disease risk, as well as the correlation between CD8+ T-cell phenotype and disease outcome. The heterogeneous phenotype, resistance to regulation and impaired regulatory function of CD8+ T cells — especially at the target organ — might contribute to the persistence of autoimmune inflammation. Moreover, newly identified populations of tissue-resident CD8+ T cells and their interaction with antigen-presenting cells might have a key role in disease pathology. In this Review, we assess the link between CD8+ T cells, autoimmune arthritis and the basis of their homeostatic changes under inflammatory conditions. Improved insight into CD8+ T cell-specific pathogenicity will be essential for a better understanding of autoimmune arthritis and the identification of new therapeutic targets.

The crucial role of CD4+ T cells in the pathogenesis and maintenance of inflammation in autoimmune diseases (AID) has been extensively studied (1); by contrast, the role of CD8+ T cell subsets has received less attention. CD8+ T cells are known as cytotoxic cells because of their ability to induce cell death via release of cytolytic granules or induction of Fas-mediated apoptosis (2), which is mediated by the recognition of the antigen on HLA class I molecules expressed by antigen presenting cells (APCs). However, similarly to CD4+ T cells, CD8+ T cells subsets with diverse immunological features have been described, including those producing pro-inflammatory cytokines and regulatory cells. In this Review, we discuss the growing body of evidence indicating that CD8+ T cells have a prominent role in the (auto)antigen-driven immune response, especially at the target sites of inflammation.

CD8+ T CELLS IN AUTOIMMUNE ARTHRITIS

A number of studies have suggested that CD8+ T cells are involved in the pathogenesis of autoimmune arthritis and could be useful as disease biomarker. CD8+ T cells have also been associated with other AID (BOX 1), indicating that this cell population has a wide role in autoimmunity.

HLA class I and disease risk

The strong association of ankylosing spondylitis (AS) susceptibility with the HLAB*27 allele initiated the investigation of CD8+ T cell autoantigen recognition (3). Diverse hypotheses have been proposed to explain how HLAB*27 contributes to AS pathogenesis. For example, killer cell immunoglobulin-like receptors expressed on effector cells might recognize HLAB*27 homodimers, or the misfolding of HLAB*27 heavy chain might cause

Box 1. CD8+ T cells in other autoimmune diseases. A tight link between organ-specific autoimmune diseases (AID) and CD8+ T cells has been described. Genome-wide association studies (GWAS) have shown strong associations of both type 1 diabetes mellitus (T1DM) and multiple sclerosis with HLA class I polymorphisms (98,99). In T1DM, autoreactive CD8+ T cells are thought to be involved in both the initiation of the disease and the destruction of β cells (100,101). This hypothesis is supported by the observation that autoreactive islet-specific CD8+ T cells are present in the β-cell infiltrate from patients with recent-onset T1DM as well as those patients with long-standing disease (100). Furthermore, cloned autoreactive CD8+ T cells from a patient with T1DM caused β-cell destruction in a humanized mouse model (101). In multiple sclerosis lesions, CD8+ T cells outnumber CD4+ T cells (102), and the specific enrichment of autoreactive CD8+ T cells in the central nervous system of patients with relapsing–remitting disease supports the idea of their involvement in disease pathology (103). Importantly, a 2010 study showed that the CD8+ T cell transcriptional profile but not the CD4+ enables the prediction of long-term outcome in individuals with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) or systemic lupus erythematosus (104) (SLE). A later study by the same group demonstrated that specific CD8+ T cell modular signatures are associated with clinical outcome in multiple AID, including AAV, SLE and inflammatory bowel disease (105).

endoplasmic reticulum (ER) stress (4). Given the association of HLAB*27-positive AS with the ER aminopeptidase 1 (ERAP1) gene locus, the biochemical functions of the latter — ER trimming of peptides and loading on HLA class I molecules — are hypothesized to be involved in AS development (5). However, specific arthritogenic peptides have not yet been identified, and whether autoantigen recognition by CD8+ T cells drives AS pathology is still unknown.

The association of HLAB*27 with AID is the strongest, but not the only, known example of HLA class I molecules influencing disease risk. Indeed, data collected from 22,647 cases of AS indicate that HLAA*02:01 and several HLAB alleles other than HLAB*27 (such as HLAB*51:01, HLAB*40:02, HLAB*13:02 and HLAB*40:01) affect susceptibility to AS (6). Also, the occurrence of psoriatic arthritis (PsA) is independently associ- ated with polymorphisms in HLAC, HLAB and HLAA molecules (7). Of note, besides the polymorphisms present in HLADRβ1 and HLADPβ1, susceptibility to rheumatoid arthritis (RA) is also associated with a single amino acid polymorphism in HLA-B (8). In juvenile idiopathic arthritis (JIA), despite HLA class II predominantly determining disease risk (9–11), a clear association of the HLAA*02:01 locus with the severe form of the disease is evident (9,10), whereas HLAA*01:01-positive individuals seem to be protected from JIA (9). The evidence that specific HLA class I molecules have a strong positive or negative association with susceptibility to AID supports the idea that CD8+ T cells have a role in autoimmunity.

CD8+ T cells and disease outcome

Clear indications of the relevance of CD8+ T cells in autoimmune arthritis come from studies showing direct correlations of disease severity and outcome with CD8+ T cell number and phenotype, either in the peripheral blood or in the inflamed tissue. In peripheral blood of patients with early RA (disease duration <1 year), the absolute number of CD8+ T cells was shown to be higher than that in peripheral blood of healthy controls (12). Conversely, the number of CD8+ T cells was found to be reduced in patients in remission compared with both healthy controls and patients with active disease (13). In addition, increased pro-inflammatory cytokine production by CD8+ T cells in peripheral blood of patients with RA normalizes in the remission phase, and production of TNF, IFN-γ and IL-17 positively correlates with 28-joint disease activity score activity score (DAS28) (13). At the same time, the expression in CD8+ T cells of negative regulators of immune responses, such as PD1 and T cell immunoglobulin mucin receptor 3 (TIM3, also known as hepatitis A virus cellular receptor 2), inversely correlates with disease activity (14,15). At the site of chronic autoimmune inflammation of arthritis — that is, in the synovial fluid aspirated from the joint — CD8+ T cell populations are expanded in patients with RA, JIA or PsA (16,17,18). However, CD8+ T cell relevance to the pathogenesis of different AID is complex: in RA, their frequency in the synovial fluid negatively correlates with DAS28 (REF. 18), but in JIA, an increased CD8:CD4 ratio was shown to correlate with more-severe disease (17).

Specific CD8+ T cell subsets have been also described to predict disease severity and outcome in autoimmune arthritis. For example, CD73, a cell surface molecule with anti-inflammatory properties (19), is expressed at low levels in CD8+ T cells from the synovial fluid of patients with oligoarticular JIA, and its expression is lower in patients with extended oligoarthritis than in those with the milder form of the disease (20). In PsA, the number of activated CD25+CD8+ T cells infiltrating psoriatic skin lesions positively correlates with the psoriasis area and severity index (PASI) disease activity score (21). Additionally, the frequency of IL-17-producing CD8+ T cells in the synovial fluid of patients with PsA positively correlates with clinical parameters of inflammatory status (such as C-reactive protein and the erythrocyte sedimentation rate) and disease activity scores (22). Correlations of CD8+ T cell frequency and phenotype with disease severity and clinical outcome not only indirectly suggest a role for CD8+ T cells in chronic inflammation, but also reveal their potential as biomarker of disease activity.

MATURATION AND DIFFERENTIATION

CD8+ T cell differentiation and subsets

Scant data are available on CD8+ T cell differentiation in human inflammatory conditions. Nonetheless, different states of CD8+ T cell differentiation can be identified on the basis of the expression of CD45RA and CCR7 (REF. 23). Within the memory T cell compartment (CD45RO+), CD8+CCR7+ central memory T (TCM) cells home to secondary lymphoid organs mounting robust recall responses, whereas the CD8+CCR7– effector memory T (TEM) cell subset is commonly found in nonlymphoid tissues. Both CD8+ TCM and CD8+ TEM cell populations are thought to continuously recirculate through blood vessels as well as lymphoid and nonlymphoid organs (24), and to convert into tissue-resident memory cells (25,26) (see discussion of this newly identified subset below).

Like CD4+ T cells, CD8+ T cells form a heterogeneous population in which different subsets and functions can be identified not only on the basis of differentiation markers, but also on the basis of cytokine production. For example, CD8+ T cell subpopulations producing IFN-γ and IL-4 (REF. 27), and more recently, those producing IL-17 and IL-22 (REFS 28,29) have been described in humans.

In human autoimmune arthritis, the phenotype of CD8+ T cell subsets in peripheral blood is skewed towards an activated or inflammatory phenotype. Indeed, the frequency of CD8+ TEM cells is increased in the peripheral blood of patients with RA compared with age- matched healthy donors, whereas terminally differentiated (CCR7– CD45RA+) CD8+ TEM cells are reduced (13,18). CD8+ T cell production of TNF, IL-17 and the cytolytic enzyme granzyme B is increased in the peripheral blood of patients with active RA(13); however, a minor increase in IL-10-producing CD8+ T cells is also observed when their expression in patients was compared with that in healthy individuals. Another study has shown that CD8+ T cell production of granzyme B and perforin 1 in peripheral blood of patients with RA and healthy controls is similar (18). Moreover, a clear enrichment of

IL-22-producing CD8+ T cells was found in the circulation of patients with PsA (30), and IL-17A-producing CD8+ T cells were shown to be elevated in skin plaques(31), which is consistent with the relevance of IL-17 in disease pathology (32).

Interestingly, in the synovium of patients with RA, CD8+ T cells have a unique profile at the site of inflammation, with pro-inflammatory and anti-inflammatory features coexisting in the same compartment (TABLE 1). These cells might undergo local differentiation into 'early' (CD27+ CD28+) and 'transitory' (CD27– CD28+) memory subsets (18), which are regarded as intermediate states of differentiation between CD8+ TCM and TEM cells (33). The synovial fluid of patients with RA comprises highly proliferative CD8+ TEM cells that express elevated levels of activation markers such as CD80, CD86, CD69 and CD25 (REFS. 13,18), Fas (TNF receptor superfamily member 6) and chemokine receptors, and cytokines such as IL-6 and TNF (13,18). At the same time, CD8+ T cells derived from synovial fluid also have anti-inflammatory properties. In RA, CD8+ T cells producing IL-4 and IL-10 are enriched in synovial fluid18, together with an increased expression of negative co-stimulatory markers such as PD1 and TIM3 (REFS 18,34); by contrast, the production of cytotoxic enzymes is reduced (18). Additionally, a subset of CD8+ T cells producing IFN-γ, but not perforin 1, was found to be enriched in the outer layer of ectopic germinal centres present in the synovial tissue of patients with RA; in this context, investigators have hypothesized that these IFN-γ-producing T cells physically interact with

Table 1. Functional heterogeneity of $CD8^+$ T cells from the synovial fluid of patients with RA

Function	Effector profile	Anti-inflammatory profile
Cytokine production	↑ IL-6	↑ IL-4
	↑ TNF	↑ IL-10
Cytotoxic enzyme release	NA	↓ Granzyme B
		↓ Perforin 1
Negative costimulation	NA	↑ PD1
		↑ TIM3
Differentiation	↑ CD45RO	NA
	↓ CCR7	
	↑ CD28	
	↓ CD127	
Activation	↑ CD25	NA
	↑ CD69	
	↑ CD80	
	↑ CD86	
Homing	↑ CCR4	NA
	↑ CCR5	
	↑ CXCR4	

NA, not applicable; TIM3, T-cell immunoglobulin mucin receptor 3 (also known as hepatitis A virus cellular receptor 2).

dendritic cells (DCs) and B cells, acting as T helpers (35). Thus, the pro-inflammatory environment, together with local autoantigen-stimulated and cytokine-induced (for example, by IL-15 (REFS 36,37)) clonal expansion, might drive CD8+ T cells into a unique status of differentiation (FIG. 1).

Clonal expansion and antigen specificity

In the 1990s, CD8+ T cells accumulating in the synovial fluid of patients with RA were shown to carry an oligoclonal T cell receptor (TCR) repertoire(38–40), which is an indication of antigen-driven clonal expansion. To date, the TCR clonality has been shown to be specific for viruses such as the Epstein–Barr virus (EBV), the cytomegalovirus (CMV) and the influenza virus (41). However, this evidence does not exclude the existence of autoantigen-specific CD8+ T cells recognizing unknown epitopes. Data obtained from next-generation sequencing confirmed the presence of a restricted TCR repertoire, which

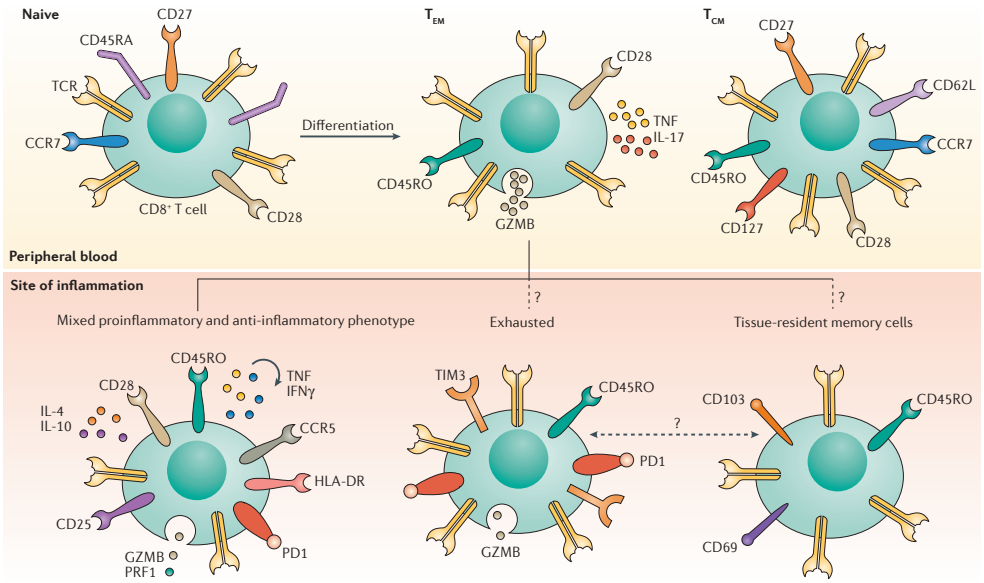

Figure 1. CD8+ T-cell differentiation in autoimmune inflammation. In the peripheral blood of patients with autoimmune arthritis, a high frequency of CD8+ effector memory T (TEM) cells producing elevated levels of pro-inflammatory cytokines differentiate from naive cells (13,18,32). CD8+ T-cell differentiation in the inflamed joint can be both antigen-driven and inflammation-driven, leading to the development of different cell populations: CD8+ TEM cells characterized by an heterogeneous phenotype (whether this population represents a single cell type with a mixed phenotype or reflects different cell subsets is unknown (13,16)); CD8+ TEM cells that upregulate negative co-stimulatory markers and might develop an exhausted phenotype (18,34) (whether this phenotype corresponds to a functional exhaustion is still unknown); or CD8+ TEM cells that reflect tissue-instructed differentiation, as in tissue-resident memory T cells (50). GZMB, granzyme B; PRF1, perforin 1; TCM, central memory T cell; TCR, T-cell receptor; TIM3, T-cell immunoglobulin mucin receptor 3 (also known as hepatitis A virus cellular receptor 2).

was mostly evident in the synovium of patients with recent-onset RA, rather than those with established disease (42). In the periphery, the TCR repertoire was polyclonal, with hardly any overlap with that seen in the synovial fluid. Interestingly, the most abundantly expanded clones were shared between different joints in the same patients (42). These data suggest that specific clones, which are probably autoreactive towards disease-relevant antigens, migrate from the periphery to the inflamed joint — even possibly recirculating among different joints — driving inflammation and disease relapse. Consistent with this hypothesis, observations in patients with PsA suggest a clear overlap of T cell clones in different target tissues such as skin and synovium (25). Identification of the most prominent clones in target organs of autoimmune arthritis might be the key to identifying potential autoreactive cells and their putative autoantigens.

Tissue-resident memory T cells

Within the memory CD8+ T cell compartment, the identification of a new subset in mice and humans — tissue-resident memory T (TRM) cells (26) — has catalysed the interest of the scientific community over the past 5 years. TRM cells are retained in organs such as lungs, intestine, skin, liver and genital mucosa, and do not recirculate in blood or lymphoid organs; instead, they have a critical role in local immuno- surveillance (43,44). In homeostatic conditions, TRM cells are enriched for the effector memory phenotype and express markers such as CD69 and CD103 but not CCR7 or CD62L (43,45–47). Scant data are available on TRM cells in human AID, particularly at sites of autoimmune inflammation (48). This cell population might adopt a quiescent inflammatory profile during the remission phase and then be periodically reactivated by unknown triggers. In support of this hypothesis, a massive infiltration of T cells expressing TRM-cell markers was shown to be present in the epidermis in active psoriasis (49). These cells were still present in the remission phase and were capable of producing cytokines known to be critical in the pathogenesis of psoriasis. In human RA, a preliminary report described the presence of TRM cells in the synovial tissue (50). Moreover, our unpublished data show that CD69+CD103-CD8+ T cells and CD69+CD103+CD8+ T cells are also present in the synovial fluid of patients with JIA. These TRM-like cells might derive from the synovial tissue and be actively shed in the synovial exudate during the active disease phase (FIG. 1). Research on TRM cells needs to be extended in humans, as the exact definition of their role in autoimmunity could radically change current dogma and drive the development of new treatments.

CD8+ T CELL REGULATION

Like CD4+ T cells, CD8+ T cell functions can be controlled by negative co-stimulatory signals and regulatory cells. The alteration of these regulatory mechanisms might sustain inflammation in autoimmune arthritis.

Inhibitory receptors and 'exhaustion'

The balance between positive and negative co-stimulation determines the strength and duration of T cell activation, acting as a thermostat for immune responses (51). Some pathways attenuate the strength of the TCR signal; this process — known as 'co-inhibition' — is used to control effector cells and limits undesired hyperactivation (52). PD1–PDL1 and CTLA4–B7 pathways induce a co-inhibitory signal in activated T cells to promote T cell anergy (53,54); this signal is also used by regulatory CD4+CD25+ T (TREG) cells as a mechanism of contact-mediated cell suppression (55,56). A similar effect has been described for the TIM3–Gal9 pathway, which can also induce inhibitory signals in T cells (57). Overexpression of negative co-stimulatory markers is also a feature of 'exhausted cells', which are currently well described only in chronic viral infection and cancer(58). Exhausted CD8+ T cells lack effector functions and upregulate inhibitory receptors such as PD1, LAG3, TIM3, CD224 and CD160 (REF. 59). In RA, the frequency of PD1+CD8+ T cells in peripheral blood has been shown to be reduced (14), or unchanged (18), compared with that in healthy controls; by contrast, the accumulation of this cell population is evident in the synovial fluid (18). Also, the expression of TIM3 in CD8+ T cells is increased in peripheral blood of patients with RA and is even higher in the synovial fluid (34). Interestingly, a CD8+ T cell signature of exhaustion in peripheral blood of patients with AID was shown to correlate with good prognosis (105). However, in a preclinical model of acute virus infection, TRM cells were shown to upregulate negative co-stimulatory markers without signs of functional exhaustion, and were found to provide enhanced protection(60). This process might also occur in chronic autoimmune inflammatory environments, where TRM cells might express negative co-stimulatory markers as a result of tissue-instructed cell differentiation, rather than as a sign of exhaustion (FIG. 1). Given that cells isolated from the synovial fluid are collected when the disease is at the peak of its activity, negative co-stimulation in this context might be overruled by positive signals or, alternatively, tissue-instructed differentiation might drive CD8+ T cells into an incontrollable pro-inflammatory state owing to an aberrant function of inhibitory signals, as shown for CD4+ T cells in synovial fluid (61). This hypothesis could explain why, despite negative co-stimulatory molecules being enriched in the inflamed joints of patients with autoimmune arthritis, CD8+ T cell homeostasis is lost at this site.

Resistance to suppression

The inability of effector T cells to be inhibited and controlled by other cell populations such as TREG cells is described as 'resistance to suppression'. This phenomenon has been described in multiple autoimmune diseases including RA (62–65), and was shown to sustain uncontrolled systemic and local inflammation. We and others have shown that TREG cell-mediated suppression is impaired in T cells isolated from the synovial fluid of patients with JIA (66,67); furthermore, both CD4+ and CD8+ effector cells are intrinsically resistant to sup- pression68. The mechanism of CD8+ T cell resistance to suppression seems to be mediated by autocrine release of TNF and IFN-γ (68) and

involves PKB hyperactivation (66,69) (FIG. 2). Of note, in CD8+ T cells in the peripheral blood of patients with JIA, susceptibility to suppression is conserved (66). Hence, despite the enrichment of TREG cells in inflamed joints (66), the pro-inflammatory response of CD8+ effector T cells is not well controlled.

CD8+ T CELLS AS IMMUNE SUPPRESSORS

CD8+ T cells have been described to have the capacity to inhibit T cell responses (2). Extensive literature shows that CD8+ TREG cells attenuate the pathology of AID, at least in preclinical models (70–72). Although the precise mechanisms of CD8+ TREG cell-mediated suppression are not fully understood, they seem to mirror those of CD4+CD25+ TREG cells: CD8+ TREG cells produce immunosuppressive cytokines, express inhibitory

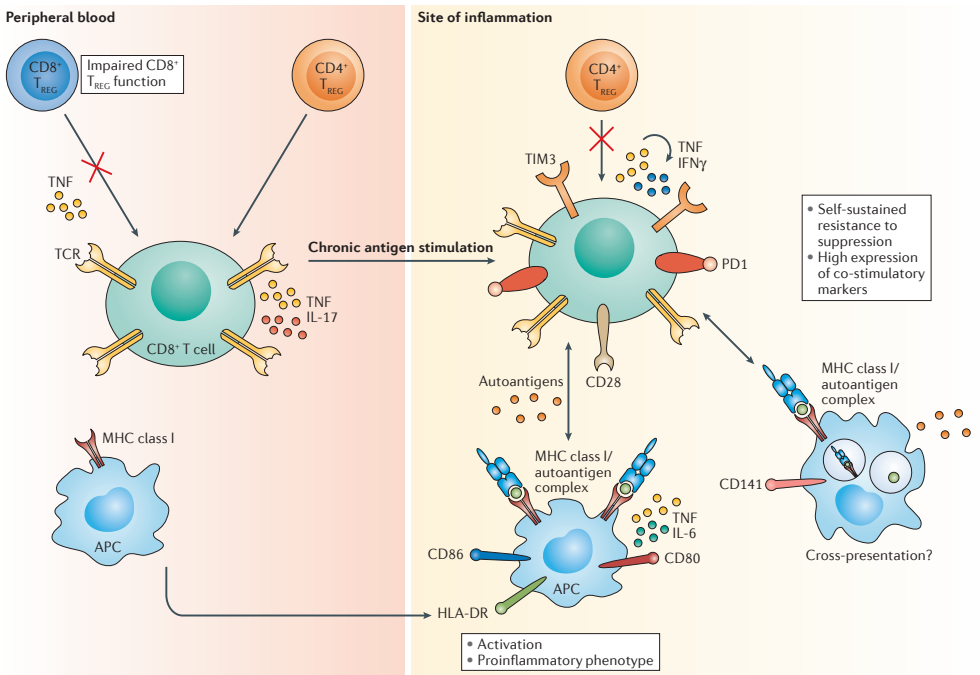

Figure 2. CD8+ T cell regulation in autoimmune arthritis. Despite an increased release of pro-inflammatory cytokines, CD8+ T cells are susceptible to regulation by CD4+CD25+ regulatory T cells (TREG) in the peripheral blood of patients with autoimmune arthritis (66). However, CD8+ TREG cells have impaired suppressive function (thus far only reported in REF. 76). In the inflamed joints, CD8+ T cells develop resistance to suppression, which is sustained by the autocrine release of pro-inflammatory cytokines (66–68). In addition, negative costimulatory molecules (PD1 and T-cell immunoglobulin mucin receptor 3 (TIM3, also known as hepatitis A virus cellular receptor 2)) are upregulated in these cells (18,34). Autoantigens might be presented or cross-presented by activated antigen-presenting cells (APCs) (90,91), which can contribute to the altered phenotypic and functional properties of CD8+ T cells. TCR, T-cell receptor.

surface molecules, release cytotoxic enzymes and degrade extracellular ATP (73). In humans, different subsets of CD8+ T cells that are able to suppress conventional T cell responses have been identified, including CD8+CD28–, CD8+CD103+ and CD8+CD122+ T cells (74). The CD8+CD28– T cell subset was shown to express high levels of FOXP3 and to suppress T cell proliferation via the production of IL-10 and TGFβ (70) as well as the upregulation of cytotoxic T-lymphocyte protein 4 (CTLA4) (75). In peripheral blood of patients with RA treated with methotrexate, CD8+CD28– TREG cells were found to be increased in frequency compared with those in healthy controls, but expressed low levels of negative co-stimulatory markers such as PD1 and ICOS (76). Furthermore, induced CD8+FOXP3+ T cells could be successfully expanded in vitro from the peripheral blood mononuclear cell fraction of patients with RA and retained high contact-dependent suppressive capability upon anti-CD3 stimulation (77). Of note, CD8+CD28– TREG cells from the peripheral blood of patients with RA treated with methotrexate but not TNF inhibitors showed an impaired function that was restored in vitro by incubation with anti-TNF drugs (76). Thus, besides restoration of CD8+ effector T cell susceptibility to suppression, other possible mechanisms of action of therapeutic agents currently used in clinical practice might include the improvement of CD8+ TREG cell function.

POTENTIAL INTERACTION WITH APCS

A unified ontogeny-based nomenclature of mouse and human DCs was proposed in 2014 (REF. 78). This nomenclature divides DCs into three subtypes: CD141+ classical type 1 DC (cDC1), CD1c+ classical type 2 DC (cDC2) and plasmocytoid DC (pDC) (78). Human monocytes have been subdivided into three main groups on the basis of CD14 and CD16 expression (79): classical (CD14high CD16–), intermediate (CD14high CD16+) — known as 'pro-inflammatory' given the elevated production of proinflammatory cytokines and potency in antigen presentation (80) — and non-classical (CD14+ CD16high). A number of reports describe highly activated total DCs and cDC2s (REFS 81,82), increased cDC2:pDC ratio (83) and enrichment in activated CD14+CD16high monocytes (84,85) in the synovial fluid of patients with RA, the latter being able to induce type 1 or type 17 T helper cells (85). However, no data on the specific interplay between APCs and CD8+ T cells in autoimmune arthritis are available.

All human DC subsets are able to cross-present in vitro if properly stimulated (79). However, cDC1s were shown to be the most efficient cross-presenting subset, especially in the cross-presentation of antigens from necrotic cells (86). cDC1s are present at very low frequency in the peripheral blood of healthy individuals (87), and have been shown to efficiently cross-present exogenous or cell-associated proteins (86) (such as autoantigens) to CD8+ T cells in preclinical models (88) and humans (89). Additional evidence indicates that human tissue-resident cDC1s are also very efficient at antigen cross-priming (90), especially of necrotic cells (86). Interestingly, cDC1s are highly enriched in the synovial fluid of patients with JIA (91) and express markers of maturation such as

CD40, CD80, CD86 and CD83 (FIG. 2). Although the role of cDC1s in disease pathology has not yet been discovered, these cells might be players in the presentation of antigens (or cross-presentation of autoantigens) to CD8+ T cells at the site of chronic autoimmune inflammation.

IMPLICATIONS FOR THERAPY

Despite the heterogeneity of AID that are characterized by joint inflammation, most patients with chronic human arthritis such as RA and JIA are still treated according to a step-up strategy. Treatment consists of an initial use of NSAIDs and/or temporary use of (intra-articular) corticosteroids, followed by DMARDs such as methotrexate and, at a later stage, biologics such as TNF or IL-6 inhibitors. Additional treatments might include B-cell depletion with rituximab or the use of CTLA4Ig (abatacept) to inhibit the CD28–B7 pathway (92,93). How do standard or experimental drugs influence CD8+ T cell frequency, phenotype and function in patients? Methotrexate treatment was shown to enhance the proliferation of circulating CD8+ T cells in patients with JIA after 6 months of therapy, without attenuating the capacity of these cells to produce pro-inflammatory cytokines (94); by contrast, no effect on the T cell compartment was found in patients with RA following treatment with a combination of methotrexate and infliximab (12). Moreover, anti-TNF (adalimumab) treatment alone was shown to decrease IFN-γ-producing CD8+ T cells in patients with RA (95). Interestingly, the impaired suppressive ability of CD8+ TREG cells observed in peripheral blood of patients with RA is rescued by in vitro co-incubation with infliximab (76). In addition, anti-TNF (etanercept) treatment was able to rescue the intrinsic resistance to suppression of CD8+ T cells derived from the synovial fluid of patients with JIA (68), and to reduce the level of phosphorylated PKB of this cell population in vivo (69). Together, these data indicate that inhibition of TNF has beneficial effects on CD8+ T cells because it dampens their pathogenic phenotype, both in peripheral blood and synovial fluid. Interestingly, clinical response to treatment with other biologic agents, such as abatacept or rituximab, has been associated with a decrease in IFNγ-producing and IL-17-producing CD8+ T cells (96), or a decrease in total CD8+ T cell counts (97), respectively. Although most of the beneficial effects of biologic drugs in autoimmune arthritis have been attributed to their ability to antagonize the function of CD4+ T cells, they also have a clear effect on the CD8+ T cell compartment.

CONCLUSIONS

Despite the huge advances in our understanding of the mechanisms underlying autoimmune arthritis made in the past decade, no cure exists and most patients require the use of lifelong therapies to control clinical symptoms. The evidence of an association between HLA class I and disease risk, as well as reports showing that CD8+ T cell frequency and phenotype correlate with disease outcome, provide support to the hypothesis that CD8+ T cells have a role in human autoimmune arthritis. In particular, at sites of chronic autoimmune

inflammation, CD8+ T cells are a heterogeneous population with both pro-inflammatory and anti-inflammatory properties, and lack susceptibility to suppression as a consequence of autocrine signalling. Apparently, effector features overrule the mechanisms set in place to dampen chronic inflammation, leading to an imbalanced immune response. In our opinion, CD8+ T cell functional properties in autoimmune arthritis deserve further investigation to define the exact contribution of this predominant T cell subset to disease pathology. In particular, the role of negative co-stimulatory markers in CD8+ T cells at the site of chronic inflammation might define either the enrichment of a population of harmless exhausted cells or a cell subset with a potentially potent detrimental effect. In this context, CD8+ T cells that permanently reside in target organs (TRM cells) might have a key role in disease pathology by driving disease relapse. Finally, further effort should be put into the study of APC cross-priming, owing to its potential relevance in CD8+ autoantigen responses. Deciphering the specific mechanisms of CD8+ T cell function — which is known to be partly different from that described for CD4+ T cells(68) — is critical to understanding disease pathology and might lead to the development of novel therapeutic approaches for patients with chronic inflammatory diseases.

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KEY POINTS

- $CD8^+$ T cell homeostasis is altered in the peripheral blood of patients with autoimmune arthritis
- The close association of MHC class I polymorphisms with disease risk and the correlation of $CD8^+$ T cell number with disease outcome support the idea that this cell population has a role in autoimmune arthritis
- At sites of chronic inflammation, the phenotype of $CD8^+$ T cells is heterogeneous and includes pro-inflammatory and anti-inflammatory features
- In inflammatory environments, $CD8^+$ T cells lose susceptibility to regulation, and this loss is sustained by autocrine release of pro-inflammatory cytokines
- Subsets of memory $CD8^+$ T cells upregulate negative co-stimulatory markers and either develop an exhausted phenotype or display a tissue-instructed differentiation, as in tissue-resident memory T cells

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Chapter 3

CD8 T CELL RESISTANCE TO SUPPRESSION AT THE SITE OF AUTOIMMUNE INFLAMMATION IS SELF-SUSTAINED AND CAN BE REVERSED BY TNF AND IFN-γ BLOCKADE

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ABSTRACT

Objective

Resistance of effector T cells (Teff) to regulatory T cell (Treg)-mediated suppression contributes to the breakdown of peripheral tolerance in the inflamed joints of Juvenile Idiopathic Arthritis (JIA) patients. However, an unanswered question is whether this resistant phenotype is self-sustained and whether $CD8^+$ and $CD4^+$ Teff share the same mechanism of resistance to suppression. Here, we investigated $CD8^+$ Teff intrinsic resistance to suppression and how this can be targeted therapeutically.

Methods

$CD8^+$ or $CD4^+$ Teff were cultured with or without antigen presenting cells (APC) in Treg-dependent and -independent suppression assays. Synovial fluid (SF)-derived Teff were cross-cultured with peripheral blood (PB) Treg from JIA patients or healthy controls. TNF-α or IFN-γ blocking agents were used to restore Teff responsiveness to suppression.

Results

Suppression of cell proliferation and cytokine production by $CD8^+$ Teff from the SF of JIA patients was severely impaired compared to PB of JIA patients, regardless of APC and $CD4^+$ Teff cell presence. Similarly to $CD4^+$ Teff, impaired suppression of $CD8^+$ Teff was shown to be an intrinsic feature of this cell population. Whereas TNF-α blockade rescued both $CD8^+$ and $CD4^+$ Teff resistance, autocrine release of IFN-γ selectively sustained $CD8^+$ Teff resistance, which could be relieved by IFN-γ blockade.

Conclusion

Unlike $CD4^+$ Teff, resistance of $CD8^+$ Teff to suppression at the site of autoimmune inflammation is maintained by autocrine release of IFN-γ and blockade of IFN-γ restores $CD8^+$ Teff responsiveness to suppression. These findings indicate a potential therapeutic value of blocking IFN-γ to restore immune regulation in JIA.

INTRODUCTION

Autoimmune diseases such as Rheumatoid arthritis (RA) and Juvenile Idiopathic Arthritis (JIA) are characterized by an aberrant immune response towards self-antigens leading to severe tissue damage (1, 2). Both diseases are featured by infiltration of the synovial tissue by immune cells, proliferation of synoviocytes and accumulation of synovial fluid (SF) in the joint causing swelling and movement limitation (2). Among other cell populations, the target organ is typically massively infiltrated by T cells, which display an activated phenotype and maintain a pro-inflammatory environment (2). In healthy conditions, latent auto-reactive effector T cells (Teff) can be successfully controlled by mechanisms of peripheral tolerance, such as regulatory T cell (Treg)-mediated suppression (3). However, in autoimmune diseases, the breakdown of peripheral tolerance (4, 5) leads to insufficient control of Teff cells and Teff cell-induced pathology.

Our group has recently shown that, Teff cells from the SF of JIA patients, but not from the peripheral blood (PB), are refractory to Treg-mediated suppression due to Akt hyperphosphorylation (6). In addition, we showed that responsiveness to suppression could be successfully restored by anti-TNF-α in vitro (7). Since antigen presenting cells (APC) are a likely source of TNF-α, we wondered whether the resistant phenotype of $CD4^+$ and $CD8^+$ Teff cells is self-sustained and can be maintained by the absence of APC in culture. Haufe et al. recently demonstrated that purified $CD4^+$ Teff cells from the SF of patients with RA were intrinsically resistant to suppression, suggesting that the resistant phenotype of $CD4^+$ Teff cells is maintained in the absence of pro-inflammatory cytokines produced by APC (8). However, it remains to be explored what mechanism underlies this self-sustainaned resistance of $CD4^+$ Teff cells to suppression.

To date, among Teff cells, the major interest has been dedicated to $CD4^+$ T cells (8, 9). However, $CD8^+$ Teff cells are relevant in arthritis, known to be enriched in the SF of patients with RA (10, 11) and contributing to pro-inflammatory cytokine production (11). SF $CD8^+$ T cells are also resistant to suppression but it remains to be explored whether this resistance to suppression is cell intrinsic, independent of the presence of $CD4^+$ Teff cells and APC and whether $CD8^+$ Teff cell resistance can be self-sustained. Defining the mediators that self-sustain Teff cell resistance at the site of autoimmune inflammation is of particular relevance in the scenario of drug-induced Treg expansion or Treg-based therapies, for which co-administration of a specific drug targeting Teff cell resistance will be a pre-requisite for successful treatment.

Here we investigated whether, similarly to $CD4^+$ Teff cells, $CD8^+$ Teff cell resistance to suppression at the site of autoimmune inflammation of JIA is intrinsic and which are the soluble mediators self-sustaining $CD8^+$ and $CD4^+$ Teff cells resistance to suppression. We found that $CD8^+$ Teff cells residing in the SF of JIA patients are intrinsically resistant to suppression, independent of the presence of $CD4^+$ T cells. TNF-α and IFN-γ redundantly sustain this resistance of $CD8^+$ Teff cells to suppression, whereas $CD4^+$ Teff cells are affected by TNF-α only. This study reveals a difference between $CD4^+$ and $CD8^+$ Teff

cells in the cytokine requirements maintaining their resistant state and demonstrates a therapeutic potential for IFN-γ blockade in restoring $CD8^+$ Teff cell responsiveness to suppression.

PATIENTS AND METHODS

Patient population

Patients with Juvenile Idiopathic Arthritis (JIA) were enrolled by the Paediatric Reumathology department at University Medical Center Centrum in Utrecht (The Netherlands) after giving written informed consent either directly and/or from parents/guardians when they were under 12 years of age. Patients with oligoarticular JIA (n=12) and patients with polyarticular JIA (n=6), according to the revised criteria for JIA (12), were included in this study. The study was conducted in accordance with local ethics committee approval (protocol Pharmachild) and the Declaration of Helsinki. The average age of the patient population was 13.8 years (range 8-17 years) and the disease duration at the time of inclusion was 5.5 years (range 1-13 years). Synovial fluid (SF) was obtained from 10 patients with active disease undergoing therapeutic joint aspiration. From n=4 patients paired blood and joint aspirate samples were collected. Peripheral blood only was withdrawn from n=4 patients. Patients were either untreated (n=4), or treated with nonsteroidal anti-inflammatory drugs (NSAIDs) alone (n=3) or in combination with methotrexate MTX (n=5), or with MTX alone (n=4) or with oral prednisolone (n=2, dose <0.3mg/Kg/day) at the time of inclusion. Paired samples were collected from patients treated with MTX alone (n=2), NSAIDs alone (n=1) and untreated (n=1). Healthy control (HC) buffy coats (n=10) from adult healthy volunteers were obtained from the blood bank (Sanquin, Amsterdam).

Cell isolation

SF Mononuclear Cells (SFMC) were incubated with Hyaluronidase (Sigma-Aldrich) for 30 min at 37°C. SFMC and PBMC were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences, AB) and frozen in foetal calf serum (FCS) (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation.

Cell culture and reagents

$CD8^+$ and $CD4^+CD25^-CD127^{hi}$ T cells (25.000 cells/100 µl) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10% human AB serum (all obtained from Invitrogen) at 37°C and 5% CO2 in round-bottom 96-well plates (Nunc). Cells were stimulated with anti-CD2/CD3/CD28 beads (Treg suppression inspector, Miltenyi) in some experiments or with $CD3^-$ cells as antigen presenting cells (APC). Each batch of Treg suppression inspector was titrated before performing experiments and adjusted at a cell to beads ratio to obtain a minimum 20% cell proliferation. In some conditions anti-human TNF-α (etanercept) and anti-human IFN-γ (eBioscience) were added at 1 µg/ml.

Suppression assay

$CD3^-$ cells (APC), $CD4^+CD25^+CD127^{low}$ Treg, $CD8^+$ and $CD4^+CD25^-CD127^{hi}$ T cells were sorted by flow cytometry on FACS Aria III (BD Biosciences) from the PB and the SF of JIA patients, and from the PB of HC. $CD8^+$ and $CD4^+CD25^-CD127^{hi}$ T cells were used as responder cells and named $CD8^+$ and $CD4^+$ Teff cells respectively throughout the manuscript. Teff cells were labelled with Celltrace Violet (CtV) fluorescent dye (Invitrogen) to measure proliferation by dye dilution. Treg were co-cultured with Teff cells at 1:2 (Treg:Teff) ratio and stimulated with either Treg suppression inspector or $CD3^-$ cells at 1:1 (APC:Teff) ratio. In cross-over assays autologous Treg from the PB of JIA patients or allogeneic Treg from HC were co-cultured with Teff cells from the SF. Treg-independent suppression of Teff cells from the SF of JIA patients and the PB of HC was performed by replacing Treg with 50ng/ml TGF-β (PeproTech). At day 4, proliferation of Teff cells was analyzed by flow cytometry and supernatant was collected to measure cytokine production.

Cytokines measurement

Supernatant was collected from suppression assays after 96-hour culture and IFN-γ, TNF-α, IL-6, IL-17 and IL-10 levels were measured by Luminex technology as previously described (6).

Flow cytometry

To detect intracellular cytokine production, cells were stimulated for 4 hours with PMA (20 ng/ml; MP Biomedicals) and ionomycin (1 μg/ml; Calbiochem), with Golgistop (1/1500; BD Biosciences) added for the last 3.5 hours of culture. Before staining, cells were washed twice in FACS buffer (PBS containing 2% FCS (Invitrogen) and 0.1% sodium azide -Sigma-Aldrich-) and subsequently incubated with surface antibodies (anti-human CD3 BV510, CD4 Alexa488, CD8 APC-Cy7). After surface staining, cells were washed twice in FACS buffer and fixed, permeabilized, and intracellularly stained using anti-human TNF-α (Fitc) and IFN-γ (Pe-Cy7). To determine Treg purity after sorting, sorted Treg were washed twice with FACS buffer, fixed, permeabilized, and intracellularly stained using anti-human FOXP3 staining set (eBioscience, according to the manufacturer's instructions).

Statistical analysis

For statistical comparison between two unpaired groups Mann Whitney test was used. We used Wilcoxon signed-rank test to analyze paired samples. P values below 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism (GraphPad Software).

RESULTS

SF CD8$^+$ Teff are intrinsically resistant to suppression, regardless of APC and CD4$^+$ T cell presence

We (6) and others (8, 13) have previously shown that Teff cells from the site of chronic autoimmune inflammation of JIA patients (i.e synovial fluid, SF) are resistant to Treg-mediated suppression of proliferation and cytokine production in the presence of APC. However, in these studies, the main focus has been on CD4$^+$ Teff cells and it has not been investigated whether CD8$^+$ Teff cells are resistant to suppression independent of CD4$^+$ Teff and APC presence. To investigate this, we cultured purified CD8$^+$ Teff cells from the SF of JIA patients and compared suppression by Treg when using either APC from the same site (i.e. SF) or anti-CD3/CD2/CD28 coated beads as stimulators. Suppression of CD4$^+$ Teff cells was used as reference. SF Treg were sorted (as shown in Supplementary Figure 1A) and tested for purity by staining for FoxP3, whose expression was confirmed to be higher as compared to the one of CD4$^+$ Teff cells (Supplementary Figure 1B). Purified CD8$^+$ Teff cells from the SF of JIA patients showed enhanced proliferation compared to cells from PB when cultured with APC, but not with beads (Fig. 1A, left panel). No differences in cell proliferation were evident in the CD4$^+$ Teff cell compartment (Fig. 1A, right panel). Suppression of CD8$^+$ Teff cell proliferation from SF was impaired compared to cells from PB both in the presence or absence of APC (Fig. 1B, left panel). However, CD8$^+$ Teff cell proliferation did not correlate with suppression levels (data not shown). In line with Haufe et al., CD4$^+$ Teff cells showed impaired suppression of cell proliferation both in the presence and the absence of APC as well (Fig. 1B, right panel). Representative histograms showing CD8$^+$ and CD4$^+$ T cell proliferation and suppression are shown in Supplementary Figure 2. To elucidate whether the impaired Treg-mediated suppression observed in APC-independent assay applies not only to Teff cell proliferation but also to cytokine release, we tested cytokine levels in the supernatant of suppression assays with sorted CD8$^+$ or CD4$^+$ Teff cells stimulated with CD3/CD2/CD28 beads (Fig. 1C left and right panel, respectively). In line with the proliferation data, cytokine production was suppressed when Treg from the PB of JIA patients were co-cultured with CD8$^+$ Teff cells from the same site (Figure 1C, left panel), but no suppression of cytokine production by CD8$^+$ Teff from SF was observed (Figure 1C, left panel). Although not statistically significant, the same trend was observed when CD4$^+$ Teff cells were used as responders (Figure 1C, right panel). IL-6 and IL-17 levels were also measured, but they were below the detection limit in most of the conditions.

Overall, these data show that, similarly to CD4$^+$ Teff cells, CD8$^+$ Teff cells from the site of autoimmune inflammation of JIA patients are intrinsically resistant to Treg-mediated suppression regardless of APC and CD4$^+$ T cell presence.

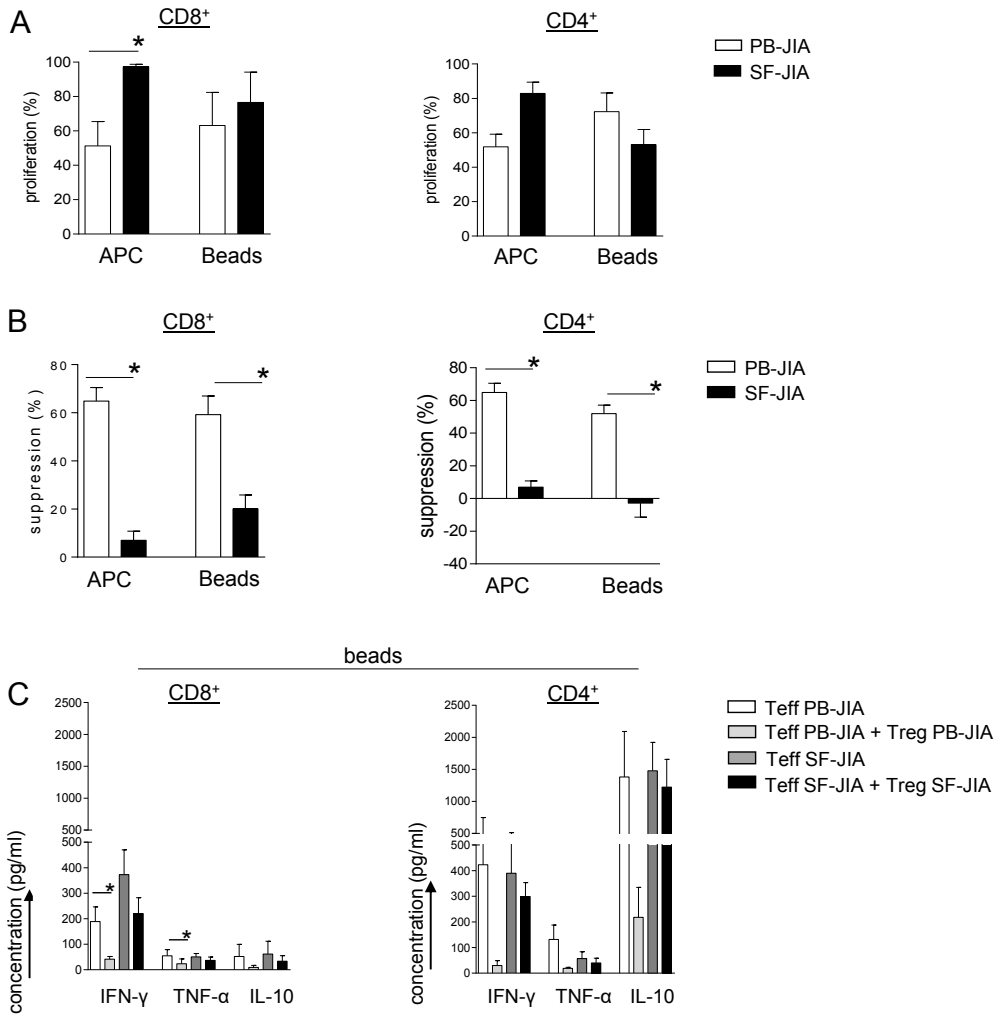

Figure 1. SF-derived CD8⁺ Teff cells display intrinsic resistance to suppression of cell proliferation and cytokine production, independent of CD4⁺ Teff cells or APC presence. (A) Proliferation of CtV-labelled CD8⁺ (left panel) and CD4⁺ (right panel) Teff cells from the PB and the SF of JIA patients in the presence of APC ($CD3^-$ cells) from the same site (i.e. PB or SF, respectively) or anti-CD2/CD3/CD28 beads as stimulators. (B) CtV-labelled CD8⁺ Teff cells (left panel) from the PB and the SF of JIA patients stimulated with APC (PB-JIA, n=6; SF-JIA, n=3) from the same site (i.e. from the PB and the SF, respectively) or with anti-CD2/CD3/CD28 beads (PB-JIA, n=5; SF-JIA, n=6) were co-cultured in the presence or the absence of Treg from the same site (i.e. from the PB and the SF, respectively). In parallel CD4⁺ Teff cell suppression was tested. Treg-mediated suppression of Teff cell proliferation after 96-hour co-culture is shown. (C) Cytokine levels in the supernatant after 96-hour co-culture using anti-CD2/CD3/CD28 beads in the presence of CD8⁺ (left panel) and CD4⁺ (right panel) Teff cells were measured by Luminex assay. Bar graphs indicate mean ± SEM. *p<0.05

CD8$^+$ Teff cells from the SF of JIA patients are intrinsically resistant to suppression, even in the presence of functional Treg or an immunosuppressive cytokine

To further confirm that the resistance to suppression of SF CD8$^+$ Teff cells is purely Teff cell-dependent and not resulting from the interplay with SF Treg, we investigated suppression of SF CD8$^+$ Teff cells by different sources of Treg. Given the ability of Treg to equally suppress when derived from the PB of JIA patients or adult HC (6), we first, performed an autologous cross-over suppression assay, using Treg from the PB of JIA patients co-cultured with paired sorted CD8$^+$ or CD4$^+$ Teff cells from the SF and stimulated with beads. In line with previously described data (6) PB derived Treg from JIA patients were perfectly capable of suppressing PB-derived CD8$^+$ Teff cells. However, even in the presence of these PB Treg, CD8$^+$ Teff cells from SF were resistant to suppression (Fig. 2A, left panel). CD4$^+$ Teff cells behaved similarly to CD8$^+$ Teff cells (Fig. 2A, right panel). Representative histograms of SF Teff cell proliferation and suppression are shown in Supplementary Figure 3. Although autologous Treg from the PB of JIA patients were able to suppress PB-derived Teff cells, we cannot exclude a reduced functionality of these cells that remains undetected due to limited sensitivity of the assay. Therefore, we also performed an allogeneic suppression assay, using functional Treg from the PB of adult HC. In 6 out of 8 SF samples CD8$^+$ Teff proliferation was not suppressed upon co-culture with functional Treg from adult HC (Fig. 2B left panel). No correlation between the restoration of suppression occurring in the n=2 SF samples and clinical features of the patients were evident. Despite donor-to-donor variability was evident in the presence of SF-derived CD4$^+$ Teff cells as well (Fig. 2B right panel), suppression by co-cultures with Treg from HC was significantly reduced compared to the reference condition, overall indicating that resistance of CD4$^+$ and CD8$^+$ Teff cells to suppression is independent of the Treg source. This was also confirmed by the absence of suppression of cytokine levels in the supernatant of the autologous (Supplementary Fig. 4A) and allogeneic (Supplementary Fig. 4B) assay. In addition, in a Treg-independent suppression assay, where Treg were replaced with TGF-β (a well-known mediator of suppression (14)), we found that SF-derived CD8$^+$ Teff cell suppression was impaired as compared to adult HC (Fig. 2C, left panel). The same was evident for CD4$^+$ Teff cells (Fig. 2C, right panel). Both CD8$^+$ and CD4$^+$ Teff cells from the SF were also found to be enriched in memory cells and showed an hyperactivated state as indicated by the increased expression of CD45RO, CD25 and CD69 when compared to the PB of JIA patients (Supplementary Figure 5).

Overall, these data show that CD8$^+$ Teff cells from the site of chronic autoimmune inflammation in JIA patients are intrinsically resistant to suppression, even in the presence of functional Treg from PB of JIA patients or healthy controls or a potent immunosuppressive cytokine such as TGF-β.

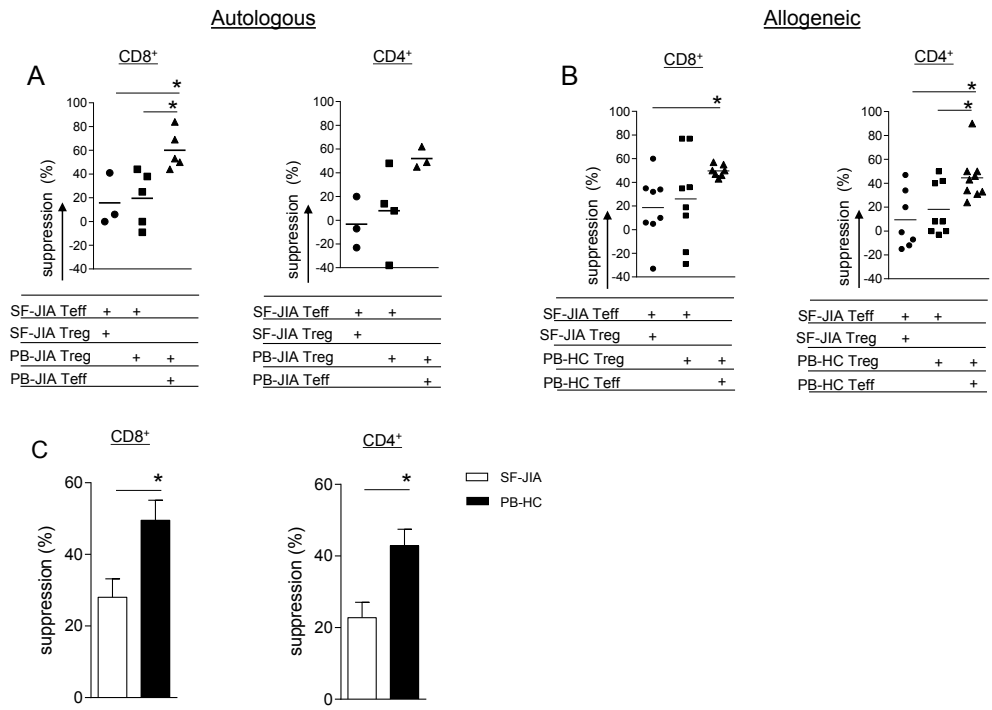

Figure 2. SF-derived CD8+ Teff cells are intrinsically resistant to suppression even in the presence of functional Treg or immunosuppressive cytokine. (A) APC-independent, Treg-mediated suppression of CD8+ Teff cells (left panel) from the SF of JIA patients was performed in the presence of autologous Treg collected from the PB of JIA patients (n=5). Reference values (third column of the graph) consisted in the suppression of Teff cells from the PB of JIA patients by autologous Treg (n=5). In parallel CD4+ Teff cell suppression was tested (right panel). (B) Treg-mediated suppression of CD8+ Teff cells (left panel) from the SF of JIA patients (n=8) was performed in the presence of allogeneic Treg collected from the PB of adult HC (n=7). In parallel CD4+ Teff cell suppression was performed (right panel). Reference values (third column of the graph) consisted in the suppression of Teff cells from the PB of HC subjects by autologous Treg. (C) Treg-independent suppression of proliferation of CD8+ Teff cells from the SF of JIA patients (n=5) and the PB of adult HC subjects (n=8) was performed by co-incubation with TGF-β. In parallel TGF-β-mediated CD4+ Teff cell suppression of proliferation was tested. Bar graphs indicate mean ± SEM. *p<0.05

Resistance to suppression of CD8+ Teff cells from the SF is self-sustained by TNF-α and IFN-γ and responsiveness to suppression can be restored by neutralizing either of these cytokines

Given the evidence that both CD8+ and CD4+ Teff cells show intrinsic resistance to suppression, we wondered whether this resistance was self-sustained by autocrine release of pro-inflammatory cytokines. TNF-α has been previously shown to contribute to resistance to suppression of CD4+ Teff cells (6) and TNF-α blockade was able to rescue Teff cell suppression in the presence of APC (7). IFN-γ is a pro-inflammatory cytokine relevant in autoimmune diseases and clearly detectable in the supernatant of our

suppression assay. In an APC-independent Treg-mediated suppression assay with SF-derived cells, co-incubation with anti-TNF-α or anti-IFN-γ led to restoration of CD8+ Teff cell suppression of proliferation (Fig 3A, left panel). Improvement of suppression was not observed in a HC suppression assay, indicating that this effect is SF T cell-specific (data not shown). However, CD4+ Teff cell proliferation was suppressed only in the presence of anti-TNF-α but not anti-IFN-γ (Fig. 3A, right panel). Of note, incubation of CD8+ and CD4+ Teff cells alone with anti-TNF-α or anti-IFN-γ had no effects on cell proliferation (Supplementary Fig. 6A and B, respectively). Co-incubation of CD8+ Teff cells with Treg and anti-TNF-α or anti-IFN-γ also restored suppression of cytokine production in the culture supernatant (Fig. 3B, left panel). Co-incubation of CD4+ Teff with Treg and anti-TNF-α resulted in a slight yet not statistically significant improvement of cytokine suppression whereas anti-IFN-γ had no effect (Fig. 3B, right panel). Then, we wondered whether the distinct effect of IFN-γ blockade on CD8+ and CD4+ Teff cells was due to the different amounts of IFN-γ released by the two cell populations. Indeed, we found that, unlike TNF-α, which was produced slightly more by CD4+ Teff cells (Figure 3C, left panel), CD8+ Teff cells produced significantly more IFN-γ then CD4+ Teff cells upon short PMA/ionomycin stimulation (Figure 3C, right panel). Overall these data show that production of TNF-α and IFN-γ contributes to intrinsic and self-sustained resistance of CD8+ Teff to suppression and that blockade of self-released TNF-α and IFN-γ restores responsiveness to suppression.

DISCUSSION

A big effort in the past years has been made to implement strategies for restoration of immunological balance between Treg and Teff cells in autoimmune diseases (15). Despite the fact that strategies to increase Treg number and function in rheumatic diseases are close to clinical application (reviewed in (16)), it is still under investigation whether resistance of Teff cells to suppression can be targeted in humans. So far, investigators have focused on the pathogenicity of CD4+ Teff showing their intrinsic resistance to Treg-mediated suppression, regardless the influence of other cell populations (i.e cytokines produced by APC or CD8+ T cell presence or Treg ability to suppress) (8).

In this study we aimed to define whether also CD8+ Teff cells from the site of autoimmune inflammation of JIA are intrinsically resistant to suppression and whether autocrine mediators are responsible for this phenotype. We found that Treg-mediated suppression of CD8+ Teff cells from the SF was impaired regardless of the presence of SF-derived APC or CD4+ Teff cells. We do not exclude the relevance of those populations in the induction/maintenance of the resistant phenotype, but our data rather indicate that CD8+ Teff cells are able to self-sustain this resistant phenotype. Being unable to rule out the contribution of SF Treg in the observed impairment of Teff cell suppression, we replaced them with Treg coming from the PB of JIA patients or healthy controls (both known to be functional (6)). Our data showed that Teff cell resistance to suppression

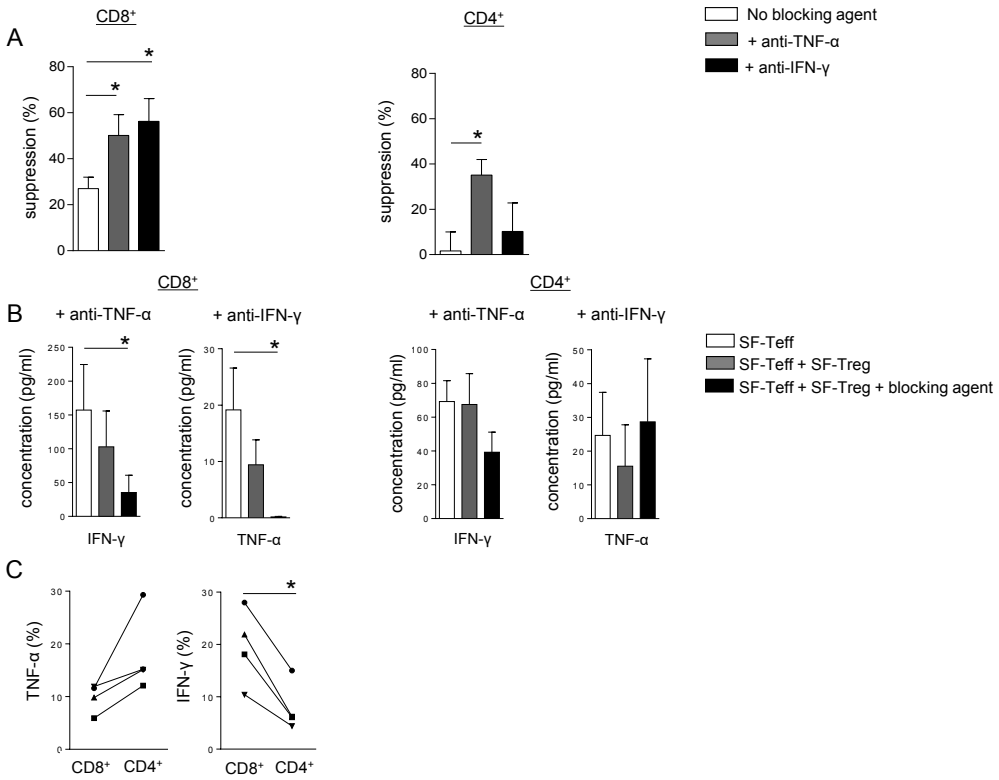

Figure 3. Resistance to suppression of CD8+ Teff cells from the SF is self-sustained by TNF-α and IFN-γ and can be restored by neutralizing these cytokines. (A) Suppression of SF-derived CD8+ Teff cell proliferation (left panel) by Treg from the same site was performed in the absence and presence of anti-TNF-α (n=6) or anti-IFN-γ (n=4). In parallel CD4+ Teff cell suppression (right panel) was performed (n=7). (B) Levels of IFN-γ and TNF-α in the supernatant where CD8+ (left panel) or CD4+ (right panel) Teff cells were co-cultured with Treg in the presence or the absence of anti-TNF-α and anti-IFN-γ, respectively are shown. (C) Frequency of TNF-α and IFN-γ-producing CD8+ and CD4+ Teff cells from the SF of JIA patients upon PMA/ionomycin stimulation. Bar graphs indicate mean ± SEM. *p<0.05

in the SF occurs regardless of SF-derived Treg suppressive abilities. Of note, a slight but consistent improvement of suppression in the presence of Treg from the PB was evident, suggesting the indirect evidence that Treg residing in the SF might have partial functional defects in an APC-independent setting (as shown in RA in (17)). Further evidence of CD8+ Teff cell intrinsic resistance to suppression was provided by their ability to retain the resistant phenotype in a Treg-independent suppression assay, as previously shown for CD4+ Teff cells (6). Resistance to suppression was found to be associated with a memory and activated phenotype of both CD8+ and CD4+ Teff cells; however, further experiments will be needed to prove that this phenotype is responsible for the lack of susceptibility to suppression observed in the SF. Interestingly, we noticed that, despite

Treg-independent susceptibility to suppression was similar for both $CD8^+$ and $CD4^+$ Teff cells (% of suppression: 25% $CD8^+$ vs. 20% $CD4^+$ Teff cells, see Figure 2C), when Treg were present, $CD4^+$ Teff cell resistance to suppression appeared to be higher than $CD8^+$ T cells (% of suppression in beads-assay: 20% $CD8^+$ vs. - 5% $CD4^+$ Teff cells, see Figure 1B). This suggest that SF-derived $CD8^+$ Teff are more susceptible to suppression than $CD4^+$ Teff cells and that Treg from the SF might have a different modality of suppression of $CD8^+$ and $CD4^+$ Teff cells. However, further studies are needed to elucidate these observations.

Subsequently, we addressed whether self-produced pro-inflammatory mediators sustained both $CD8^+$ and $CD4^+$ Teff cell resistance to suppression. Thus, we focused on TNF-α and IFN-γ, both autoimmune diseases-relevant pro-inflammatory cytokines clearly detectable in our APC-independent assay. We showed that anti-TNF-α treatment, which we previously demonstrated to rescue Teff cell resistance to suppression in the presence of APC (7), was sufficient to restore $CD8^+$ as well as $CD4^+$ Teff cell suppression in the absence of APC. However, IFN-γ neutralization was effective only in rescuing Treg-mediated suppression of $CD8^+$ but not $CD4^+$ Teff cells. This is likely to occur due to the major contribution of $CD8^+$ Teff cells to IFN-γ release: being $CD8^+$ Teff cells the major producers of IFN-γ, they are probably also more susceptible to its activity. Previously described mechanisms by which TNF-α impairs suppression in the SF are hyperphosphorylation of the AKT/PkB pathway in Teff cells (6) as well as FoxP3 dephosphorylation in Treg (17). IFN-γ-induced resistance to suppression is not mediated by AKT/PkB pathway (data not shown), however it would be of interest understanding which are the pathway(s) involved in the failure of $CD8^+$ Teff cell regulation.

In this study we show that $CD8^+$ behave differently from $CD4^+$ Teff cells at the site of autoimmune inflammation of JIA and that autocrine IFN-γ release selectively sustains $CD8^+$ Teff cell resistant phenotype. The different behavior of $CD8^+$ T cells suggests that this population is endowed with a peculiar effector function, which should be investigated independently of $CD4^+$ T cells. This reinforces the idea that new therapeutic approaches targeting the effector cell compartment in autoimmunity should take into account differences within T cell subsets.

To conclude, we believe that different players of the adaptive immune response (namely APC, $CD4^+$ Teff, $CD8^+$ Teff cells and Tregs) contribute to the defective regulation occurring at the site of chronic autoimmune inflammation, ultimately leading to the breakdown of tolerance. The self-sustained resistance of Teff cells to suppression is a piece of the puzzle with important therapeutic implications: pre-requisite for an effective therapeutic strategy for the treatment of autoimmune arthritis, is the targeting of Teff cell intrinsic ability to escape regulation.

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SUPPLEMENTARY MATERIALS

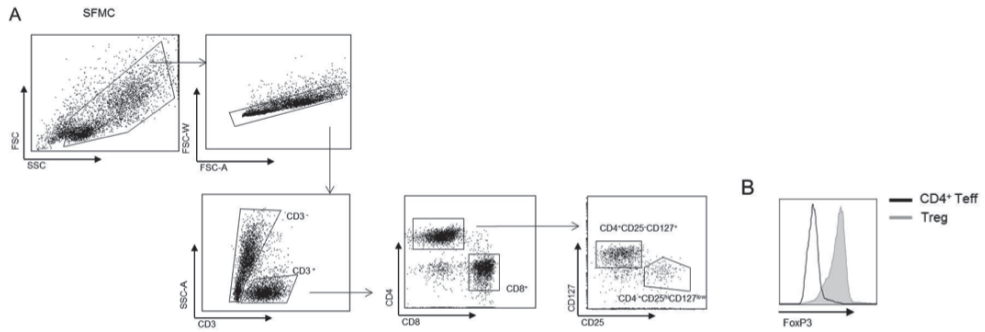

Figure S1. Gating strategy of sorted T cell populations and purity of SF Treg. (A) Representative dot plots of the gating strategy used to sort Treg, $CD8^+$ and $CD4^+$ Teff cells from the SF. (B) Representative histogram (our of n=5) of FoxP3 expression on sorted SF Treg and $CD4^+$ Teff cells.

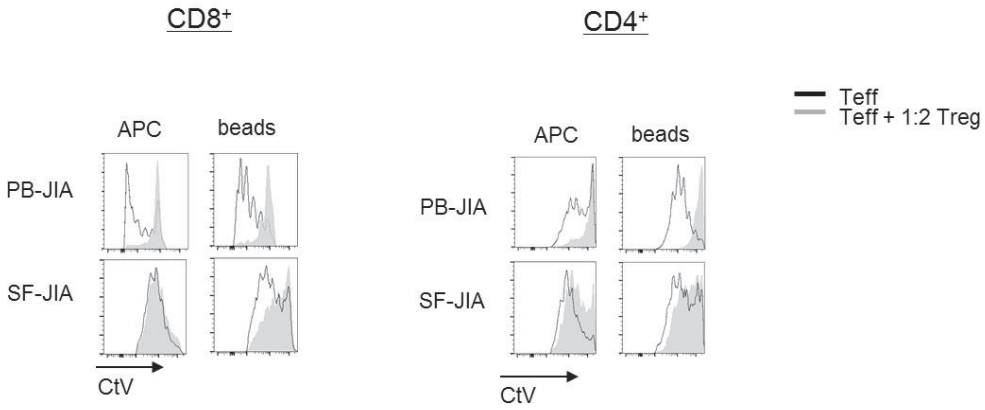

Figure S2. Representative histograms of $CD8^+$ and $CD4^+$ Teff cell proliferation and Treg-mediated suppression. CtV-labelled $CD8^+$ Teff cells from the PB and the SF of JIA patients stimulated with APC from the same site or with anti-CD2/CD3/CD28 beads were co-cultured in the presence or the absence of Treg from the same site. In parallel $CD4^+$ Teff cell suppression was tested. Representative histograms indicating $CD8^+$ (left panel) and $CD4^+$ (right panel) Teff cell proliferation (black empty line) and Treg-mediated suppression (grey filled line) after 96-hour co-culture are shown.

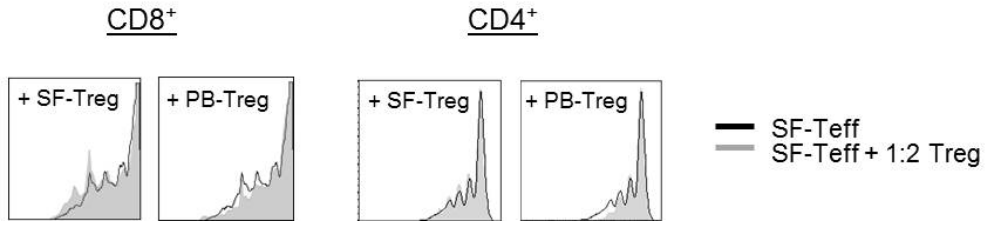

Figure S3. Representative histograms of CD8+ and CD4+ Teff cell proliferation and Treg-mediated suppression in the autologous cross-over assay. CtV-labelled CD8+ Teff cells from the SF of JIA patients stimulated with anti-CD2/CD3/CD28 beads were co-cultured in the presence or the absence of Treg from the SF or the PB of the same patient. In parallel CD4+ Teff cell suppression was tested. Representative histograms indicating CD8+ (left panel) and CD4+ (right panel) Teff cell proliferation (black empty line) and Treg-mediated suppression (grey filled line) after 96-hour co-culture are shown.

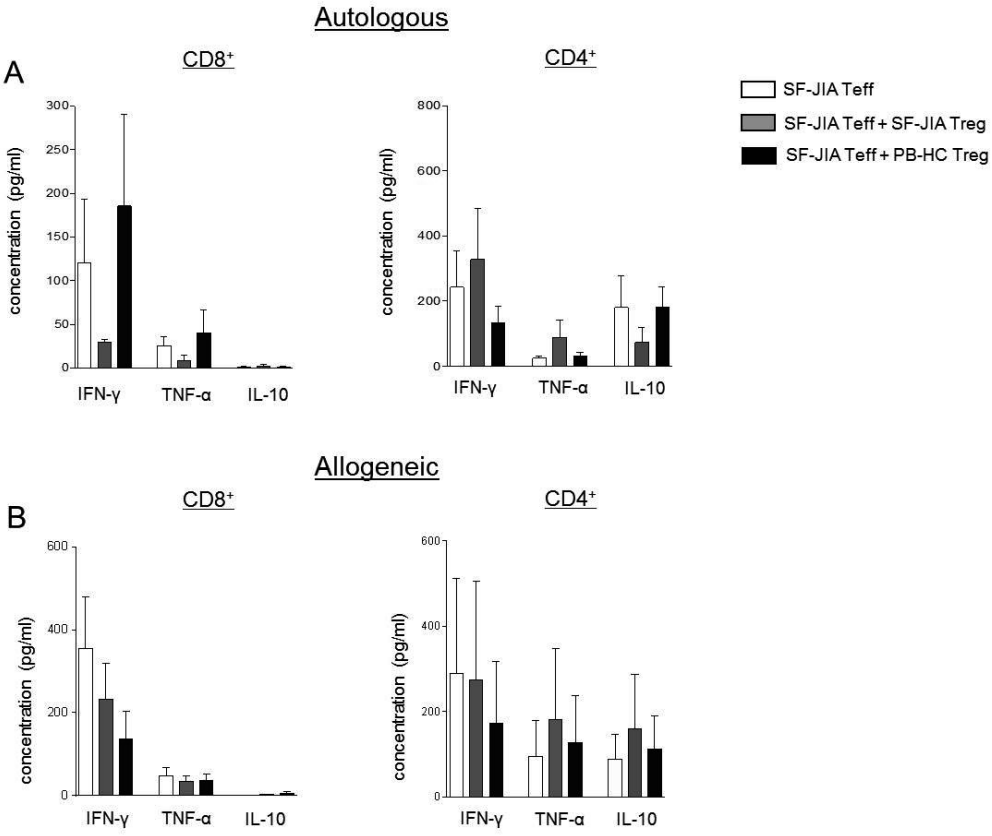

Figure S4. Treg-mediated suppression of cytokine levels in APC-independent assay. (A) Cytokine levels in the supernatant of the Treg suppression assay performed in Figure 2A and 2B, where CD8+ Teff cells from the SF where co-cultured with autologous (A) or allogeneic (B) PB-derived Treg from JIA patients and healthy controls (left panels). In parallel suppression of cytokine levels in the presence of CD4+ Teff cells was performed (right panels).

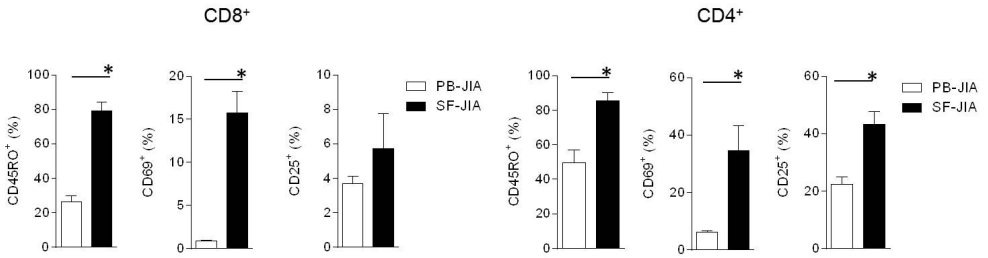

Figure S5. Expression of memory and activation markers. Expression of CD45RO (marker memory cells), CD69 and CD25 (surface markers of cell activation) on CD8+ and CD4+ Teff cells from the PB and the SF of JIA patients were tested by flow cytometry.

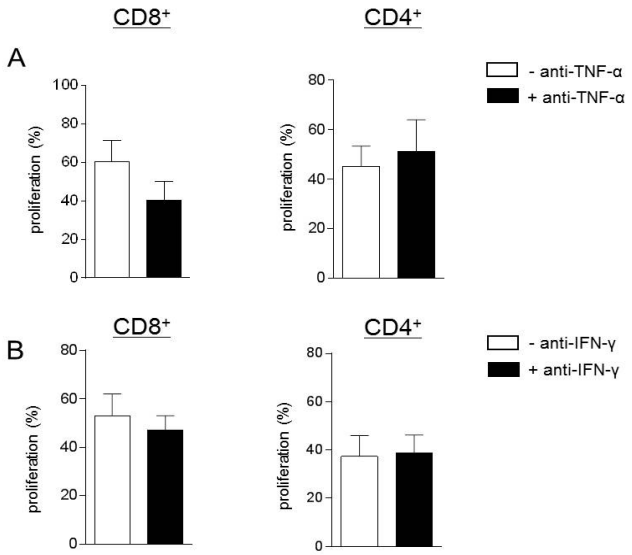

Figure S6. Proliferation of CD8+ and CD4+ Teff cells upon incubation with anti-TNF-α and anti-IFN-γ. (A) Proliferation of CD8+ (left panel) and CD4+ (right panel) Teff cells from the SF of JIA patients in the presence or absence of anti-TNF-α (1μg/ml) upon anti-CD2/CD3/CD28 bead stimulation. (B) Proliferation of CD8+ (left panel) and CD4+ (right panel) Teff cells from the SF of JIA patients in the presence or absence of or anti-IFN-γ (1μg/ml).

Chapter 4

PD-1+ CD8 T CELLS ARE A UNIQUE POPULATION OF CLONALLY EXPANDING EFFECTORS IN HUMAN CHRONIC INFLAMMATION

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Submitted

ABSTRACT

Chronic inflammatory diseases are characterized by recurrent inflammatory attacks in the tissues mediated by auto-reactive T cells. At the target site of inflammation, identity and dynamics of T cells is still largely unknown and, despite CD8 T cells can potentially play a key role, their functional programming remains to be elucidated in inflamed tissues. In the synovial fluid (SF) of Juvenile Idiopathic Arthritis (JIA) patients, a model of chronic inflammation, an overrepresentation of PD-1^{+}CD8 T cells was found. Gene expression profiling, gene set enrichment analysis and extracellular flux analysis identified PD-1^{+}CD8 T cells as metabolically active effectors, with no sign of exhaustion. Furthermore, PD-1^{+}CD8 T cells showed increased clonal expansion compared to the PD-1^{-} counterpart. Interestingly, the PD-1^{+} cells were enriched for a tissue-resident memory (TRM) cell transcriptional profile and we observed also an increase of CD8^{+}PD1^{+} cells in target tissues of other chronic inflammatory diseases (atopic dermatitis and inflammatory bowel diseases). These data indicate that local chronic inflammation drives the induction of a unique subset of CD8 T cells endowed with potential detrimental properties, thus laying the basis for investigation of PD-1-expressing CD8 T cell targeting strategies in human chronic inflammatory diseases.

INTRODUCTION

Chronic inflammatory diseases, such as Inflammatory Bowel Disease (IBD), Juvenile Idiopathic Arthritis (JIA) and atopic dermatitis (AD), are heterogeneous clinical disorders representing a major public health issue (1). Current treatments suppress but do not cure disease, and there is still a fundamental gap in our understanding of how inflammation persists. Assessment of the immunological profile in the peripheral blood may provide only a partial picture of the mechanisms triggering and maintaining organ-specific chronic inflammatory diseases. Local T cells, instead, may play a major role in the development of disease chronicity; however, identity and dynamics of T cells as well as their functional differentiation in local inflammatory environments are still largely unexplored. CD8 T cells have all features that can potentially play a key role in inflamed tissues: they can be cytotoxic and pro-inflammatory, react to self-antigens upon cross-presentation, develop regulatory properties, and can be retained and maintained in the tissues (2). However, for a long time, CD8 T cells have been neglected in chronic inflammatory diseases, and their functional programming at the target site of inflammation has yet to be investigated.

An overrepresentation of the CD8 T cell subset is found in the brain of Multiple Sclerosis (MS) patients (3), and the synovium of patients with Rheumatoid Arthritis (RA) (4), Juvenile Idiopathic Arthritis (JIA) (5) and Psoriatic Arthritis (PsA) (6). In this study, as a model to investigate inflammation-instructed CD8 T cell functional specialization, we used the target site of inflammation of JIA (i.e. the synovium). Synovial fluid (SF)-derived CD8 T cells have been shown to display a mixed pro- and anti-inflammatory phenotype (4, 7), as well as intrinsic resistance to regulation by regulatory T cells (Treg) (8, 9). Additionally, they up-regulate negative co-stimulatory markers, such as PD-1 and TIM-3 (4, 10), typically overexpressed in environments characterized by chronic antigen-driven stimulation (11). Up-regulation of negative co-stimulation on CD8 T cells, indeed, has been previously associated with exhaustion, meaning loss of effector function, in cancer as well as infectious diseases (11, 12). In this context, effector functions can be restored via PD-1/PDL-1 pathway inhibition (13, 14), and this is confirmed by the evidence that anti-PDL-1 agents are considered promising therapies in a wide range of malignancies (15). PD-1 expression has been shown to be elevated on T cells obtained from the SF of patients with inflammatory (4) but not non-autoimmune arthritis (i.e. osteoarthritis) (16), raising the question whether this subset represents an effector rather than a functionally impaired cell subset. However, in chronic inflammatory diseases, it appears counterintuitive for PD-1$^+$ CD8 T cells to be impaired in function and cytotoxic activity, given that they derive from the site with actively on-going inflammation.

In this study, we investigated PD-1-expressing CD8 T cells from chronically inflamed tissue sites in great detail, using several different techniques to evaluate their transcriptional and metabolic phenotype as well as T cell receptor repertoire. We here demonstrate that, at the target site of human chronic inflammatory diseases, a specific subset of highly activated PD-1-expressing CD8 T cells are induced, enriched for both

an effector and tissue-resident memory (TRM) T cell transcriptional profile. Furthermore, we show that this unique subset is locally clonally expanding and may therefore have detrimental effects in human organ-specific chronic inflammatory diseases. Together these data provide the rational for investigation of therapeutic strategies targeting local PD1-expressing CD8 T cell subsets in chronic inflammation.

RESULTS

Transcriptional profiling identifies a highly activated subset of PD-1$^+$ CD8 T cells at the target site of inflammatory arthritis

As a model to investigate specific CD8 T cell identity at the site of chronic inflammation we collected SF from JIA patients (SF-JIA), which is an exudate accumulating in the joint of patients during the active disease state. CD8 T cells in SF-JIA were increased in frequency compared to the peripheral blood PB of JIA patients (PB-JIA), but not to the PB of healthy children (PB-HC-Ch) or healthy control adults (PB-HC-Ad) (Fig. S1A). In SF-JIA, effector memory CD8 T cells (CD8 T_{EM}) were the predominant subset followed by terminally differentiated effector memory (CD8 T_{TEMRA}) and by small fractions of central memory (CD8 T_{CM}) and naïve CD8 T cells (CD8 T_N) (Fig. S1B), similarly to what was previously described in RA patients (4). Of note, the differentiation state of CD8 T cells in PB is not age-dependent given that the expression of CD28, CD27 and CD127 was similar between PB-HC-Ad and PB-JIA (Fig. S2).

PD-1-expressing CD8 T cells were highly enriched in SF-JIA (Fig. 1A) when compared to the PB of JIA patients, healthy control children (HC-Ch) and healthy control adults (HC-Ad) (Fig.1A). Additionally, PD-1$^+$ CD8 T cells from SF were almost exclusively memory cells (i.e. CD45RO$^+$), while the only CD8 T_N cells present in SF were found within the PD1$^-$ compartment (Fig. 1B). Similarly, PD-1$^+$ CD8 T cells showed a predominant memory phenotype in PB of both JIA patients and HC-Ad, while PD1$^-$ cells were mainly CD8 T_N (Fig. 1B).

To further investigate the phenotype of PD-1-expressing CD8 T cells enriched at the site of inflammation, whole-transcriptome sequencing analysis was performed on sorted PD-1$^+$ and PD1$^-$ CD8 T cells from SF of JIA patients and PB of HC-Ad (from now on referred as PB-HC). As expected, the hierarchical clustering showed a cut-off separation between PB-HC and SF-JIA samples (Fig. S3). Principal Component Analysis (PCA) confirmed this data, additionally showing a better-defined segregation between PD-1$^+$ and PD-1$^-$ CD8 T cells in SF rather than PB (Fig. 1C). Interestingly, a much higher number of differentially expressed genes between PD-1$^+$ and PD-1$^-$ CD8 T cells was found in SF (i.e. n=436, red dots in Fig. 1D, left panel) compared to PB-HC (i.e. n=29, red dots in Fig. 1D, right panel), suggesting that PD-1 defines a unique CD8 T cell subset in SF of JIA patients and less so in PB of control subjects.

A cluster of n=173 genes was found to be selectively up-regulated in the PD-1$^+$ subset from SF when compared to PD-1$^-$ cells from SF, PD-1$^+$ and PD-1$^-$ cells from PB-HC

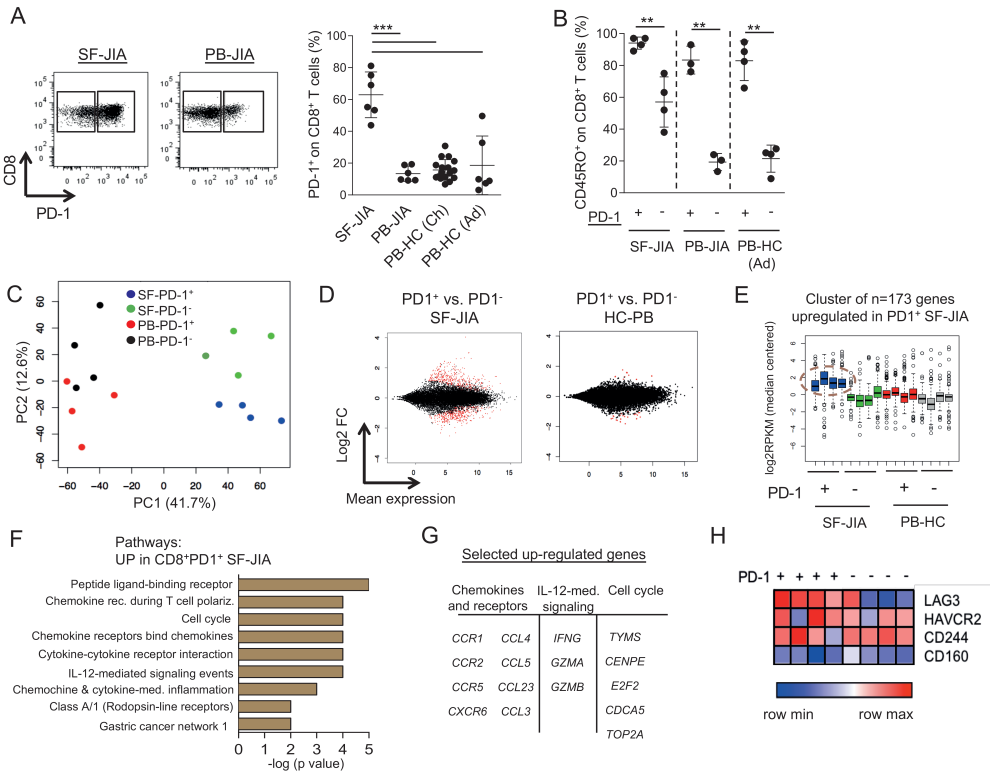

Figure 1. PD1-expressing CD8 T cells are highly activated at the target site of inflammatory arthritis. (A) PD-1 expression on CD8 T cells is shown at the site of inflammation of JIA patients (i.e. the synovial fluid –SF-), the peripheral blood (PB) of JIA patients, healthy control children and adults. Representative dot plots are shown in the left panel. Data are means ± SD. ***p<0.0001, One-way Anova. (B) CD45RO expression on PD-1$^+$ and PD-1$^-$ CD8 T cells in indicated samples. Data are means ± SD. **p<0.01, Paired Student's t-test. (C) PD-1$^+$ and PD-1$^-$ CD8 T cells were sorted from SF of JIA patients and PB of healthy control adults. Clustering of SF vs. PB PD-1$^+$ and PD-1$^-$ CD8 T cells by Principal Component Analysis (PCA) is shown. (D) Differentially expressed genes (red dots) between PD-1$^+$ and PD-1$^-$ CD8 T cells in SF and PB are depicted in MA plots. (E) K-means analysis identifies a set of genes specifically up-regulated in PD-1$^+$ CD8 T cells from SF. (F) Pathways specifically enriched in PD-1$^+$ CD8 T cells from SF are listed. (G) Selected genes up-regulated in PD-1$^+$ CD8 T cells from SF are shown. (H) The heatmap shows color-coded gene expression levels of negative co-stimulatory markers typically upregulated in exhausted CD8 T cells in PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. *SF-JIA: synovial fluid from Juvenile Idiopathic Arthritis patients; PB-JIA: peripheral blood from Juvenile Idiopathic Arthritis patients; PB-HC (Ch): peripheral blood of healthy control children; PB-HC (Ad): peripheral blood of healthy control adult; UP: up-regulated*

(Fig. 1E, left panel). Interestingly, up-regulated genes in PD-1$^+$ CD8 T cells from SF-JIA were significantly enriched in pathways associated with activated cells, such as cell cycle regulation, chemokine and cytokine signalling as well as IL-12 signalling (Fig. 1F). Selected genes up-regulated in the PD-1$^+$ subset from SF are shown in Fig. 1G, and include chemokine receptors and ligands (e.g. *CCR1*, *CCR2*, *CCR5*, *CXCR6*, *CCL4* and *CCL5*), IL-

12-mediated signalling molecules (i.e. *IFNG*, *GZMA*, *GZMB*) and proteins directly involved in the cell cycle (i.e. *TYMS*, *E2F2*, *TOP2A*). Fig. S4 shows expression levels of selected genes. Interestingly, gene expression of negative co-stimulatory markers typically elevated in exhausted T cells such as *LAG3*, *HAVCR2*, *CD160* and *CD244* are not consistently higher in PD-1$^+$ CD8 T cells from SF compared to the PD-1$^-$ subset (Fig 1H). A total of n=179 genes, instead, was found downregulated in PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF-JIA. Genes downregulated in PD-1$^+$ CD8 T cells include killer cell-associated activating and inhibitory receptors such as NCRs, KIR2DLs and KIR3DLs. (Fig. S5A-B).

These data show that PD-1-expressing cells enriched at the site of inflammatory arthritis are a specific subset of CD8 T cells endowed with an activated phenotype.

PD-1-expressing CD8 T cells from the target site of inflammatory arthritis are enriched for an "effector" and not an "exhaustion" transcriptional profile

To investigate the possibility that PD-1-expressing CD8 T cells from SF of JIA patients are exhausted, we compared their gene expression profile with the known expression signature of exhausted cells using gene set enrichment analysis (GSEA) (17). We showed that the gene signature of previously described exhausted CD8 T cells from HIV patients with progressive disease (18) was enriched neither in PD-1$^+$ or PD-1$^-$ CD8 T cells from SF, demonstrating that PD-1$^+$ CD8 T cells from SF-JIA do not display an exhausted profile (Fig. 2A, left panel). Instead, the signature of effector CD8 T cells (19) was found significantly enriched in PD-1$^+$ CD8 T cells (Fig. 2A, right panel), as well the set of genes featuring proliferating cells (Fig. 2B). This data, together with the elevated intracellular expression of the marker of cell proliferation Ki-67 (Fig. 2C), strongly suggest that SF-derived PD-1$^+$ CD8 T cells are not exhausted. Complementary analysis confirmed these data showing that genes up-regulated in PD-1$^+$ vs. PD1$^-$ CD8 T cells in SF (Fig. S6A) or shared between PD-1$^+$ SF-JIA vs. PD-1$^+$ PB-HC and PD-1$^+$ vs. PD-1$^-$ SF-JIA (Fig. S6B) were consistently enriched in pathways associated with effector cells. Of note, although the overall proliferation of CD8 T cells in the PB of JIA patients was lower than in SF, PD-1$^+$ cells showed higher expression of Ki-67 compared to PD-1$^-$ cells also at this site (Fig. 2C), while no differences in cell proliferation were evident between the two subsets in the PB of HC subjects (Fig. 2C). This suggests that physiologically occurring PD1$^+$ CD8 T cells from healthy controls represent a different cell type than the population isolated from patients with an inflammatory condition and, in particular, from the site of inflammation.

Immune cell differentiation and function depends on the activation of specific metabolic pathways: quiescent cells generate energy (i.e. adenosine triphosphate, ATP) primarily in their mitochondria via oxidative phosphorylation, while effector cells engage anaerobic glycolysis, converting glucose into lactate (20, 21). To further support the evidence that PD-1$^+$ CD8 T cells from SF-JIA are not exhausted, we analysed the level of glucose consumption in these cells by measuring the *ex vivo* extracellular acidification rate (ECAR) with extracellular flux (XF) technology (Seahorse Bioscience). In the exhaustion

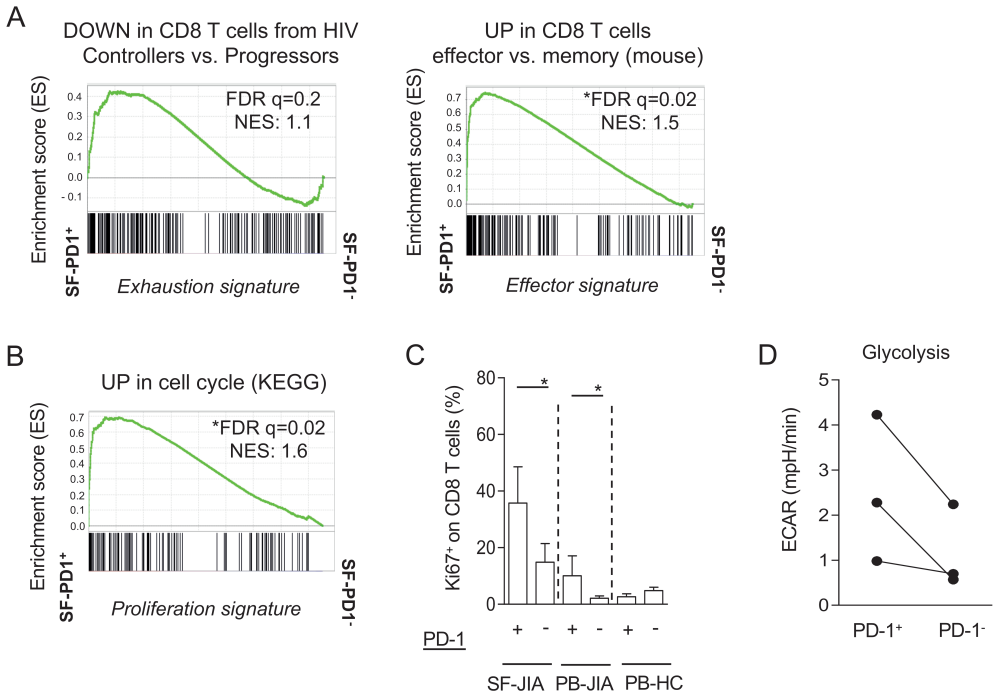

Figure 2. PD1-expressing CD8 T cells are effector, metabolically active and not exhausted cells at the target site of inflammatory arthritis. (A) Enrichment of previously published gene signatures of CD8 T cell exhaustion (described in (18), showed in the left panel) and effector CD8 T cells (described in (19), showed in the right panel) was tested on PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF by Gene Set Enrichment Analysis (GSEA). (B) Enrichment of genes linked to cell cycle (obtained from the KEGG database) was tested on PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF. (C) Assessment of cell proliferation was performed by Ki67 staining on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF and PB of JIA patients as well as PB of healthy donors (n=5 per group). Data are means ± SD. *p<0.05, Paired Student's t-test. (D) The metabolic phenotype of PD-1$^+$ and PD-1$^-$ CD8 T cells from SF was tested by XF technology (Seahorse Bioscience). Glycolysis was calculated as the difference between levels of Extracellular Acidification Rate (ECAR) upon exposure to glucose vs. exposure to the glycolysis inhibitor 2-DG. *ns*, paired Student's t-test. *SF-PD1$^+$: synovial fluid-derived PD1$^+$CD8 T cells; SF-PD1$^-$: synovial fluid-derived PD1$^-$CD8 T cells; SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-JIA: peripheral blood of Juvenile Idiopathic Arthritis patients; PB-HC: peripheral blood of healthy control adults; ECAR: Extracellular Acidification Rate; UP: up-regulated; DOWN: down-regulated*

setting, PD-1 is known to regulate metabolism by inhibiting glycolysis *(22)*. Consistent with the effector cell phenotype that we found in SF, PD-1$^+$ CD8 T cells showed increased glycolysis compared to the PD-1$^-$ subset from the same site (Fig. 2D).

Taken together, these data provide evidence that this cell subset is endowed with typical features of effector but not exhausted cells at the transcriptional as well as the metabolic level.

PD-1-expressing CD8 T cells at the target site of inflammation of different human chronic inflammatory diseases display a TRM profile

We then investigated the expression of CD69, an activation marker (23) whose expression is known to be strongly associated with residency in tissues (24), on CD8 T cells in SF. Tissue-resident memory T cells (TRM) have been recently described as a resting memory cell subset expressing CD69, stably residing in tissues, with a critical role in local immunosurveillance (24-26), and characterized by the expression of PD-1 and other negative co-stimulatory markers (such as CTLA-4, ICOS and TIGIT) in mice (27) and humans (28).

In our setting, $CD69^+$ CD8 T cells were present at a high frequency in SF compared to PB of the same patients (Fig. 3A) and PD-1 expression was higher on total $CD69^+$ compared to $CD69^-$ CD8 T cells in SF (Fig. 3B). Moreover, $CD69^+CD103^+$ and $CD69^+CD103^-$, two distinct subsets of CD8 TRM cell (29), were both present in SF, with a prevalence of the $CD69^+CD103^-$ fraction (Fig. S7A). Interestingly, barely any $CD103^+$ CD8 T cell was present in the PD-1^- subset (Fig. S7B).

GSEA showed that PD-1^+ CD8 T cells from SF-JIA, but not PD-1^-, were enriched with the signature of TRM cells (27) (Fig. 3C). Moreover, increased expression levels of signature genes of TRM cells were found in SF-derived PD-1^+ cells (Fig. 3D) and this was confirmed by the increased frequency of CTLA-4^+, $TIGIT^+$ and $GzmB^+$ cells (Fig. 3E) and the decreased expression of *S1PR1* (encoding for S1P1, sphingosine 1-phosphate receptor, whose down-regulation is required for the establishment of resident CD8 T cells (30)) observed in PD-1^+ cells (Fig. 3F). This data shows an overlapping profile between PD-1-expressing CD8 T cells in SF and TRM cells, suggesting that inflammation may be the driver of the upregulation of retention molecules and the TRM-associated phenotype observed at this site.

We then examined whether similar features were evident in tissues obtained from patients with chronic inflammatory diseases, such as atopic dermatitis (AD) and inflammatory bowel diseases (IBD). Immunohistochemistry on histological sections of the lesional skin of n=3 patients with AD showed that a large fraction of CD8 T cells present in the dermis co-expressed PD-1. Moreover, single positive CD8 cells and PD-1 cells could be also identified (Fig. 3G, upper panels). Rarely, in those sections, CD8 cells were localized in the epidermis. Serial sections from the same AD patients were stained for the TRM cell marker CD69 showing its ubiquitous expression in lesional infiltrates (Fig. 3G, lower panels). Approximately half of TRM cells present in the dermis co-expressed PD-1. Interestingly, no cells expressing PD-1 alone were found, indicating that PD-1^+ cells are all CD69-expressing TRM. Sections of non-lesional skin were stained as well, but very few $CD8^+$ cells were present (Fig. S8). Similar results were also obtained from the site of inflammation of IBD, i.e. the colonic mucosa. In the macroscopically inflamed gut mucosa of IBD patients, PD-1 was almost exclusively expressed by TRM $CD8\alpha^+CD4^-$ T cells (i.e. $CD69^+$ cells) and not by recirculating CD8 T cells (i.e. $CD69^-$ cells) (Fig. 3H). Importantly, the frequency of $CD69^+$PD-1^+ CD8 T cells was consistently higher in inflamed vs. non-inflamed gut mucosa, suggesting a potential effector property of this subset in this context.

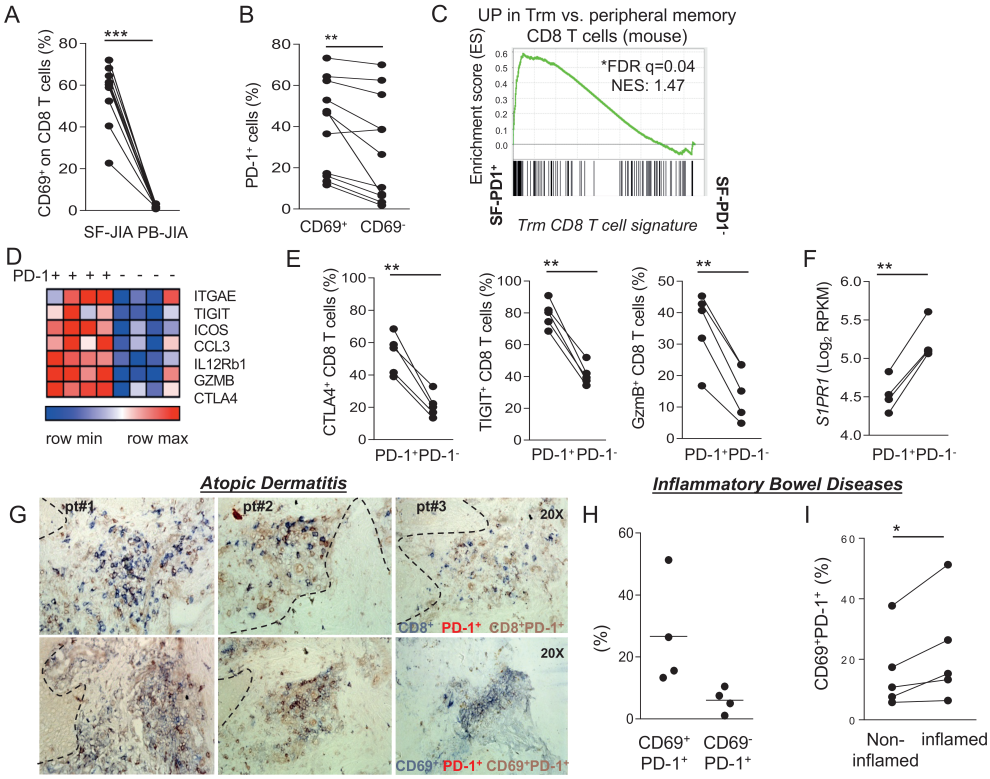

Figure 3. PD1-expressing CD8 T cells display a TRM profile at the target sites of human chronic inflammatory diseases. (A) Frequency of CD69$^+$ CD8 T cells in SF compared to PB of JIA patients is shown. ***p<0.0001, Paired Student's t-test. (B) PD-1 expression on TRM (CD69$^+$) compared to recirculating (CD69$^-$) CD8 T cells is shown. **p<0.01, Paired Student's t-test. (C) Enrichment of previously published gene signatures of TRM CD8 T cells (described in (27)) was tested on PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF by GSEA. (D) The heatmap shows color-coded gene expression levels of signature genes of TRM cells in PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. (E) Protein expression levels of CTLA-4, TIGIT and GzmB were tested on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. **p<0.01, Paired Student's t-test. (F) Expression of *S1PR1* at mRNA level on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. **p<0.01, Paired Student's t-test. (G) Double immunohystochemistry was performed on skin biopsies from inflamed tissue of patients with Atopic Dermatitis (AD). Single positive cells for CD8 (blue) and PD-1 (red) as well as double positive cells for CD8 and PD-1 (brown) are shown (top panels). Serial sections were stained for CD69 (marker of tissue-resident memory - TRM - cells) and PD-1. Single positive cells for CD69 (blue) and PD-1 (red) as well as double positive cells for CD69 and PD-1 (brown) are shown in the bottom panels. (H) Immune cells were isolated from inflamed gut mucosa of patients with Inflammatory Bowel Diseases (IBD). PD-1 expression on TRM (CD69$^+$) and recirculating (CD69$^-$) CD8a$^+$CD4$^-$ T cells is shown. (I) The frequency of CD69$^+$PD-1$^+$ CD8 T cells was tested in both inflamed and non-inflamed gut mucosa from IBD patients. *p=0.04, Paired Student's t-test. *SF-PD1$^+$: synovial fluid-derived PD1$^+$CD8 T cells; SF-PD1$^-$: synovial fluid-derived PD1$^-$CD8 T cells; UP: up-regulated*

Overall, these data show that PD-1$^+$ CD8 cells are present and enriched in both tissue and exudate of human chronic inflammatory diseases and display a TRM profile, suggesting that inflammation might be a driver for the local development of this effector resident cell subset.

PD-1-expressing CD8 T cells from the target site of inflammatory arthritis represent a specific clonally expanded population

In the absence of a known auto-antigen in JIA, we asked whether the inflammation-associated enrichment in PD1$^+$ CD8 T cells was due to a random influx of PD1$^+$ CD8 T cells or to a specific clonal expansion of this population at this site.

Next-generation TCRBV CDR3 sequencing was performed on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. The analysis of amino acid sequences showed that the top 80% TCR repertoire of PD-1$^+$ CD8 T cells from SF-JIA was less diverse compared to that of PD-1$^-$ cells (Fig. 4A, left panel). For instance, in patient #4 (Fig. 4A, right panel), 52% of the TCR diversity is explained by n=28 clones in the PD-1$^-$ fraction, but only n=4 clones in the PD-1$^+$ fraction. When we tested the presence of shared unique TCR clones between PD-1$^+$ and PD-1$^-$ cells, we surprisingly found that their frequency was extremely low compared to the total number of identified clones (Venn diagrams in Fig. 4B), suggesting that PD-1$^+$ CD8 T cells present in SF represent a specific population of cells, distinct from PD-1$^-$ cells.

To further confirm that PD-1$^+$ CD8 T cells are clonally expanded, we tested the expression of TNFRSF9 (i.e. CD137), described to regulate CD8 T cell clonal expansion (31), as well as the length of telomeres, known to shorten along with cell division and differentiation (32). PD-1$^+$ CD8 T cells showed higher mRNA expression level of *TNFRSF9* (Fig. 4C, left panel) and, despite the overall low detection level, higher intensity was evident in PD-1$^+$ compared to PD-1$^-$ cells at a protein level (Fig. 4C, right panel). Additionally, telomere length analysis showed shorter telomeres of PD-1$^+$ compared to PD-1$^-$ CD8 T cells from SF in 3 out of 4 samples (Fig. 4D). In 1 patient the telomere had such a short length already, that no differences between the PD-1$^+$ and PD-1$^-$ subset could be detected (Fig. 4D).

In n=1 JIA patient (i.e. #5) we were able to obtain PD-1$^+$ and PD-1$^-$ CD8 T cells from two different inflamed joints involved during active disease. A clear increased overlap of the top 100 TCR sequences was found in PD-1$^+$ but not PD-1$^-$ cells (55% and 5.8% respectively, Fig. 4E), indicating that the expansion occurring at different sites of inflammation in the PD-1$^+$ subset is driven by common antigen(s).

Together, these data show that PD-1-expressing CD8 T cells represent a distinct population of cells undergoing clonal expansion towards specific antigen(s) selectively at the target sites of human inflammatory arthritis.

DISCUSSION

CD8 T cells localizing at the target site of chronically inflamed tissues undergo local differentiation, which may be driven and influenced by different players such as the inflammatory state and the type of antigen(s). To date, little is known about the functional specialization that CD8 T cells develop at the target sites of inflammation and, consequently, it is yet undetermined whether standard or experimental therapies are able to reshape or even resolve the inflammatory responses occurring at these sites. Elucidating these mechanisms might open new therapeutic avenues for clinical application.

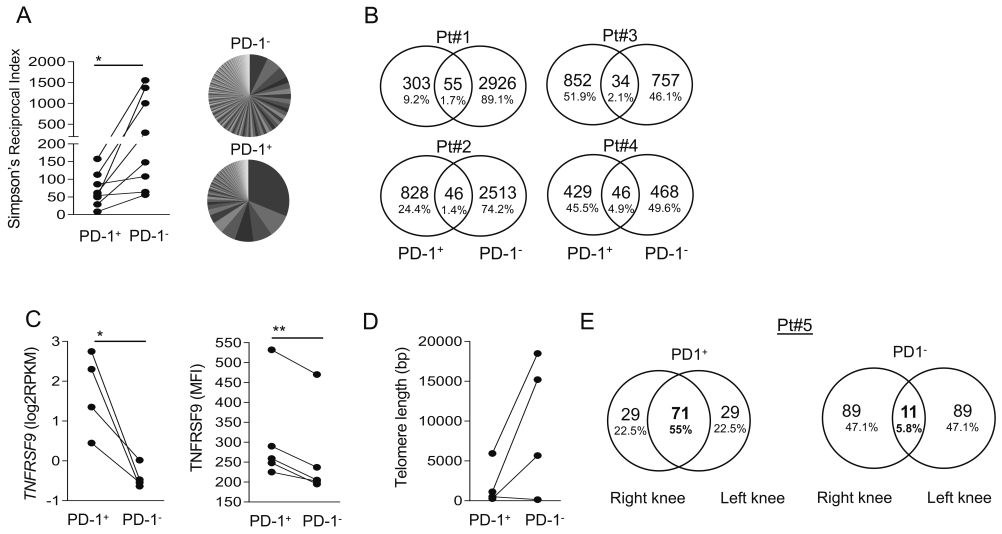

Figure 4. PD1-expressing CD8 T cells are a unique subset undergoing antigen-driven clonal expansion at the target site of inflammatory arthritis. Next-generation TCRBV CDR3 sequencing was performed on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. (A) The Simpson's Reciprocal Index was assessed as indicator of TCR diversity (left panel). *p<0.05, Paired Student's t-test. Representative pie charts show the distribution of unique clones. (B) Numbers of unique clones and sequences overlapping between PD-1$^+$ and PD-1$^-$ CD8 T cells are shown for patients #1-4 by Venn Diagrams. (C) The expression of the surrogate marker of antigen-specificity TNFRSF9 (i.e. CD137) was assessed in PD-1$^+$ and PD-1$^-$ CD8 T cells from SF both at mRNA (left panel) and protein (right panel) level, *p<0.05, **p<0.01, Paired Student's t-test. (D) Telomere length was tested on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. *ns*, paired Student's t-test. (E) In n=1 patient PD-1$^+$ and PD-1$^-$ CD8 T cells from 2 joints were sorted and TCR sequencing was performed. 55% of the top 100 sequences overlap between the two sites of inflammation in the PD-1$^+$, while only 5.8% in the PD-1$^-$ compartment.

In cancer and infectious diseases, also characterized by a state of chronic stimulation, CD8 T cells undergo a well-described tumour or viral antigen-driven process of differentiation characterized by PD-1 up-regulation and progressive loss of effector properties (described as exhaustion) (11, 12), which can be efficiently rescued by inhibiting the PD-1/PDL-1 pathway (13-15). However, recent studies suggest that this may not be a pure loss of function, but more a sign of functional adaptation to the chronically inflamed milieu (11, 33). In this study, by dissecting the role of PD-1-expressing CD8 T cells, we aimed to explore whether similar processes of CD8 T cell specialization do occur also at the target site of human chronic inflammatory diseases.

PD-1$^+$ CD8 T cells from SF of JIA patients clearly showed up-regulation of pathways associated with effector but not exhausted phenotype, which was confirmed by GSEA and by the elevated proliferative capacity of PD-1$^+$ CD8 T cells from SF. Moreover, PD-1$^+$ CD8 T cells from SF didn't upregulate the whole spectrum of negative co-stimulatory markers that are typically enriched in exhausted CD8 T cells (34). Additionally, PD1-

expressing CD8 T cells from SF showed increased usage of the glycolytic pathway, which is required to meet the increased bioenergetic demands occurring in effectors but not exhausted CD8 T cells (22). This finding rules out the hypothesis that PD-1-expressing CD8 T cells are exhausted in this context of chronic inflammation, as instead described in cancer and chronic viral infections (11). PD-1 is a well-known marker of activation (35) and it has been shown to identify a population of oligoclonal CD8 T cells endowed with tumor-specific effector properties in human melanoma metastasis (36). Few reports also show a positive correlation between PD-1 and Ki67 in the periphery of virally infected macaques and humans (37, 38). Interestingly, chemokines such as CCL3 and CCL4, which are upregulated in PD-1-expressing CD8 T cells from SF, are known to be highly released by effector HIV-specific cells from HIV long-term non progressors (symptom-free patients who don't require antiretroviral therapy) (39). Therefore, PD-1 rather than being a marker of exhaustion or activation, seems to define a subset of antigen-experienced cells whose functional properties are influenced by the location and determined by the environment they are exposed to. This was supported by the finding that PD-1 expression was found elevated on CD8 T cells localized at the target sites of different chronic inflammatory diseases, such as the synovium of JIA patients, the inflamed skin of AD patients and the gut of patients with IBD. Confirming previous reports (11, 33, 40), our data indicate that, in the target site of chronic inflammatory diseases, an exhausted phenotype doesn't mean complete loss of function, but rather functional adaptation and development of expansion capacity and possible memory features.

To assess whether PD-1-expression is only driven by the inflammatory milieu or depends on an antigen-driven component as well, we examined SF-derived PD-1$^+$ and PD-1$^-$ CD8 T cell clonality. PD-1$^+$ CD8 T cells displayed a clonal TCR compared to the diverse PD-1$^-$ counterpart from the same site. The evidence that few clones were shared between PD-1$^+$ and PD-1$^-$ cells suggests that both cell subsets might only in part derive from common progenitors. Therefore, it is possible that CD8 T cells in the SF originate from clones present in the periphery, which have specifically expanded in the synovium. Indirect evidence was provided by the increased expression of a surrogate marker of antigen-specific CD8 T cells (i.e. TNFRSF9), and by the shortened telomere length, indicating local clonal expansion towards cognate antigen(s) at the site of inflammation. Antigen-driven clonal expansion was confirmed by the evidence that, in the PD-1$^+$ compartment only, different sites of inflammation within the same patient display elevated TCR sequences overlap. This is in line with data in autoimmune diseases showing that self-reactive cells with the highest self-antigen affinity express the most PD-1 (41).

PD-1 expression was found elevated especially on CD69$^+$ CD8 T cells. Growing literature has described CD69$^+$ T cells localized in tissues as tissue-resident memory (TRM) cells, i.e. a subset of experienced T cells stably residing in tissues and providing local protection upon re-exposure to pathogens (42, 43). Features of TRM cells such as the transcriptional profile (27, 28), expression of typical surface (retention) markers

(i.e. CD69 and CD103), down-regulation of the receptor for sphingosine 1-phosphate *S1PR1* (30) and up-regulation of negative co-stimulatory markers as well as the cytotoxic enzyme GzmB (27, 28) could be found in PD-1-expressing CD8 T cells enriched in the SF of JIA patients. Our data indicate that local inflammation drives the development of this unique population of tissue-restricted PD-1-expressing CD8 T cells. The cells we describe in this paper are localized in the synovial fluid; however, PD-1-expressing T cells (*19*) and CD8 TRM cells (44) have been previously described in the synovial tissue of patients with RA. Therefore, it is possible that immune cells present in SF are actually originating from the synovial tissue infiltrate, fall off the tissue and shed in the fluid.

This locally adapted cell subset is endowed with a constitutively pro-inflammatory/cytotoxic profile and lie in a functional re-activated effector state. This hypothesis is reinforced by the evidence that in the gut mucosa of IBD patients, a consistent higher frequency of TRM PD-1-expressing CD8 T cells was observed in inflamed vs. non inflamed tissue indicating a potential detrimental role of this subset in inflamed sites. This is also supported by the observation that, in human skin, $CD103^+$ TRM cells (in which PD-1 expression was found enriched) represent the subset with more pronounced effector functions compared to $CD103^-$ TRM cells (29).

Using a variety of techniques, in this study we have shown that PD-1-expressing CD8 T cells enriched at the site of autoimmune inflammation are antigen-experienced cells, have undergone tissue-instructed differentiation and clonal expansion and are actively proliferating towards a cognate antigen, likely a self-antigen. A lot of research is currently directed towards PD-1 agonists as a novel treatment for chronic inflammatory diseases (16, 45, 46). However, given the evidence that PD-1 deletion (47, 48) or immune checkpoint inhibitors (49-51) may facilitate the development of autoimmune-like phenotypes, systemic depletion of PD-1-expressing cells might be a risky approach, due to the regulatory role that peripheral $PD\text{-}1^+$ cells might display *in vivo*. Halting migration to the site of inflammation or local depletion of resident effector cells showing tissue-specific harmful potential might have substantial therapeutic implications in chronic inflammatory diseases.

METHODS

Patient characteristics

Patients with Juvenile Idiopathic Arthritis (JIA) were enrolled by the Paediatric Rheumatology Department at University Medical Center Centrum of Utrecht (The Netherlands). A total number of n=46 JIA patients were included in this study, n=36 with oligoarticular JIA and n=10 with polyarticular JIA, according to the revised criteria for JIA (52). The average age of the patient population was 11.7 years (range 2.5-23.8 years) and the disease duration at the time of inclusion was 5.9 years (range 0.5-19.5 years). Synovial fluid (SF) was obtained from patients with active disease undergoing therapeutic joint aspiration. From n=10 patients paired blood and joint aspirate samples were collected. Peripheral blood

from adult healthy volunteers (HC, n=27) was obtained from the Mini Donor Service at our Institute. Peripheral blood from n=17 healthy children volunteers was obtained from a cohort of control subjects for a case-control clinical study. Adult patients (n=3) affected by atopic dermatitis (AT) underwent skin biopsies of lesional and non-lesional skin. Adult patients affected by either Crohn's disease or Ulcerative Colitis underwent endoscopy for surveillance, and biopsies were taken from macroscopically inflamed and non-inflamed colonic mucosa (n=5).

Cell isolation

SF Mononuclear Cells (SFMC) were incubated with Hyaluronidase (Sigma-Aldrich) for 30 min at 37°C. SFMC and PBMC were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences, AB) and frozen in foetal calf serum (FCS) (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation. Intestinal biopsies were incubated for 1 hour at 37°C with 1mg/ml Collagenase IV (Sigma) in RPMI medium (supplemented with 10% FCS, 100 U/ml penicillin-streptomycin and 0.2% Fungizone), then forcefully resuspended through a 18.5G needle, filtered with 70 mm cell strainer (Costar), counted and used for flow cytometry experiments.

Flow cytometry and cell sorting

200.000 SFMC and PBMC were plated in round bottom 96-well plates in FACS buffer (PBS containing 2% FCS (Invitrogen) and 0.1% sodium azide -Sigma-Aldrich-) and subsequently incubated with surface antibodies: anti-human CD3 BV510, CD4 Alexa488, CD8 APC-Cy7, PD-1 PE and APC, CD45RA FITC, CD45RO PB, CCR7 APC, TIGIT Percp-eF710, CD69 APC, CD103 FITC. To determine cell dead the Fixable Viability Dye eFluor® 506 (eBioscience) was used. To determine Ki67 and CTLA-4, after surface staining, cells were fixed and permeabilized by using Fixation and Permeabilization Buffers (eBioscience Cat. No. 00-8333), and stained with anti-human Ki67 FITC or CTLA-4 APC. The Apoptosis Detection kit (eBioscience) was used to detect Annexin V (PE) expression. For intracellular cytokine production, cells were incubated with surface antibodies, and then fixed, permeabilized, and intracellularly stained with anti-human GzmB FITC. In the gut of IBD patients cells were stained with CD3 BV510, CD4 APC, CD8aα PB, TCRγδ PE, PD-1 Percp-Cy5.5 monoclonal antibodies. In some experiments, after surface staining, cells were sorted by flow cytometry with FACS Aria III (BD Biosciences) and further experiments were performed.

Whole transcriptome sequencing and analysis

Total RNA was isolated using the RNAeasy plus Universal Mini kit (Qiagen, Hilden, Germany) as specified by the manufacturer's instructions and stored at -80°C. RNA yield was assesses by Qubit RNA BR assay kit and then integrity was determined by Bioanalyzer. mRNA was selected using poly(A) purist MAG kit (Life Technologies, AM 1922) and

additionally purified with a mRNA-ONLY™ Eukaryotic mRNA Isolation Kit (Epicentre). Transcriptome libraries were then constructed using SOLiD total RNA-seq kit (Applied Biosystems Life Technologies) and sequenced using 5500 W Series Genetic Analyzer to produce 40-bp-long reads. Sequencing reads were mapped against the reference human genome (hg19, NCBI37) using BWA (-c –l 25 –k 2 –n 10) (53). Data were analyzed using DEseq2 (54) and custom Perl and R (www.r-project.org) scripts. Principal Component Analysis (PCA) was performed by selecting variable genes with fold change between samples on 15th and 85th quantile at least 1 log2 RPKM and expression at least 1 log2 RPKM in the sample with the maximal expression. Hierarchical clustering was performed using unsupervised Pearson correlation of the variably expressed genes with min 2 log2 fold change between samples with highest and lowest expression and min 2 log2 RPKM expression value in the sample with the highest expression. Differentially expressed genes with adjusted p value < 0.05, baseMean >2 and log2 fold change at least 1 are shown as red dots in MA plots. For K-means clustering, genes with fold change between samples on 20th and 80th quantile at least 1.5 log2 RPKM and expression at least 1 log2 RPKM in the sample with the maximal expression were shown. Pathway enrichment analysis was performed using ToppGene Suite (55) publicly available online portal. Pathways were considered significantly enriched when the p-value corrected for multiple hypothesis testing using the Bonferroni method was < 0.05. Gene set enrichment analysis (GSEA) (17) was used to assess whether specific signatures were significantly enriched in the PD-1$^+$ or PD-1$^-$ subset. One thousand random permutations of the phenotypic subgroups were used to establish a null distribution of enrichment score against which a normalized enrichment score and FDR-corrected q values were calculated. Gene-sets were either obtained by analysing raw data using GEO2R (NCBI tool) or downloaded from the Molecular Signature Database of the Broad Institute software. In particular, the following data sets were used: *exhaustion* signature: gene set GSE24081; effector CD8 T cell signature: gene set GSE4142; proliferating cell signature: downloaded from the KEGG pathway database, http://www.genome.jp/kegg/pathway.html; tissue-resident memory cell signature: gene set GSE39152.

Seahorse Assays

PD-1$^+$ and PD-1$^-$ CD8 T cells from the SF of JIA patients were sorted and plated in XF-24 Cell Culture Microplates (Seahorse Bioscience) in XF Base Medium Minimal DMEM (0mM glucose) supplemented with 2 mM L-glutamine, and 1 mM sodium pyruvate. Extracellular acidification rates (ECAR) was measured under basal conditions and in response to glucose 30mM on the Seahorse XF-24 Extracellular Flux Analyser (Seahorse Bioscience). ECAR for each time point was normalized on ECAR levels measured in the negative control wells filled with XF Base Medium alone. Cell ability to respond to glucose (glycolysis) was calculated as ECAR in the 30 mM glucose condition minus ECAR measured when 2-deoxyglucose (2-DG) -glycolysis inhibitor- was added.

Double immunohistochemistry

Frozen sections (6 µm) of lesional skin biopsies of AD patients were fixed in acetone + H2O2 (0.1%) for 10 minutes and then incubated for 30 min in 10% horse serum in 1% BSA/PBS solution. After blow-off, sections were incubated with primary antibodies: mouse anti-human CD8, (Dako #M7103 1:100), or mouse anti-human PD1 (AbCam #ab52587, 1:50) in 1% horse serum, 1%BSA/PBS for 1 hour at room temperature (RT) and then with a biotinylated anti-mouse IgG (Vector, #BA-2000, 1:300) for 30 min at RT. Afterwards, slides where incubated in 10% sheep serum for 30 min, and then with the second primaries: mouse anti-human CD69-Fitc (Miltenyi Biotec, #130-092-166, 1:50), or mouse anti-human CD8-Fitc (Dako # F0765, 1:100) in 1% sheep serum 1% BSA/PBS for 1 hour at RT. Sections were then incubated with a sheep anti-Fitc-AP antibody (Roche cat #14973500, 1:300) in ABC-HRP solution (ABC-HRP kit, Vectastain # PK-6100) for 30min at RT. Slides where stained with Vector Blue Substrate Kit (Vector #SK-5300) to detect Alkaline Phosphatase activity, and Levamisole (Dako, #X3021) was added to block endogenous AP during the Fast Blue incubation. The reaction was stopped after monitoring for the desired staining intensity under the microscope (~10min) in demi-water. Slides where stained subsequently with AEC chromogen (AEC staining Kit, Sigma # AEC101) to detect peroxidase activity until the desired colour development was reached (~5min). Slides where finally covered with a water base mounting solution (Imsol Mount) and then hard mounted in Entellan (Merck #HX399193). Single staining with anti-human CD8 antibody was performed in non lesional skin sections from the same AD patients, following the protocol with the ABC-HRP kit and AEC detection method described above. Sections were counterstained with Mayer's Hematoxylin before mounting.

Next Generation T cell receptor β chain (TCR β) sequencing analysis

TCR β chain sequencing was performed as previously described (56). A total of 100,000–900,000 PD-1$^+$ and PD-1$^-$ CD8 T cells were isolated from the SF (n=4) of JIA patients. Cells were washed with PBS and frozen at -80°C. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), following the instructions of the manufacturer. cDNA was synthesized using the SMARTER-RACE SMARTer™ RACE cDNA Amplification Kit (Clontech). Amplification of the TCR β VDJ region was performed using previously described primers and amplification protocols(57). PCR product were analysed with a QIAxcel Advanced System (Qiagen). Upon successful amplification, end repair was performed with the ClaSeek Library Preparation Kit, Illumina compatible (Thermo Scientific) according the recommendations of the manufacturer. Subsequently, TruSeq Barcode adapters (Illumina) were ligated using the ClaSeek Ligation Mix (Thermo Scientific). Cleanup of the samples was performed with The Agencourt AMPure XP system (Beckman Coulter). Next generation sequencing was performed on an Ilumina MiSeq system 500 (2x250bp) (Illumina). Sequencing data were analyzed with the MiTCR program(58). The MiTCR output file was used to calculated the Simpson's Index (D), , in which n= total number of specific sequence and N= the total number of all sequences. Data are presented as the Simpson's Reciprocal Index of

Diversity = 1/D. Simpson's Reciprocal Index of Diversity = 1 indicates monoclonality, whereas the higher the value, the greater the diversity.

Telomere length

DNA was collected using the AllPrep DNA/RNA plus kit (Qiagen) from PD-1^{+} and PD-1^{-} CD8^{+} T cells from the SF of JIA patients. Telomere repeats (TTAGGG) (Telomere Length Assay kit, Cat No. 12 209 136 001, Roche Applied Science, Penzberg, Germany) were quantified using qualitative real-time polymerase chain reaction (qPCR). The qPCR was modified with synthetic standard of telomere repeats enableing the absolute quantification of telomere length which was additionally normalized by the house keeping gene (36B4), quantified based on the same method(59). Telomere length is represented according to the absolute number of the quantified base paires.

Statistics

For analysis of variance between more than two groups One-way ANOVA was used. As post-hoc analysis, the Bonferroni's multiple comparison test was performed. Paired sample t-test was used to compare matched samples, while unpaired Student's t-test was applied when samples were not matched. Data are shown as mean ± SD. P values below 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism (GraphPad Software).

Study approval

Patients were enrolled after giving written informed consent either directly and/or from parents/guardians when they were under 12 years of age. Informed consent was obtained after the nature and possible consequences of the study were explained. The study was conducted in accordance with local ethics committee approval (protocol Pharmachild) and the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

A.P. and F.W. are responsible for experimental design, data interpretation and manuscript preparation; G.M., D.H.K., J.M., B.G., E.M.G., N.V. contributed to acquire and analyze data; J.C.B., D.J.H, B.O., P.C., S.J.V., B.J.P, K.G., E.S. and M.M. contributed to data interpretation and manuscript preparation; K.G. provided a reagent.

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SUPPLEMENTARY MATERIALS

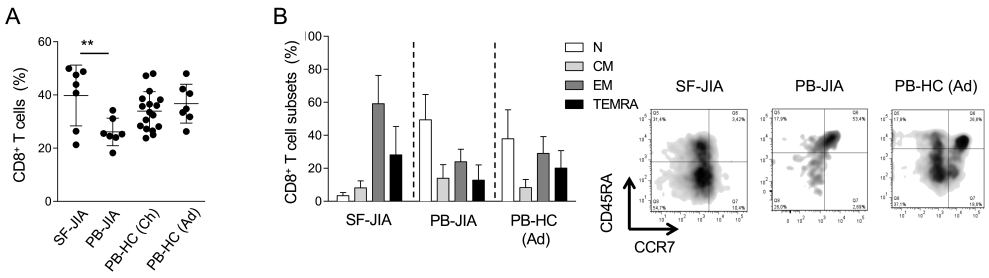

Figure S1. CD8 T cells are enriched in SF and display an effector memory phenotype. (A) CD8 T cell frequency was tested at the site of inflammation of JIA patients (i.e. the synovial fluid –SF-), in the peripheral blood (PB) of JIA patients and healthy control children and adults. Data are means ± SD. **p<0.01, One-way Anova. (B) Frequency of different subsets of CD8 T cells based on CCR7 and CD45RA expression were tested in the SF and PB of JIA patients as well as PB of healthy control adults (left panel). Data are means ± SD. Representative density plots of CD8 T cell subsets in indicated samples are shown (right panel) *SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-JIA: peripheral blood of Juvenile Idiopathic Arthritis patients; PB-HC (Ch): peripheral blood of healthy control children; PB-HC (Ad): peripheral blood of healthy control adults. N: naive; CM: central memory; EM: effector memory; TEMRA: terminally differentiated effector memory.*

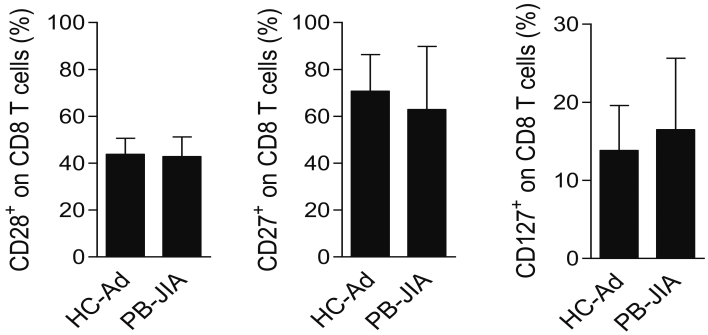

Figure S2. Similar differentiation state is evident between PB of JIA patients and HC adults. The frequency of $CD28^+$, $CD27^+$ and $CD127^+$ cells on CD8 T cells was tested in the PB of JIA patients and HC adults. (n=4 per group). Data are means ± SD. *ns*, unpaired Student's t-test. *HC-Ad peripheral blood of healthy control adults; PB-JIA: peripheral blood of Juvenile Idiopathic Arthritis patients.*

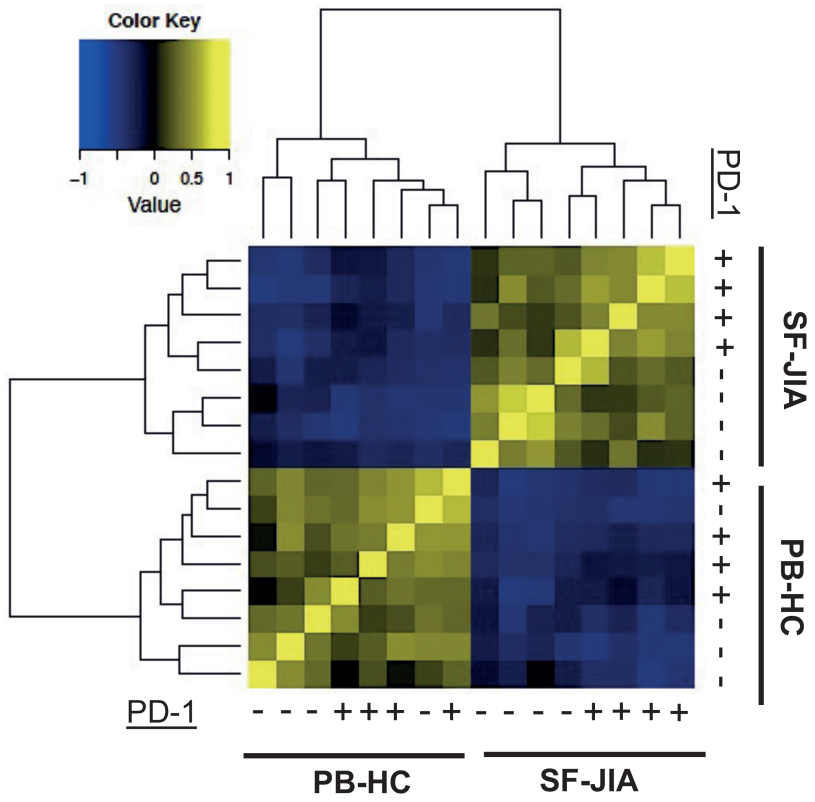

Figure S3. Heatmap from hierarchical clustering of variable genes. PD-1^{+} and PD-1^{-} CD8 T cells were sorted from SF of JIA patients and PB of healthy control adults and whole transcriptome sequencing was performed. Hierarchical clustering of variable genes is shown. *SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-HC: peripheral blood of healthy control adults.*

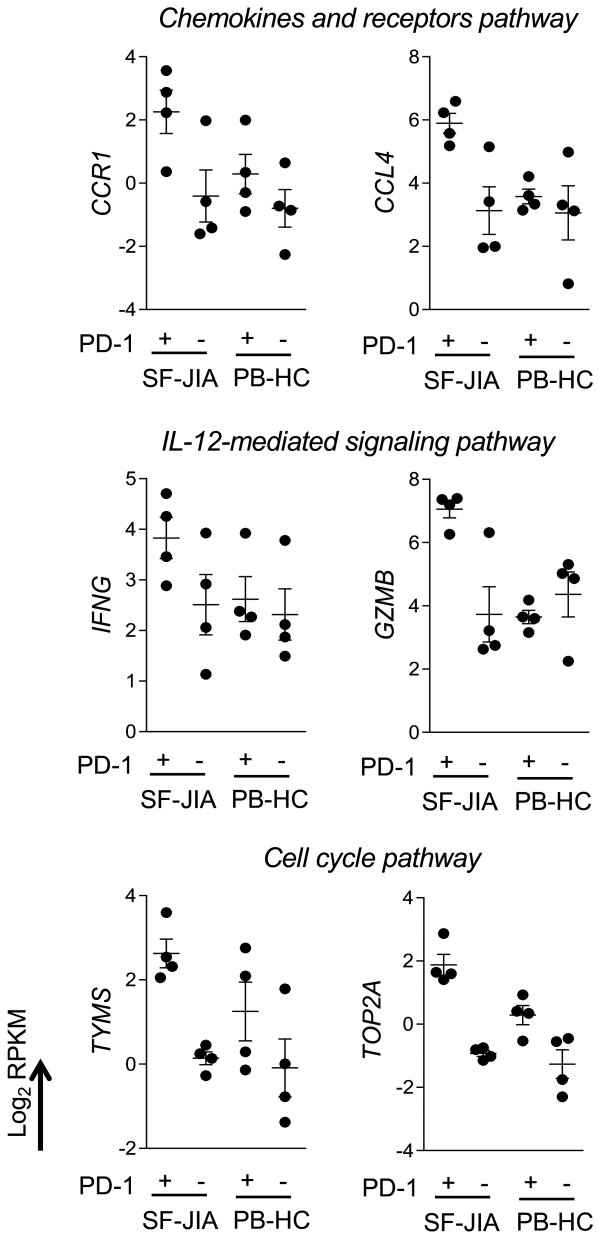

Figure S4. Expression levels of genes involved in cell activation. PD-1^{+} and PD-1^{-} CD8 T cells were sorted from SF of JIA patients and PB of healthy control adults and whole transcriptome sequencing was performed. Expression levels of selected genes up-regulated in PD-1^{+} CD8 T cells and associated with pathways involved in cell activation are shown. *SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-HC: peripheral blood of healthy control adults.*

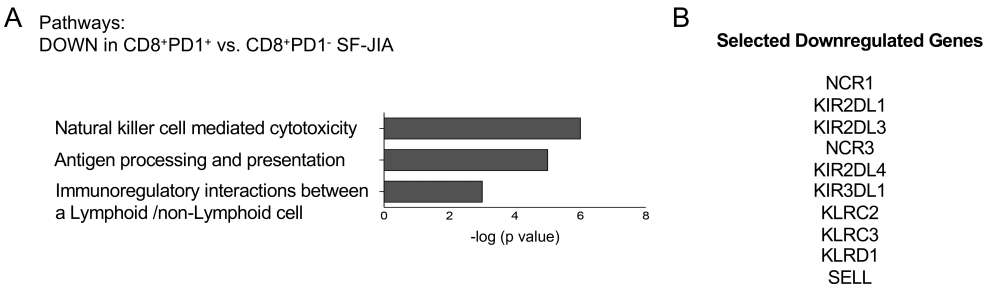

Figure S5. Activating and inhibitory killer cell receptors are downregulated in PD-1$^-$ CD8 T cells from SF-JIA. (A) Pathways enriched when genes down-regulated in PD-1$^+$ compared to PD-1$^-$ CD8 T cells from SF (n=179) were selected. (B) Selected genes from this analysis are shown. *SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients.*

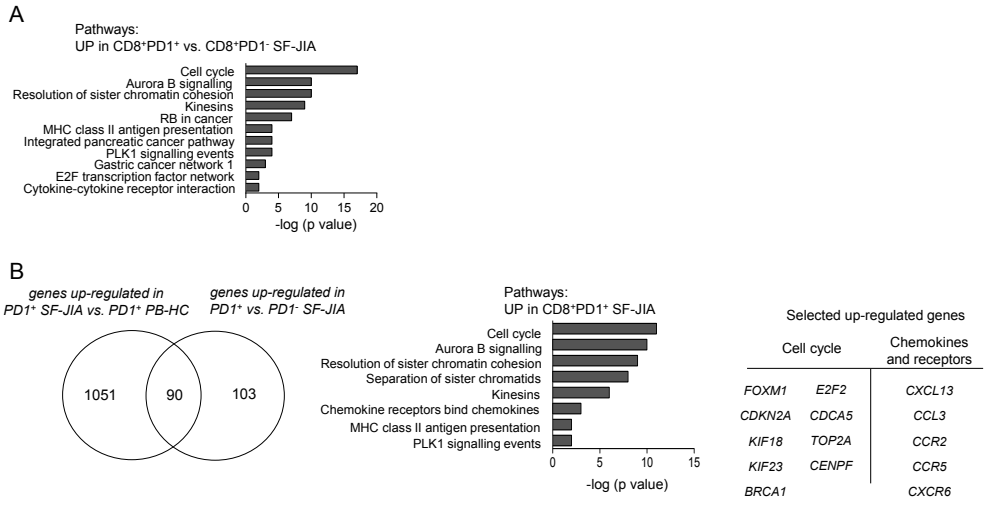

Figure S6. Enrichment of pathways involved in cell activation are evident in PD-1$^+$ CD8 T cells from SF. (A) Pathways enriched when genes up-regulated in PD-1$^+$ compared to PD-1$^-$ CD8 T cells from SF were selected. (B) n=90 genes were shared between the following comparisons: genes up-regulated in PD-1$^+$ from SF-JIA vs. PD-1$^+$ from PB-HC and genes up-regulated in PD-1$^+$ vs. PD-1$^-$ from SF-JIA (left panel). Pathway enrichment analysis based on the n=90 genes is shown (middle panel). Selected genes from this analysis are shown in the right panel. *SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-HC: peripheral blood of healthy control adults.*

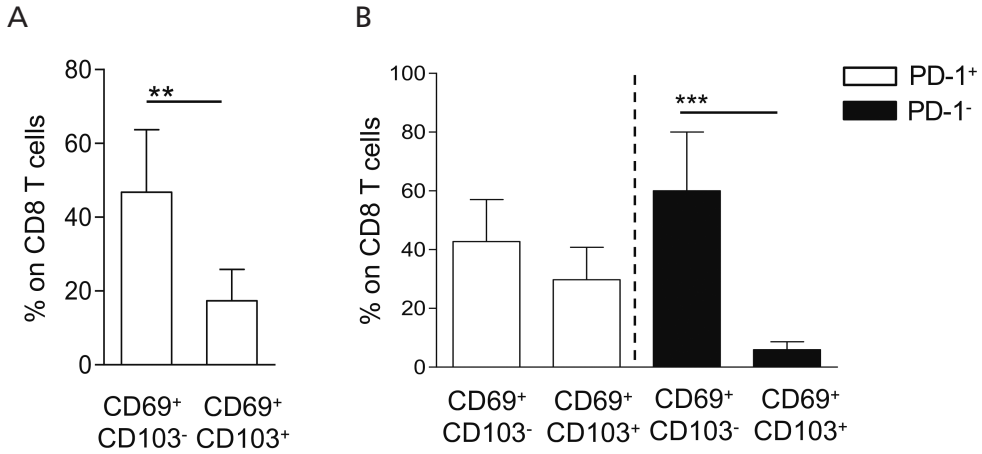

Figure S7. Tissue residency markers on CD8 T cells from SF. (A) The frequency of CD69+CD103- and CD69+CD103+ CD8 T cell subsets in SF is displayed (n=11 per group). Data are means ± SD.**p<0.01, Paired Student's t-test. (B) PD-1 expression on CD8 T cells expressing tissue residency markers in SF is described (n=11 per group) (right panel). Data are means ± SD. ***p<0.0001, Paired Student's t-test.

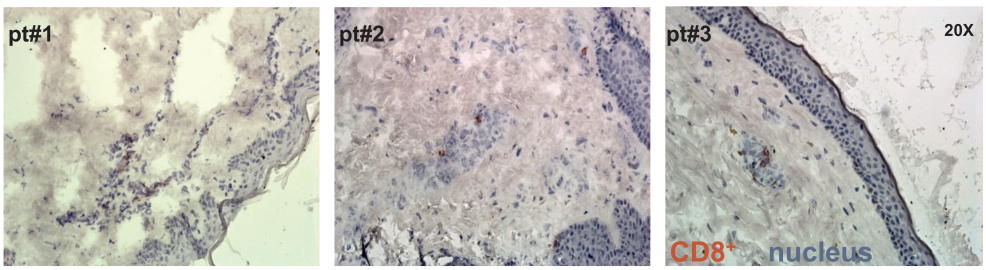

Figure S8. Few CD8 T cells are evident in non lesional skin of AD patients. (A) Immunohystochemistry was performed on skin biopsies from non inflamed tissue of patients with Atopic Dermatitis (AD). CD8 cells are shown in red. Counterstaining with Mayer's Hematoxylin (blue) indicates cell nuclei.

Chapter 5

IL-27 CONTRIBUTES TO THE TH1 SIGNATURE PRESENT AT THE SITE OF CHRONIC AUTOIMMUNE INFLAMMATION IN JUVENILE IDIOPATHIC ARTHRITIS

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In preparation

ABSTRACT

Objective

The target site of inflammation of autoimmune arthritis is enriched with IFN-γ-producing (i.e. TH1) CD4 T cells; however, it is still unexplored what molecule(s) contribute to this phenotype. Here, we investigated the contribution of IL-27 to the differentiation of CD4 T cells at the site of inflammation (i.e. SF) of Juvenile Idiopathic Arthritis (JIA) patients.

Methods

CD4 T cell signature in SF was assessed by open gene array and flow cytometry. IL-27 and IL-12 levels were measured in SF plasma and peripheral blood (PB) serum from JIA patients by Luminex assay, while their expression in antigen presenting cells was assessed by rtPCR. The effect of IL-27 signaling on CD4 T cell differentiation was determined by flow cytometry and Luminex assay upon *in vitro* cultures.

Results

CD4 T cells (Treg excluded) from SF showed a TH1 but not TH17 (IL-17-producing CD4 T cell) signature when compared to PB of healthy children and JIA patients. IL-27, but not IL-12, levels were elevated in SF plasma compared to paired PB of JIA patients. In SF, IL-27 was expressed by monocytes as well as conventional, but not plasmacytoid, dendritic cells. Co-culture of naïve CD4 T cells from PB of JIA patients with IL-27 induced a TH1 phenotype only in combination with IL-12. Exposure of SF-derived CD4 T cells to IL-27 increased survival and IFN-γ production, reduced IL-17 secretion and induced STAT1 phosphorylation.

Conclusion

Elevated IL-27 levels in SF from JIA patients contribute to the local TH1 signature, suggesting that IL-27 might be a promising therapeutic target for JIA.

INTRODUCTION

Autoimmune arthritis, such as Rheumatoid arthritis (RA) and Juvenile Idiopathic Arthritis (JIA), is characterized by recurrent inflammation targeting one or more joints (1). Among T cell subsets, IFN-γ-producing CD4 T cells (TH1) cells are predominantly present in the synovial fluid (SF) of inflamed joints (2); however the driver(s) of TH1 cell differentiation at the target site of chronic autoimmune inflammation are still unknown.

IL-27 has all the features that can potentially contribute to the CD4 T cell phenotype observed at the target site of inflammation. Indeed, IL-27, immunomodulatory cytokine secreted by activated antigen presenting cells (APC), is endowed with both pro- and anti-inflammatory properties (3). It drives inflammation by promoting proliferation of naive CD4 T cells as well as their differentiation into IFN-γ-producing T cells, via T-bet and the STAT1 signaling (3). Additionally, IL-27 inhibits TH2 and TH17 cell differentiation via suppression of GATA3 and RORγ and induces IL-10 production (3). Growing literature is posing IL-27 as a regulator of the immune response in autoimmune arthritis (4-6); however, no consensus on its effects on the local inflammatory milieu in this context has been found yet.

Given that IL-27 skews CD4 T cell differentiation towards IFN-γ-producing T cells, we hypothesized that, in combination with IL-12, a well-known inducer of the TH1 phenotype, IL-27 contributes to the enrichment of the TH1 phenotype observed in SF. Here we show that IL-27 is elevated in SF of JIA patients, is mainly expressed by intermediate monocytes (iMo) and conventional dendritic cells (cDC) and, via STAT1 phosphorylation, contributes to the maintenance and survival of TH1 cells in SF. This study reveals that IL-27 has a pro-inflammatory effect on CD4 T cells residing at the site of chronic inflammation of autoimmune arthritis, indicating a potential detrimental role for IL-27 in JIA pathology.

METHODS

Patient population

Juvenile Idiopathic Arthritis (JIA) patients were enrolled by the Paediatric Reumathology Department at University Medical Center of Utrecht (UMCU) after giving written informed consent either directly or from parents/guardians when they were under 12 years of age. A total of n=41 JIA patients were included in the study. For Luminex assay, paired peripheral blood (PB) serum and synovial fluid (SF) plasma was obtained from patients with active disease with oligoarticular (n=12) and polyarticular (n=5) JIA. The average age of JIA patients at the time of inclusion was 13 years (range 3-18 years) with disease duration of 6 years (range 0,3-14,5). Patients were either untreated (n=8), or treated with NSAIDs (n=7). For *ex vivo* or *in vitro* experiments, patients with oligoarticular (n=18), polyarticular (n=4) and monoarticular (n=2) JIA were included in this study. The median age of the patient population was 11 years (range 2-19 years) and the disease duration at the time of inclusion was 7 years (range 0-14 years). Patients were untreated (n=7), treated with nonsteroidal anti-inflammatory drugs (NSAIDs) alone (n=3), with methotrexate (MTX)

(n=7), prednisone (n=2), Cell Cept (n=1), Leflunomide (n=1) and Adalimumab (n=3). The study was conducted in accordance with local ethics committee approval (protocols Pharmachild) and the Declaration of Helsinki. PB from healthy donor children (n=4) was obtained from volunteers at the Urology and Orthopedics department at our Institute after giving written informed consent.

Cell isolation and sorting

PB and SF mononuclear cells (i.e. PBMC and SFMC, respectively) and stored in liquid nitrogen. Thawed PBMCs or SFMCs were stained with monoclonal anti-human CD3-BV510, CD4-Percp-Cy5.5, CD45RO-PB (Biolegend) and CD45RA-FITC (eBiosciences) or CD141-APC (Miltenyi), CD123-Percp-Cy5.5, HLADR-Pe-Cy7, CD14-FITC, CD16-PE, CD11c-V450, Lin (CD3/CD19/CD59)-Amcyan (BD). Cell subsets were sorted with the fluorescence activated cell sorter FACSAria III (BD Biosciences) and used for *ex vivo* and *in vitro* studies.

Cell culture

For *in vitro* co-culture assays, $CD4^+CD45RA^+CD45RO^-$ or $CD4^+CD45RA^-CD45RO^+$ T cells were stimulated with coated 1 µg/ml anti-CD3 (OKT3 clone, eBioscience) alone or in combination with anti-CD28 (eBioscience). In some experiment cells were cultured in the presence of recombinant human (rh) IL-27 (10 ng/ml, R&D Systems) or rhIL-12 (10 ng/ml, BD) or both combined. Cells were incubated at 37°C for 5 days.

Flow cytometry

Flow cytometry was performed on PBMCs and SFMCs *ex vivo* and after *in vitro* culture. Cells were first stained with fixable viability dye eFluor 506 (eBiosciences) and then with the following monoclonal anti-human antibodies: CD3-BV510, CD4-PerCP-Cy5.5, CD45RO-PB, CCR5-PE (eBioscience), CXCR3-APC (BD), IL-27Rα-AF488 and gp130-PE (R&D Systems). For intracellular staining, cells were stimulated for 4 hours with Phorbol Myristate Acetate (PMA) (40 ng/ml, Sigma) and Ionocymcin (2.5 µg/ml, Calbiochem) in the presence of Golgistop (1:1500, BD Biosciences). Cells were fixed, permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained with anti-human monoclonal antibody IFN-γ-PE-Cy7 (eBioscience) and T-bet-FITC (Santa Cruz). To test STAT1 phosphorylation (anti-STAT1 (pY701)-AF647, BD Phophoflow™), cells were first starved in serum-free medium for 30 minutes and then incubated in Cytofix buffer and Perm Buffer III (BD Biosciences). Data were analysed using FlowJo (Tristar, V10).

TaqMan human inflammation panel open array

$CD4^+CD25^-CD127^+$ T cells were sorted from n=4 SF of JIA patients and n=4 PB of healthy children. Total RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) and stored at -80°C. RNA yield was assessed by Qubit® RNA BR

Assay Kit and integrity was determined by Bioanalyzer. cDNA synthesis was performed (cDNA Reverse Transcription kit, iScript, BioRad) and 18,75ng cDNA was pre-amplified (PreAmplification master Mix, LifeTech) for 12 cycles. The gene expression was analyzed by using the TaqMan® OpenArray® Human Inflammation Panel (Life Technologies) that covers 586 genes involved in the inflammatory cascade plus 21 endogenous control genes. After global normalization, differentially expressed genes were identified based on the 2^-ddCt method, by using the ExpressionSuite Software (Life Technologies). Genes with a fold change higher than 2.5 or lower than 0.4 and with a p-value of 0.05 were considered differentially expressed. Pathway enrichment analysis was performed using ToppGene Suite publicly available online portal.

Polymerase chain reaction

To assess gene expression, $CD14^{+}CD16^{+}$ intermediated monocytes (iMo), $CD1c^{+}CD141^{-}$ conventional DC (CD1c cDC), $CD1c^{-}CD141^{+}$ cDC (CD141 cDC) and $CD123^{+}$ plasmacytoid DC (pDC) were sorted and dissolved in TRIzol (Invitrogen). Total RNA was extracted and cDNA synthesis was performed. Real-time PCR (rtPCR) reactions were performed with QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific) using the following primers: IL27A FW: CTCCCTGATGTTTCCCTGAC, RV: TCCTCTCCATGTTGGTCCAG; EBI3 FW: AGCTTCGTGCCTTTCATAACAG, RV: AGTGAGAAGATCTCTGGGAAGG; p35 FW: AGGGCCGTCAGCAACATG, RV: TCTTCAGAAGTGCAAGGGTAAAATTC; p40 FW: GGACATCATCAAACCTGACC, RV: AGGGAGAAGTAGGAATGTGG. Relative gene expression was determined by the ΔCt method, resulting in fold changes normalized to internal control genes (GAPDH, β2M, GUSB).

Luminex

Paired PB serum and SF plasma samples from JIA patients were thawed and IL-27 and IL-12 levels were measured by Luminex assay. IL-10, IL-17 and IL-13 levels were tested on supernatants obtained by cell cultures.

Statistical Analysis

The paired T-test was used to compare the mean of matched groups. The Mann-Whitney test were used to compare 2 unpaired groups. For analysis of variance among more than two groups One-way ANOVA was used. Results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism software (V6). p-values less than 0.05 were considered significant.

RESULTS

CD4 T cells from SF of JIA patients show a TH1 but not TH17 gene signature when compared to different control groups

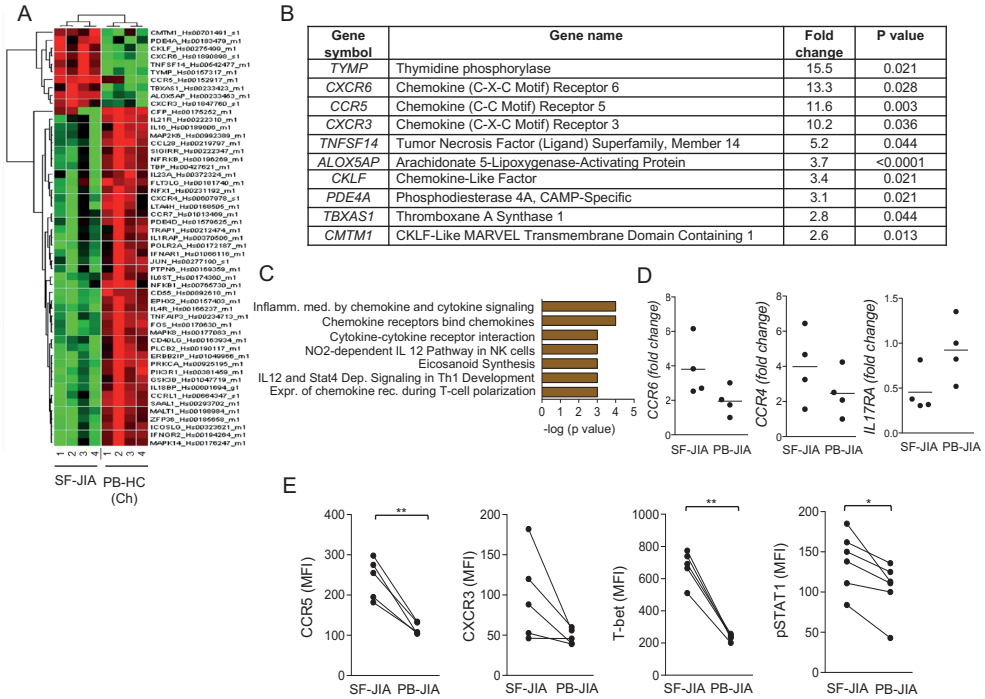
Previous studies have shown an enrichment of IFN-γ-producing CD4 T cells in SF of patients with autoimmune arthritis at a protein, epigenetic and mRNA level (2, 7). However, it is still under debate whether TH17 cells are similarly enriched at this site and what role they play in this context (8). We first investigated the signature of SF-derived CD4 T cells, removed of the Treg subset, compared to PB collected from age-matched healthy children or the same JIA patients.

An open array for the study of pre-selected 586 inflammatory genes was performed on sorted $CD4^+CD25^-CD127^+$ T cells from SF of JIA patients and PB of healthy children, showing a clear-cut clustering of 43 differentially expressed genes (Fig. 1A). A total of 10 genes were found up-regulated in SF (Fig. 1B), which were mainly involved in pathways enriched in TH1 cells (Fig. 1C). Interestingly, IL-17 levels were undetectable and typical TH17 cell-associated markers, such as *CCR6*, *CCR4* and *IL17RA* were found expressed at similar levels in SF and PB (Fig. 1D). The TH1 signature in SF was also confirmed at a protein level, based on the expression of typical TH1 marker such as CCR5, CXCR3, T-bet and phosphorylation of STAT1 (pSTAT1), when compared to PB of the same JIA patients (Fig. 1E).

These data confirm that CD4 T cells residing in SF of JIA patients are enriched with the TH1 but not TH17 signature.

IL-27 is elevated in SF plasma of JIA patients and is expressed by specific APC subsets

Both IL-27 and IL-12 induce differentiation into IFN-γ-producing T cells (3) potentially contributing to the enrichment of the TH1 signature observed in SF. Therefore, we measured the levels of both cytokines in SF plasma and PB serum of the same JIA patients. While levels of IL-27 were significantly increased in SF (Fig. 2A), IL-12 levels were very low in all the samples tested, and no difference between SF and PB was evident (Fig. 2B). To determine the source of IL-27 in SF, gene expression levels of the two IL-27 subunits (i.e. *IL27A* and *EBI3*) were investigated in different APC subsets. Expression of *IL27A* was elevated in inflammatory monocytes (iMo), compared to CD1c and CD141 conventional dendritic cells (cDC) (Fig. 2C). *IL27A* was almost undetectable in plasmocytoid DC (pDC) (Fig. 2C, left panel). *EBI3*, instead, was mainly expressed by CD1c cDC, with lower expression levels in iMo and CD141 cDC (Fig. 2C, right panel). *EBI3* was undetectable in pDC as well (Fig. 2C, right panel). The two subunits of IL-12, *IL12p35* and *IL12p40*, despite the overall low expression levels, were expressed by all APC subsets (Fig 2D). These results indicate that high levels of IL-27, but not IL-12, are present in SF, and that IL-27 may be mainly produced by iMo and CD1c cDC, to a small



Gene symbol	Gene name	Fold change	P value
TYMP	Thymidine phosphorylase	15.5	0.021
CXCR6	Chemokine (C-X-C Motif) Receptor 6	13.3	0.028
CCR5	Chemokine (C-C Motif) Receptor 5	11.6	0.003
CXCR3	Chemokine (C-X-C Motif) Receptor 3	10.2	0.036
TNFSF14	Tumor Necrosis Factor (Ligand) Superfamily, Member 14	5.2	0.044
ALOX5AP	Arachidonate 5-Lipoxygenase-Activating Protein	3.7	<0.0001
CKLF	Chemokine-Like Factor	3.4	0.021
PDE4A	Phosphodiesterase 4A, CAMP-Specific	3.1	0.021
TBXAS1	Thromboxane A Synthase 1	2.8	0.044
CMTM1	CKLF-Like MARVEL Transmembrane Domain Containing 1	2.6	0.013

Figure 1. CD4 T cells from SF of JIA patients show a TH1 but not TH17 signature when compared to controls. (A) TaqMan Human Inflammation Panel Open Array for the study of 586 inflammatory genes was performed on CD4+CD25$^-$CD127$^+$ T cells from SF of JIA patients (n = 4) and PB of healthy children (n = 4). (B) Genes up-regulated in SF of JIA are listed. (C) Pathway analysis was performed by using the ToppGene Suite portal. (D) Expression levels of *CCR6*, *CCR4* and *IL17RA* (TH17-related genes) extracted from the gene array. (E) Expression levels of TH1 markers were assessed by flow cytometry in SF and PB of JIA patients. *SF-JIA: synovial fluid from Juvenile Idiopathic Arthritis patients; PB-JIA: peripheral blood from Juvenile Idiopathic Arthritis patients; PB-HC (Ch): peripheral blood of healthy control children; MFI: geometric mean fluorescence intensity. P value: *<0.05, **<0.01*

IL-27 sustains the TH1 phenotype observed in SF

To investigate whether IL-27 contributes to the differentiation of CD4 T cells from PB towards TH1 cells, naïve CD4$^+$CD45RO$^-$CD45RA$^+$ T cells from PB of JIA patients were incubated with rhIL27 or rhIL12 or both cytokines upon TCR stimulation. Unlike rhIL12, which induced a massive increase of IFN-γ producing cells, rhIL27 alone did not induce IFN-γ production or T-bet expression in naïve CD4 T cells (Fig. 3A). However, when cells were co-incubated with both cytokines, a synergistic induction of TH1 cells was observed (Fig. 3A), indicating that IL-27 may cooperate with IL-12 in the development of the TH1 phenotype in JIA.

However, CD4 T cells accumulating in SF have already been differentiated. Therefore, we tested the effect of IL-27 on cells chronically exposed to the inflammatory milieu, i.e.

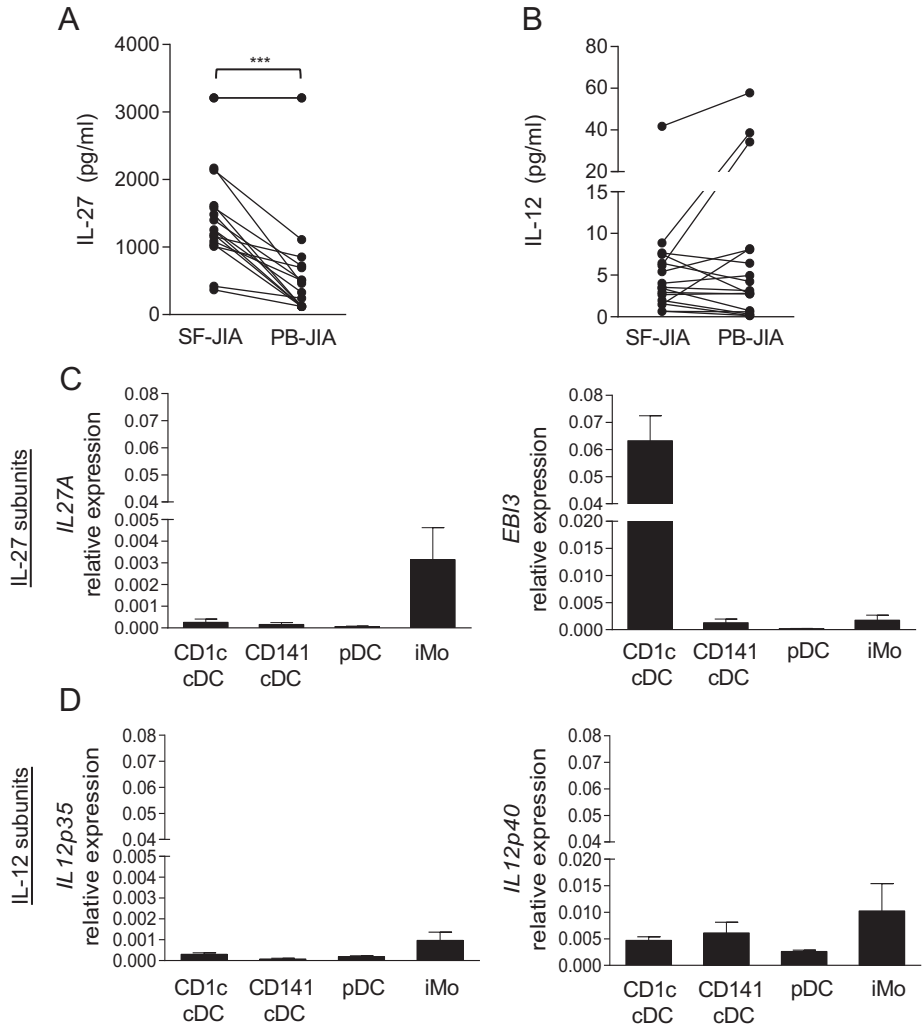


Figure 2. IL-27 is elevated in SF plasma of JIA patients and is produced by iMo, CD1c cDC, but not pDC. (A) IL-27 and (B) IL-12 levels were tested in SF plasma and paired PB serum of n=17 JIA patients by luminex assay. (C) The expression of IL-27 subunits, IL27A (left panel) and EBI3 (right panel) were tested in SF-derived iMo, CD1c cDC and pDC by rtPCR (n=4). (D) The expression of IL-12 subunits, IL12p35 (left panel) andIL12p40 (right panel) were tested in iMo, CD1c cDC and pDC by rtPCR in SF of JIA patients (n=4). *iMo: intermediated monocytes, CD1c cDC: CD1c$^+$ conventional DC, CD141 cDC: CD141 convenstional DC, pDC: CD123$^+$ plasmacytoid DC. P value: ***<0.001*

memory CD4 T cells from SF. The already elevated frequency of IFN-γ^+ CD4 T cells from SF further increased in the presence of rhIL-27 (Fig. 3B). When cytokines such as IL-10, IL-17 and IL-13 were tested in the supernatant of the co-culture, no differences in IL-10, while a reduction of IL-17 levels were found in the presence of rhIL-27 (Fig. 3C); while IL-13 was undetectable in the assay. Interesting, in the presence of rhIL-27, an increased

frequency of live cells was observed (Fig. 3D). Overall, these data indicate that IL-27 sustains maintenance and survival of TH1 cell in SF.

We then assessed IL-27 signaling by investigating the expression level of the two IL-27 receptor subunits, gp130 and IL27Rα, as well as pSTAT1 response to rhIL-27. While gp130 showed lower expression levels in SF compared to PB, higher expression of IL27Rα was evident (Fig. 3E). This data might be interpreted in the light of the evidence that IL-27Rα can homodimerize in inflammatory environments and signal independently of gp130 (9). In parallel, pSTAT1 expression was tested upon stimulation with rhIL27 showing that, both in SF and PB, the pathway is functional and responsive to the cytokine (Fig. 3F).

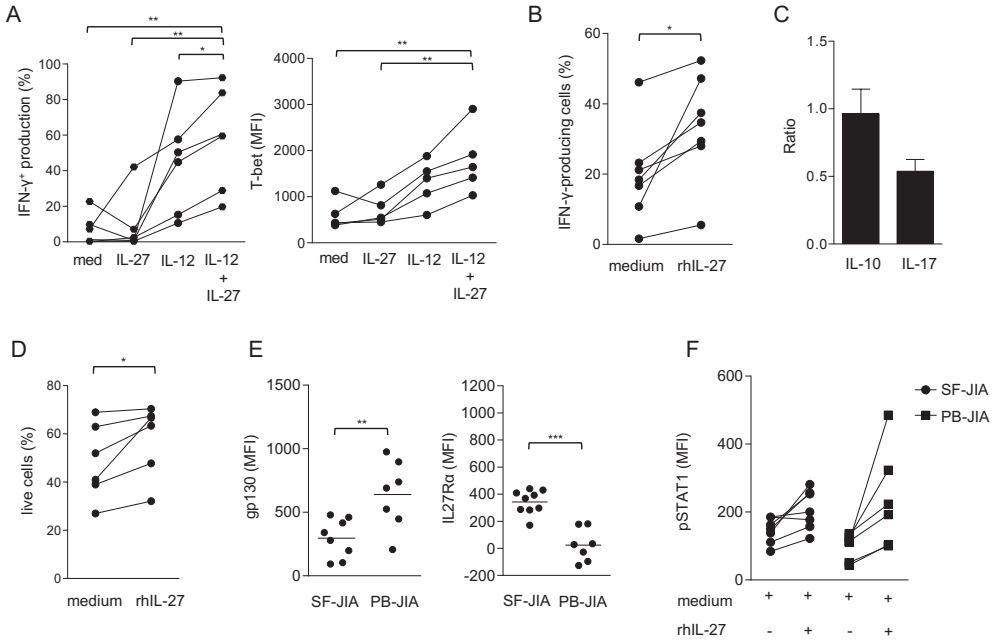


Figure 3. IL-27 sustains maintenance and survival of TH1 cell from SF. (A) Naïve CD4 T cells from PB of JIA patients were sorted and incubated with rhIL-27 (10ng/ml) or rhIL-12 (10ng/ml) or both recombinants combined. After 5 day culture, the frequency of IFN-γ-producing cells (left panel) and T-bet expression (right panel) were tested in the different conditions by flow cytometry. (B) Memory CD4 T cells from SF of JIA patients were sorted and incubated with rhIL-27. The frequency of IFN-γ-producing cells was tested after culture. (C) In parallel, IL-10 and IL-17 levels were measured in the supernatant. For each sample, data are expressed as the ratio of levels measured in the presence/absence of rhIL-27. A ratio of 1 means no difference between the two conditions. (D) In the same experiment, the frequency of live cells was assessed by staining with fixable viability dye. (E) Expression levels of IL-27 receptor subunits gp130 (left panel) and IL27Rα (right panel) were measured on CD4 T cells from SF and PB of JIA patients. (F) Phosphorylated STAT1 (pSTAT1) expression levels were assessed upon co-culture with rhIL-27 in SF and PB of JIA patients (left panel). *SF-JIA: synovial fluid from Juvenile Idiopathic Arthritis patients; PB-JIA: peripheral blood from Juvenile Idiopathic Arthritis patients; MFI: geometric mean fluorescence intensity. P value: *<0.05, **<0.01, ***<0.001*

These data indicate that IL-27, in combination with IL-12, induces a robust TH1 phenotype in naïve CD4 T cells from PB. Moreover, with the contribution of the STAT1 signalling, IL-27 promotes selective maintenance of TH1 cells in SF.

DISCUSSION

IL-27 is a cytokine that modulates the immune response in inflammatory conditions skewing T cell differentiation towards the TH1 profile (3). However, its role in autoimmune arthritis and, in particular, at the site of inflammation of JIA, is still unexplored. In this study we investigated if and how IL-27 participates in the induction of the TH1 signature present in SF of JIA patients.

Our data confirmed the enrichment of the TH1 signature in SF of JIA patients (2, 7), which was still evident when Treg were excluded from the analysis. Interestingly, the TH17 signature, previously described in SF from JIA patients (*) was undetectable. TH17 cells, despite present in SF, apparently represent a very small fraction with potentially relevant complementary role, which deserves further investigation. While it is clear that IL-27 contributes to the induction of TH1 cells, its role in autoimmune arthritis is still controversial as well as its effect on lineage committed CD4 T cells coming from the target site of autoimmune inflammation. In preclinical models, IL-27 was found to be protective as well as accelerate disease onset depending on the model used (5, 6). In humans, levels of IL-27 in SF plasma were shown to be either elevated (10) or similar (11) a heterodimeric cytokine, has been reported to be involved in the pathogenesis of autoimmune diseases through mediating differentiation of TH1 or TH17 cells and immune cell activity or survival. However, the origin and effects of IL-27 in joints of rheumatoid arthritis (RA in RA patients compared to PB. In this study we found almost 3-fold increase of IL-27 levels in SF plasma compared to PB of paired JIA patients, while no difference in IL-12 levels were observed. These data are also in line with a recent report showing increased IL-27 levels in SF of patients with ankylosing spondylitis (12).

Since APC are considered the main producers of IL-27 (3), we tested which APC subset mainly expressed IL-27 subunits in SF. Unlike IL-12, which was expressed at low levels by all APC subsets, IL-27 subunits were mainly expressed by SF-derived iMo and CD1c cDC, at a lower extent by CD141 cDC, and were undetectable in pDC. Therefore, specific local APC subsets producing IL-27 may contribute to the TH1 skewing observed in SF.

Then, we wondered what would be IL-27 effect on CD4 T cells already committed to the TH1 phenotype and chronically exposed to the inflammatory milieu. In SF, upon exposure to IL-27, the frequency of TH1 cells was further increased, IL-10 levels were stable and IL-17 levels were reduced. As IL-27 is described to induce IL-10 (3), it was unexpected that IL-10 levels were unaffected by IL-27 stimulation. This might be due to the commitment that CD4 T cells already have to the TH1 lineage in SF, which seems to be stable and protected from plasticity in this context. Additionally, an increased cell survival of SF-derived CD4 T cells was observed in the presence of IL-27, as previously observed also in memory mouse-derived CD4 T cells (13), indicating that IL-27 has

a crucial role in maintaining and sustaining the TH1 phenotype. We also explored whether IL-27 is endowed with system effects on CD4 T cells in JIA, showing that IL-27 synergizes with IL-12 to induce IFN-γ production in naïve CD4 T cells from PB of JIA patients. This same effect was previously described in HC (14), indicating that PB-derived CD4 T cells from JIA patients behave similarly to HC-derived cells and that a functional specialization is specifically acquired by CD4 T cells once they reach the target site of autoimmune inflammation.

To elucidate the mechanisms by which IL-27 mediates these effects in SF, we investigated IL-27 signaling showing a higher IL-27Rα and lower gp130 expression on CD4 T cells. This confirms previous findings where gp130, also subunit of IL-6 receptor, was shown to be internalized upon continuous IL-6 stimulation (15). Interestingly, IL-27Rα has been described to homodimerize in inflammatory environments and signal independently of gp130 (9), possibly explaining the response of CD4 T cells to IL-27 observed in SF. Finally, we analyzed IL-27-mediated intracellular signaling via STAT1 phosphorylation. Similar to PB, SF-derived CD4 T cells showed increased pSTAT1 upon IL-27 stimulation, indicating that STAT1 drives IL-27 signaling, thus being involved in the development of the TH1 profile observed in SF.

To conclude, we were able to show that elevated IL-27 levels are present at the site of chronic autoimmune inflammation of JIA and contribute to the maintenance and stabilization of the TH1 signature observed at this site via the engagement of the STAT1 signaling pathway. Taken together, this study indicates an active participation of IL-27 in the defective regulation occurring at the site of chronic inflammation in autoimmune arthritis, suggesting a role for IL-27 as a potential therapeutic target in this context.

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AUTHOR'S CONTRIBUTION

All authors were involved in revising the article critically and all authors approved the final version to be published.

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Chapter 6

GENERATION OF DONOR-SPECIFIC T REGULATORY TYPE 1 CELLS FROM PATIENTS ON DIALYSIS FOR CELL THERAPY AFTER KIDNEY TRANSPLANTATION

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ABSTRACT

Background

T regulatory type 1 (Tr1) cell-mediated induction of tolerance in preclinical models of transplantation is remarkably effective. The clinical application of such a therapy in patients on dialysis undergoing kidney transplantation should take into account the possible alterations of the immune system observed in these patients. Herein, we aimed at testing the ability to generate donor-specific Tr1-cell enriched lymphocytes from patients on dialysis on the waiting list for kidney transplantation.

Methods

Tr1-cell enriched lymphocytes were generated by co-culturing IL-10-producing dendritic cells (DC-10) obtained from healthy donors with peripheral blood mononuclear cells (PBMC) of patients on dialysis, following the same protocol used in a previous cell therapy clinical trial to prevent graft-versus-host disease (GvHD). Alternatively, purified $CD4^+$ T cells were used instead of total PBMC. The ability to generate clinical-grade Tr1-cell enriched products was defined by testing the reduced response to re-stimulation with mature DC generated from the original donor (i.e., anergy assay).

Results

Tr1-cell enriched medicinal products generated from PBMC of patients on dialysis showed a low anergic phenotype, incompatible with their eventual clinical application. This was irrespective of HLA-matching with the donor or the intrinsically reduced ability to proliferate in response to alloantigens. On the contrary, the use of purified $CD4^+$ T cells isolated from patients on dialysis led to the generation of a highly anergic donor-specific medicinal product containing an average of 10% Tr1 cells.

Conclusions

Tr1-cell enriched medicinal products can be efficiently generated from patients on dialysis by carefully tailoring the protocol on patients' immunological characteristics.

INTRODUCTION

Kidney transplantation is the only curative treatment for patients suffering from end-stage renal disease on dialysis (1, 2). Nowadays, combined immunosuppressive treatments have decreased the incidence of acute rejection achieving 1-year graft survival rates above 90% in many transplant centers (3). Unfortunately, the efficacy of immunosuppressive drug treatment is counter-balanced by undesired side-effects such as nephrotoxicity (4), metabolic disorders (5), cardiovascular diseases (6), infections (7) and malignancies (8). Therefore, drug minimization and induction of donor-specific tolerance is a key clinical goal (9).

T regulatory cells (Tregs) have been shown to induce tolerance after transplantation in several preclinical models (10, 11). Tregs are categorized into three major subgroups based on their ontogeny (12, 13): thymus-derived Tregs (tTreg), which develop in the thymus and are present in healthy individuals from birth, peripheral Tregs (pTreg), which are generated in the periphery under various tolerogenic conditions and *in vitro*-induced Tregs (iTregs). T regulatory type 1 (Tr1) cells are a subset of pTreg characterized by elevated production of IL-10 selectively in response to the antigen they have been primed with (14). Tr1 cells are induced by antigen (Ag) stimulation via an IL-10-dependent process *in vitro* and *in vivo* (14). Our group performed the first-in-man clinical trial infusing IL-10-anergized donor T cells containing Tr1 cells specific for the host Ags to prevent graft-versus-host disease (GvHD) after hematopoietic stem cell transplantation (HSCT) (ALT-TEN trial) (15). The medicinal product containing host-specific Tr1 cells and memory T cells able to respond to pathogens (named MLR/10) was infused in patients with haematological cancers following HSCT. The long-term follow-up showed no safety concerns relating to the cell therapy, complete immune reconstitution in 5/12 patients, disease remission and reduced severity of acute GvHD in 4 patients (15). Ag-specific Tr1 cells have been also used in patients with Crohn's disease (16).

The ONE Study (www.onestudy.org) is a cooperative international multicenter clinical trial aiming at developing and testing the safety of different immunoregulatory cell products in living donor kidney transplant recipients (17). Our group participates to this study to test the tolerogenic ability of a donor-specific Tr1-cell–enriched medicinal product. The fundamental pre-requisite for the clinical use of Tr1 cells in kidney transplanted patients is the development of an effective method for their *ex vivo* generation from patients on dialysis. The immune system of these patients is characterized by several immunological alterations such as: (i) lymphopenia (18), (ii) presence of activated pro-inflammatory monocytes (19, 20), (iii) reduced frequency of highly immunogenic circulating dendritic cells (DC) (21-23); (iv) defective humoral immunity (24), (v) hyporesponsiveness to T-cell priming (25) and (vi) high levels of plasmatic pro-inflammatory molecules (25-27). Importantly, tTregs expanded *in vitro* from patients on dialysis are less suppressive and more plastic (i.e., more prone to produce IL-17) as compared to those generated from control healthy subjects (28). Conversely, few data are available on pTreg cells generated

from patients on dialysis (29). Berglund et al. reported donor-specific IL-10 production from a cell population generated by co-culturing T cells from patients on dialysis with immature dendritic cells (iDC) from healthy subjects (30). Our group previously characterized a subset of IL-10-producing human dendritic cells (DC), termed DC-10, which can be induced in vitro from circulating monocytes in the presence of IL-10 (31). Co-culture of *in vitro* generated DC-10 with allogeneic PBMC for 10 days in the presence of exogenous IL-10 (MLR/DC-10) was shown to be an effective way of generating Tr1-cell enriched products (32). Accordingly, we expect to use donor-derived DC-10 for the generation of Tr1-cell enriched medicinal product in The ONE Study. However, no data on the functional characterization of the MLR/DC-10 from patients on dialysis has been reported. Herein, we aim at testing whether the MLR/DC-10 protocol previously developed by our group (32) is suitable for generating donor-specific Tr1-cell enriched product to be used in The ONE Study. Our findings indicate that the MLR/DC-10 product by using total PBMC shows low anergic phenotype when obtained from patients on dialysis. Therefore we optimized the protocol by depleting non-CD4$^+$ cells and obtained a cell product endowed with the safety features required for clinical application.

MATERIALS AND METHODS

Healthy donors and patients

Peripheral whole blood was collected from patients with end-stage renal disease on dialysis waiting for kidney transplantation at the San Raffaele Hospital (n=13) and from family-related kidney donors (n=5) or from healthy volunteer blood donors (n=11). Buffy coats from healthy volunteer blood donors (n=25) were also obtained. Kidney living donors and healthy volunteer blood donors were both considered healthy control subjects in this study and named "*controls*". Our goal was to test the protocol for Tr1-cell generation using a representative patient population likely to be enrolled in The ONE Study clinical trial at our clinical center. Therefore, patients were not stratified for dialysis modality, pathogenesis of kidney failure, or uremic vs. dialysis but rather all patients on waiting list for kidney transplantation at our Institute were included in this study. Characteristics of patients and healthy controls are listed in Tables 1 (includes donors tested for MLR/DC-10 protocol) and 2 (includes donors tested for CD4$^+$/DC-10 protocol). All subjects enrolled in this study provided written informed consent prior to blood withdrawal, in accordance with the local ethics committee's approval (protocol PERIBLOOD) and with the Declaration of Helsinki.

Dendritic cell (DC) generation

IL-10 producing DC (DC-10) and mature DC (mDC) were generated from control subjects as previously described (31, 32). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from peripheral whole blood or buffy coats collected from control subjects by density-gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). CD14$^+$

Table 1. Characteristics of subjects used as responders for the generation of the MLR/DC-10 product in Figure 2A. Data are median (interquartile range).

	Patients (n=8)	Symbol	Controls (n=8)
Age (yrs)	50.5 (31-59)		45 (36-56)
Sex (M)	5/8		4/8
Time of dialysis (yrs)	3.1 (1.3-4.7)		N/A
Cause of CKD	Hypertension (n=1) IgA nephropathy (n=1) PKD (n=1) T1D (n=1) CAKUTs (n=1) Unknown (3)	● ■ ▲ ▼ ◆ ⬣ ◒ ⊗	N/A
Dialysis modality	HD: n=7 PD: n=1	 ⊗	N/A

CKD: chronic kidney disease; PKD= Polycystic kidney disease; T1D: type 1 diabetes; CAKUT: Congenital anomalies of the kidney and urinary tract; HD: hemodialysis; PD: peritoneal dialysis.

monocytes were isolated by autoMACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and cultured in RPMI 1640 (BioWhittaker) supplemented with 10% FCS (BioWhittaker) and 100 U/ml penicillin-streptomycin (BioWhittaker) with 10 ng/mL rhIL-4 (R&D Systems, Minneapolis, MN, USA) and 100 ng/mL rhGM-CSF (R&D Systems, Minneapolis, MN, USA) for 7 days in the presence (DC-10) or absence (mDC) of 10 ng/mL rhIL-10 (CellGenix GmbH, Freiburg, Germany). mDC were matured on day 5 with lipopolysaccharide (LPS) from *E. coli* (1 mg/mL, Sigma Chemicals, St Louis, MO, USA).

MLR/DC-10 and CD4/DC-10 generation

Irradiated DC-10 generated from controls were used as stimulators, while total PBMCs (for MLR/DC-10 generation) or immunomagnetic positively selected $CD4^+$ T cells (for CD4/DC-10 generation) from controls or patients on dialysis were used as responders with a responder:stimulator ratio of 10:1. PBMCs or $CD4^+$ T cells were co-cultured with DC-10 in X-VIVO 15 medium (BioWhittaker, Verviers, Belgium) supplemented with 5% human AB serum (BioWhittaker) and 100 U/ml penicillin-streptomycin for 10 days with addition of exogenous IL-10 as previously described (32). As reference, in each experiment, irradiated mDC from control subjects were generated in parallel to DC-10 and co-cultured with PBMCs (for MLR/mDC generation) or $CD4^+$ T cells (for CD4/mDC generation) for a total of 10 days without IL-10 supplementation.

Anergy assay

At the end of the co-culture, MLR/DC-10 and MLR/mDC or $CD4^+$/DC-10 and CD4/mDC were collected, washed and plated in a secondary MLR with donor-derived mDC at 10:1 ratio (responder:stimulator) to test their anti-donor responsiveness. 1μCi/well of ^{3}H-thymidine (Sigma-Aldrich) was added 48 hours after culture for the last 12 hours. Anergy is defined as donor-specific hypo-responsiveness and it is calculated as follows: [MLR/mDC – MLR/DC-10 : MLR/mDC] x 100 (or [CD4/mDC – CD4/DC-10 : CD4/mDC] x 100). In the ALT-TEN trial, the anergy cut-off value for MLR/10 - below which the cell product was not considered safe to be infused into patients - was 67%. The anergy cut-off value for CD4/DC-10 product instead, was set in this study at 60%. Those values were defined based on the average anergy – 2* standard deviation (SD) values obtained from experiments performed in more than 30 control subjects (15). The anergy assay was the release criterion for the MLR/10 medicinal product used in the ALT-TEN trial (15).

Flow cytometry

The phenotype of *in vitro* generated mDC and DC-10 was evaluated by flow cytometry. The expression of the following surface markers was tested after culture: CD1a (anti-CD1a Alexa488), CD14 (anti-CD14 APC-H7), CD16 (anti-CD16 APC-H7) and CD86 (anti-CD86 PE). All monoclonal antibodies (mAbs) were obtained from BD Pharmingen (San Jose, CA, USA). Cells were washed two times with FACS buffer (PBS, 0.5-1%, 10% FBS, 0.1% NaN3 sodium azide) and incubated at room temperature (RT) for 30 min, then washed two more times with FACS buffer. Tr1 cell enrichment in the CD4/mDC and CD4/DC-10 cell products was evaluated with the following mAb: CD3 PerCp-Cy5.5 (Biolegend), CD4 Pe-Cy7 (BD Bioscience), CD45RO PacificBlue (Biolegend), LAG-3 PE (R&D System), CD49b Alexa488 (Biolegend). Cells were incubated at 37°C for 30 min instead of RT. Samples were acquired using the BD FACSCanto II (Becton Dickinson, San Jose, CA, USA) and data were analysed with FlowJo software.

Enzyme-linked immunoassorbent assay (ELISA)

Supernatant was collected from the MLR/DC-10 and CD4/DC-10 co-culture 96 hours after plating (primary MLR) or 48 hours after re-stimulation with donor-derived mDC (secondary MLR) to test IFN-γ levels. Levels of IFN-γ were determined by capture enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA) (33). The detection limit of IFN-γ was 60 pg/mL.

Statistical analysis

Comparisons between groups were performed using the Mann-Whitney U test. For all analyses, a two-tailed P value ≤ 0.05 was considered significant. Data are shown as medians. Statistical analyses were performed using the statistical software GraphPad Prism 6.0 (San Diego, CA, USA).

RESULTS

The MLR/DC-10 culture generated from patients on dialysis displays low anergic phenotype

We first tested the feasibility of generating Tr1-cell enriched products from the peripheral blood (PB) of patients on dialysis using the MLR/DC-10 protocol (32). DC-10 and mDC were generated *in vitro* from $CD14^+$ monocytes purified from PBMC of control subjects. After 7 days of culture in the presence of polarizing cytokines, DC-10 and mDC were collected and analyzed by flow cytometry. Both cell subsets had the expected phenotype (31). Namely, DC-10 were $CD1a^-CD14^+CD16^+CD86^+$ while mDC were $CD1a^+CD14^-CD16^-CD86^{high}$ (Figure 1).

The MLR/DC-10 cell product was obtained by a 10-day co-culture of DC-10 generated from whole blood or buffy coats of control subjects (stimulators) with PBMC obtained from whole blood of patients on dialysis or control subjects (responders) in the presence of exogenous IL-10. In parallel, as control, the MLR/mDC culture was generated. Characteristics of the subjects enrolled in the study and used to generated data shown in Figures 1-4 are listed in Table 1. Controls and patients on dialysis were matched for age and sex. Patients on dialysis were on renal replacement treatment (n=7/8 on hemodialysis and n=1/8 on peritoneal dialysis) for a median of 3.1 years and the pathogenesis of chronic kidney disease was variable (including autoimmune diseases, hypertension, genetic and congenital diseases or unknown diseases). To test the anergy of the MLR/DC-10 cell product, cells were re-stimulated with donor-derived mDC and their proliferative capacity was compared to that of the MLR/mDC product re-stimulated with the same donor-derived mDC. We first tested the anergic phenotype of MLR/DC-10 obtained from co-culturing PBMC from patients on dialysis and DC generated from the PB of family related control subjects (Figure 2A, samples 1-5). As previously defined by our group, the cut-off anergy value for MLR/DC-10 products was set at 67% (15). Such anergic value was not achieved in 3 out of the 5 generated MLR/DC-10 cell products. To exclude that the reduced anergy observed in the MLR/DC-10 products was due to the partial HLA-matching between patients on dialysis and the family related control donors, MLR/DC-10 were generated with PBMC of patients on dialysis and DC-10 generated from the PB of unrelated healthy control donors who were assumed to be fully HLA mismatched. A limited anergic value was also detected in MLR/DC-10 cultures generated with fully mismatched pairs (Figure 2A, samples 6-8) suggesting that the level of HLA-match does not impact anergy induction. On the other hand, highly anergic MLR/DC-10 cultures were generated from all except one control subjects, confirming our previous findings (Figure 2B) (32). Overall, MLR/DC-10 cultures generated from patients on dialysis showed reduced anergic phenotype as compared to those generated from control subjects (Figure 2C).

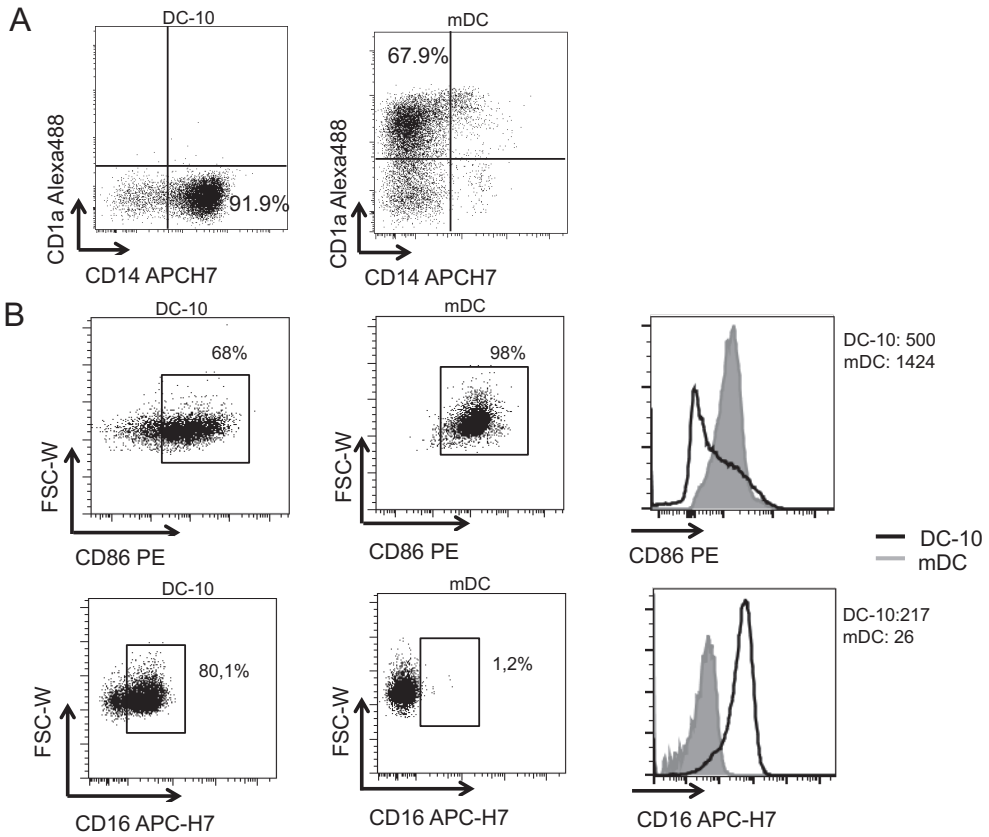

Figure 1. Phenotypic characterization of DC-10 and mDC generated from healthy control subjects. (A) Magnetically isolated CD14⁺ cells were cultured for 7 days in presence of rhIL-10 obtaining a population of IL-10 producing dendritic cells (DC-10) that retains CD14⁺ expression but lack the expression of CD1a *(one representative dot plot is shown out of 5)*. Mature dendritic cells (mDC) were cultured in absence of rhIL-10 and matured with LPS at day 5. They lose CD14 and acquire CD1a expression *(one representative dot plot is shown out of 5)*. (B) Further characterization shows that CD86 expression is higher in terms of frequency and median fluorescence intensity (MFI) on mDC than DC-10 *(one representative dot plot and histogram is shown out of 5)* and that DC-10 express high levels of CD16 while mDC are negative for this marker *(one representative dot plot and histogram is shown out of 5)*. Histograms show direct comparison of CD86 and CD16 on DC-10 (empty) and mDC (filled). Numbers indicate MFI of the tested marker.

The MLR/mDC culture is a proper reference to test donor-specific anergy

It is known that T cells isolated from patients on dialysis display reduced cell proliferation in vitro upon culture with allogeneic PBMCs (i.e., in primary MLR assays) ((25) and data not shown). Given that the proliferation of MLR/mDC is our reference value in the anergy assay, a possible explanation for the low anergic phenotype observed in the MLR/DC-10 cultures generated from patients on dialysis is that the MLR/mDC culture is an improper

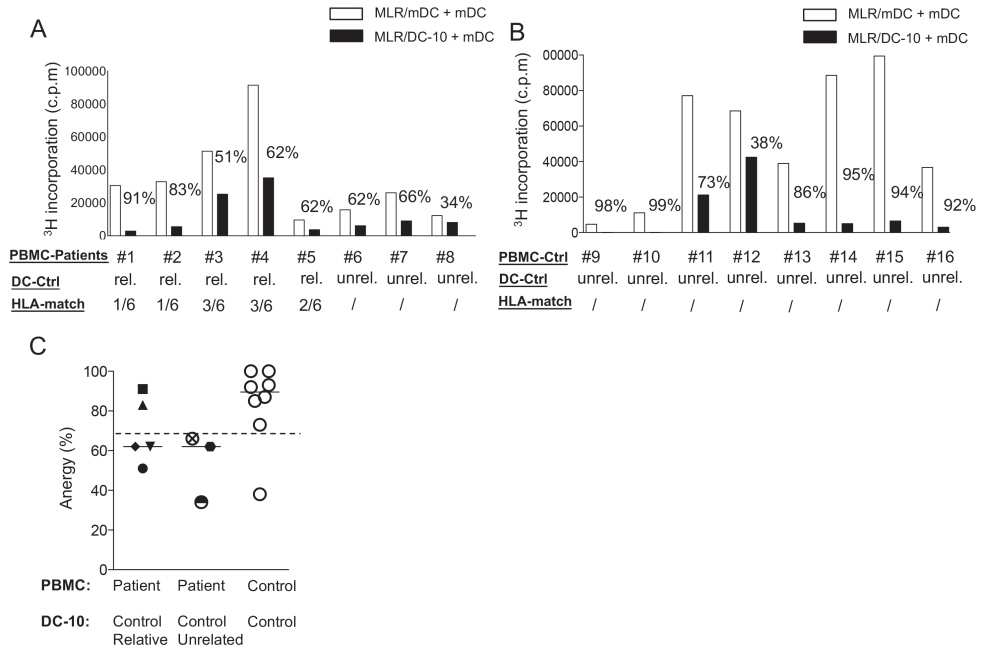

Figure 2. Anergic phenotype of MLR/DC-10 cell product generated from dialysis patients and healthy controls. (A) MLR/DC-10 obtained by co-culturing PBMC from the PB of dialysis patients with PB-derived DC-10 of healthy controls (n= 5 family related and n=3 unrelated donors) was re-stimulated with donor-derived mDC and cell proliferation was assessed by ^{3}H incorporation. MLR/mDC proliferation upon re-stimulation with donor-derived mDC was used as a reference value for cell proliferation. The anergy value is shown as percentage on top of the bar graphs. Patients and controls characteristics are listed in Table 1. (B) MLR/DC-10 and MLR/mDC were generated by using DCs coming from buffy coats of healthy controls and PBMCs from the PB of unrelated healthy subjects. The anergy value is shown as a percentage on top of the bar graphs. Donor characteristics are not available. (C) Anergy values of MLR/DC-10 products obtained from patients on dialysis (by co-culture with DC-10) and healthy controls are shown. The dotted line is set at 67%, which is the cut-off value to consider the cell product safe for infusion into patients. Each symbol represents one patient. Control subjects were tested in parallel. Lines represent median value of each data-set (*ns*, Mann-Whitney-U test). *C.p.m: count per minute; MLR: mixed lymphocytes reaction; mDC: mature dendritic cells; DC-10: IL-10-producing dendritic cells; PBMC: peripheral blood mononuclear cells; Ctrl: healthy controls; rel: relative; unrel: unrelated donor; HLA-match: human leukocyte antigen matching.*

cell culture reference. Interestingly, both IFN-γ levels measured in the culture supernatants (Figure 3A) and cell proliferation (Figure 3B), were similar in MLR/mDC cultures generated from patients on dialysis and from control subjects. This data suggests that the MLR/mDC is a proper reference culture to test the MLR/DC-10 culture responsiveness.

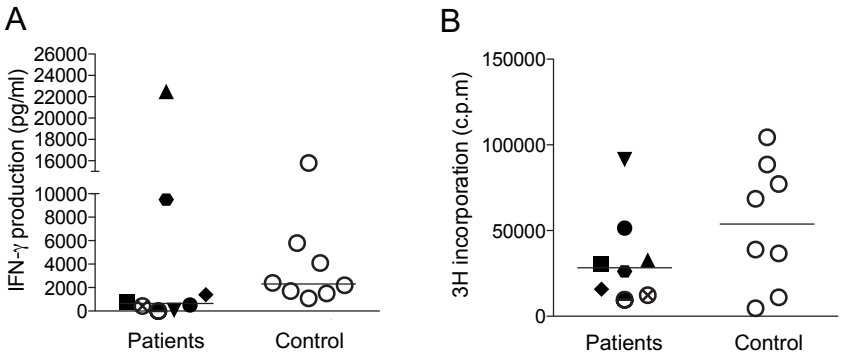

Figure 3. Characterization of the activation status of the MLR/mDC reference product. MLR/mDC products obtained from dialysis patients and healthy controls were re-stimulated with donor-derived mDC. After 48-hour stimulation, IFN-γ levels in the supernatant (A) and cell proliferation by ^{3}H incorporation (B) were assessed. Each symbol represents one patient (listed in Table 1). Control subjects were tested in parallel. Lines represent median value of each data-set (*ns*, Mann-Whitney-U test).

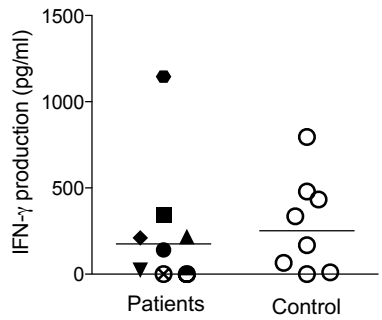

Figure 4. IFN-γ release from the MLR/DC-10 products. After 10-day co-culture, MLR/DC-10 were re-stimulated with mDC of the original donors and IFN-γ levels were tested in the supernatant obtained from patients on dialysis and healthy controls were assessed. Each symbol represents one patient (listed in Table 1). MLR/DC-10 from control subjects were tested in parallel. Lines represent median value of each data-set (*ns*, Mann-Whitney-U test).

The low anergic phenotype of MLR/DC-10 cultures from patients on dialysis is not due to the enrichment of IFN-γ–producing cells

It is known that PBMC from patients on dialysis produce more pro-inflammatory cytokines, such as IL-2 (25, 34) and IL-12 (35). Thus, we tested whether the reduced anergic phenotype detected in MLR/DC-10 cultures generated from patients on dialysis was due to enrichment in IFN-γ–producing cells during the 10-day co-cultures. IFN-γ levels measured in the culture supernatants were similar in patients on dialysis and control subjects (Figure 4), thus suggesting that the reduced anergy observed was likely not due to enrichment in IFN-γ–producing cells.

Development of a novel protocol for Tr1-cell enriched medicinal product tailored on patients on dialysis

An alternative explanation for the low MLR/DC-10 anergic phenotype observed when cells from patients on dialysis were used is the presence, in the starting PBMC, of cells that could hamper the *in vitro* differentiation of $CD4^+$ T cells into Tr1 cells. To test this, we set up a new protocol in which purified $CD4^+$ T cells (rather than total PBMC) from the peripheral blood of patients on dialysis or buffy coats from control subjects were cultured with DC-10 generated from buffy coats of control unrelated subjects in the presence of exogenous IL-10 to generate anergic Tr1 cell-enriched cultures (named

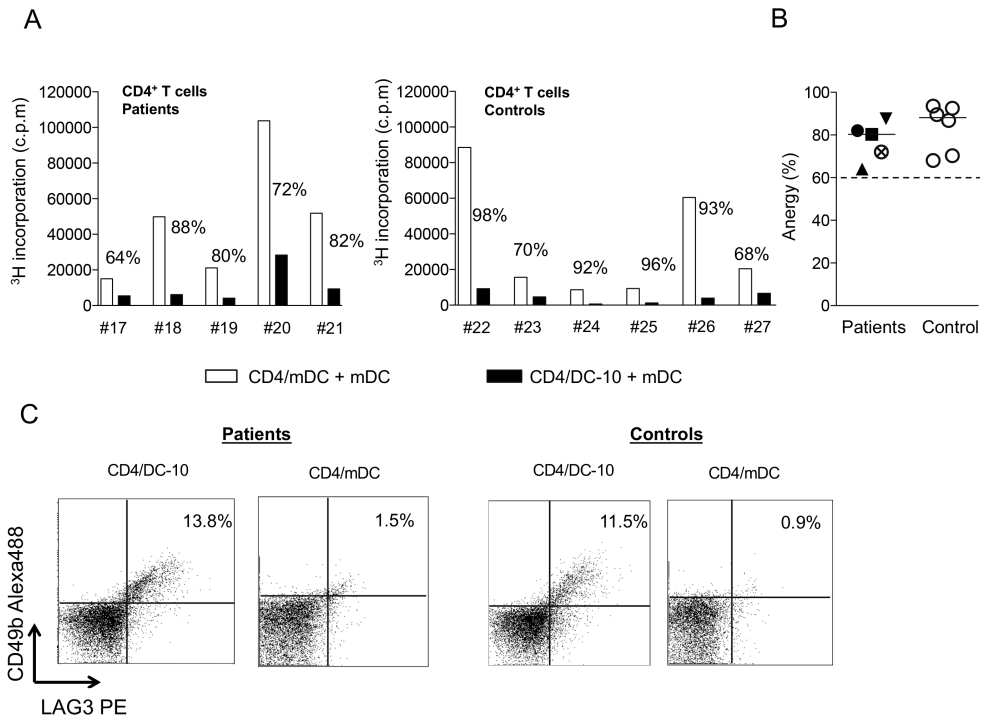

Figure 5. Characterization of the CD4/DC-10 cell products generated from patients on dialysis and healthy controls. (A) CD4/DC-10 obtained by co-culturing $CD4^+$ T cells from patients on dialysis (left) or healthy controls (right) with healthy-control–derived DC-10 were re-stimulated with donor-derived mDC and cell proliferation was assessed by ^3H incorporation. CD4/mDC proliferation upon re-stimulation with donor-derived mDC was used as reference value of proliferation. The anergy value is shown as percentage on top of the bar graphs. (B) Anergy values of CD4/DC-10 products obtained from dialysis patients and healthy controls are shown (*ns*, Mann-Whitney-U test). The dotted line is set at ~~67%~~ 60%, cut-off value to consider the CD4/DC-10 cell product safe for infusion into patients. (C) Tr1 cell enrichment in the CD4/DC10 generated from patients on dialysis and healthy controls is shown as LAG-3^+CD49b^+ cells on $CD4^+$CD45RA^- cells in both CD4/DC-10 and CD4/mDC cell product. One representative dot plot out of 3 is shown. *C.p.m: count per minute; mDC: mature dendritic cells; DC-10: IL-10-producing dendritic cells; Ctrl: healthy controls.*

Table 2. Characteristics of patients used as responders for the generation of CD4/DC-10 product shown in Figure 5A. Data are median (interquartile range).

	Patients (n=5)	Symbol
Age (yrs)	45 (26-48)	
Sex (M)	3/5	
Time of dialysis (yrs)	2.5 (4)	
Cause of CKD	Hypertension (n=1) SLE (n=1) PKD (n=1) T1D (n=1) Unknown (1)	● ■ ▲ ⊗ ▼
Dialysis modality	HD: n=4 PD: n=1	 ⊗

CKD: chronic kidney disease; SLE: systemic lupus eritematosus; PKD: Polycystic kidney disease; T1D: type 1 diabetes; HD: hemodialysis; PD: peritoneal dialysis

CD4/DC-10). In parallel, the control culture was obtained by co-culturing $CD4^+$ T cells with mDC generated from buffy coats of control subjects (named CD4/mDC). Characteristics of this group of patients on dialysis used to generate data shown in Figure 5 are listed in Table 2.

The set-up of the CD4/DC-10 protocol performed on healthy control subjects confirmed that 1:10 DC-10:$CD4^+$ cell ratio was optimal to obtain an anergic cell product and reproducible results (data not shown). In addition, the anergy cut-off value of the CD4/DC-10 product optimized on healthy control subjects was set at 60% (data not shown). The CD4/DC-10 cell product was then tested for final cell composition and it was confirmed to be purely composed of $CD4^+$ T cells (data not shown). The anergy assay was performed by re-stimulating CD4/DC-10 with donor-derived mDC and by comparing their proliferative capacity to that of CD4/mDC re-stimulated with the mDC of the same donors. This modified protocol led to the generation of a CD4/DC-10 product from patients on dialysis equally-anergic to that obtained from control subjects (Figure 5A-B).

The Tr1-cell content in the CD4/DC-10 cultures was tested by measuring the frequency of $CD4^+CD45RA^-LAG\text{-}3^+CD49b^+$ T cells, according to the surface markers recently identified (36). An average frequency of 10% of Tr1 cells in the CD4/DC-10 cultures was observed - while approximately only 1% of Tr1 cells in the CD4/mDC cultures - irrespective of whether obtained from patients on dialysis or controls (Figure 5C for one representative plot). This data indicates that culturing $CD4^+$ T cells from patients on dialysis with DC-10 generated from healthy control subjects in the presence of exogenous IL-10 is an efficient method for the generation of Tr1-cell enriched medicinal product to be used in cell therapy trials.

DISCUSSION

Our results show that anergic donor-specific Tr1-cell–enriched medicinal products for cell therapy application in the context of The ONE Study cannot be generated from dialysis patients using the MLR/DC-10 protocol previously developed by our group (32). Thus, we set up a novel protocol by using purified $CD4^+$ T cells instead of total PBMC. This change led to the generation of a cell product suitable for cell therapy in patients with kidney failure.

The immune system of patients on dialysis has been extensively characterized and it is currently recognized to be altered. Patients on renal replacement therapy are characterized by a pro-inflammatory peripheral status (21, 25, 37). Nonetheless, T-cell responsiveness to *in vitro* alloantigen stimulation is impaired (23). Given the well-known immunological alterations occurring in patients on dialysis, as a pre-requisite for the initiation of cell therapy arm of The ONE Study clinical trial (17), we tested whether the generation of anergic donor-specific Tr1-cell enriched lymphocytes was feasible in these patients.

To date, only one group reported the generation of Tr1-cell enriched products from patients on dialysis (29). However, in this study, the cell product was generated by using iDC rather than DC-10 as donor-derived cells (29). It is now well accepted that DC-10 are more effective than iDC for Tr1-cell induction (31). In addition, despite the fact that IL-10 production was shown to be alloantigen-specific, no assessment of the cell product anergic phenotype (fundamental requirement for safety concerns) or Tr1-cell frequency was reported. Thus, there was a strong need for the development of a clinical-grade protocol for the generation of donor specific Tr1 cells from patients on dialysis.

The protocol previously developed by our group (32), which envisaged the use of total PBMC as starting cells, did not lead to the generation of a clinical-grade product. A Tr1-cell enriched product endowed with a low anergic phenotype, indeed, represents a possible risk for the recipient due to the potential proliferation *in vivo* upon encountering donor antigens after kidney transplantation. The reduced ability to generate anergic Tr1-cell enriched products from patients on dialysis with the MLR/DC-10 protocol might be due to the use of an improper reference for anergy assessment (i.e. MLR/mDC proliferation). Indeed, the well-known T cell hyporesponsiveness of dialysis patients towards alloantigens in a primary MLR (25) might also affect their responsiveness in a secondary MLR. However, our data indicate that both cell proliferation of and IFN-γ production by MLR/mDC cell product re-stimulated with mDC in a secondary MLR were not different from those in healthy donors, making the MLR/mDC-proliferation a reliable reference value for defining MLR/DC-10 anergy. In addition, given the alteration of the immune system of patients on dialysis featured by the increase of pro-inflammatory cytokines such as IL-2 and IL-12 (25, 34, 35), we excluded that the generation of highly anergic Tr1 cells was hampered by an increased IFN-γ production in the MLR/DC-10 product. Thus, we hypothesized that a pro-inflammatory cell fraction residing in the bulk PBMC population of patients on dialysis would hamper Tr1-cell enriched product generation. DC from patients on dialysis have for instance been described to induce high proliferation of T cells from healthy

subjects (23). Moreover, monocytes produce high levels of pro-inflammatory cytokines upon stimulation (20). Thus, $CD4^+$ T cells of patients on dialysis were purified and co-cultured with donor-derived DC-10 in the CD4/DC-10 protocol. Thanks to this novel approach, we were able to obtain a medicinal product with the following characteristics: (i) highly anergic towards donor-derived mDC; (ii) enriched in Tr1 cells (around 10%); and (iii) purely composed of $CD4^+$ T cells (whereas the MLR/DC-10 product was contaminated by a small fraction of $CD8^+$ T cells and NK cells, data not shown). Overall, the CD4/DC-10 cell product is endowed with the characteristics of safety that are required for clinical application.

Given the overwhelming uremia, continuous exposure to the dialysis filter membrane and nutritional deficiencies, the immune system of dialysis patients lays in a pro-inflammatory status. This might affect the *in vitro* generation of Tr1-cell enriched lymphocytes, rendering the MLR/DC-10 protocol not suitable for patients on dialysis, contrary to what observed in patients with hematological cancer disease (15). One explanation for the different ability to generate clinical grade Tr1-cell enriched product between patients on dialysis and those with hematological cancer (15) may resides in the fact that, in the ALT-TEN study, T cells were collected from healthy donors and DC were generated from patients in the aim to prevent GvHD (15). It might be possible that the generation of DC is less "patient-dependent" than is that of Tr1 cells for patients on dialysis.

The major limitation of our study is the inclusion of patients with a variety of primary and secondary kidney diseases that eventually all lead to renal replacement therapy. The limited number of patients enrolled in our study did not allow a direct correlation between disease etiology and lack of anergy in the MLR/DC10 products. Further studies enlarging the patient population to stratify for disease etiology are required to get hints on the mechanisms underlying the failure of Tr1-cell generation upon usage of total PBMCs.

A relevant point of discussion is the in vivo effect of immunosuppressive drugs used in The ONE Study (i.e mycophenolate mofetil and FK506) on Tr1 cells. Although data is too preliminary to draw definitive conclusions, immune monitoring performed so far on patients enrolled in the reference group trial of The ONE Study, shows no negative effect of the abovementioned immunosuppressive drugs on Tr1 cells (data not shown).

An additional important issue is the number of Tr1 cells needed to be transferred to generate donor-specific tolerance. Data in preclinical models of islet transplantation suggests that $2x10^6$ of $CD4^+$ T cells, containing an average 10% Tr1 cells, induce graft tolerance in immunocompetent mice in the absence of active immunosuppression (10). Direct translation from mouse to human is hard but one could anticipate that 10% of Tr1 cells in the final medicinal product should be enough to induce tolerance. Only data from clinical trials will hopefully elucidate this important matter.

To conclude, we want to emphasize the necessity of carefully screening the immune system of patients before enrolling in cell-therapy–based clinical trials. We believe in

the relevance of setting up disease-tailored protocols aiming at minimizing possible negative outcomes and finally safeguarding patient health.

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Chapter 7

DEVELOPING TRANSLATIONAL MEDICINE PROFESSIONALS: MARIE SKŁODOWSKA-CURIE ACTION MODEL

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ABSTRACT

End goal of Translational Medicine is to combine disciplines and expertise to eventually promote improvement of the global healthcare system by delivering effective therapies to individuals and society. Well-trained experts of the Translational Medicine process endowed with profound knowledge of biomedical technology, ethical and clinical issues, as well as leadership and teamwork abilities are essential for the effective development of tangible therapeutic products for patients. In this article we focus on education and, in particular, we discuss how programs providing training on the broad spectrum of the Translational Medicine continuum have still a limited degree of diffusion and do not provide professional support and mentorship in the long-term, resulting in the lack of well established Professionals of Translational Medicine (TMPs) in the scientific community. Here, we describe the Marie Skłodowska Curie Actions program ITN-EUtrain (EUropean Translational tRaining for Autoimmunity & Immune manipulation Network) where training on the Translational Medicine machinery was integrated with education on professional and personal skills, mentoring, and a long-lasting network of TMPs.

THE NEED FOR TRANSLATIONAL MEDICINE PROFESSIONALS (TMPS)

The 20th century has been revolutionary in terms of biomedical discoveries and innovations in technologies, e.g. high-throughput screenings and omics. The large amount of knowledge so derived has great potential to prolong human life and enhance its quality. However, there is considerable concern of an insufficient translation of this knowledge into tangible products with clear clinical impact (1). This gap in the translational process is described as the valley of death. The valley represents the challenges that clinicians and scientists are facing in the process of developing effective therapies for patients, resulting in only a small fraction of all efficacious pharmacological agents in preclinical models being licenced after Phase III clinical testing (2). Possible explanations are: (i) animal models do not correctly represent human diseases due to significant differences in biology (3), (ii) overestimation of drug effects in preclinical models due to the higher probability of positive results to be published (4), (iii) limited well-designed clinical trials in the era of precision medicine (5) and (iv) irreproducibility of scientific findings (6), which is only to a small extent caused by scientific misconduct (7). Despite translational medicine is a priority for the scientific community, professional figures specifically trained to facilitate the complex processes of the translational medicine continuum remain scarce. Translation requires a bi-directional effort starting from the identification of clinical needs and ending with the development of technology/products to be brought back into the clinic. Lack of translation results in waste of public money and resources, delay in scientific progress, and therefore, loss of potential benefits for human health. One proposed solution to bridge the gap between research and clinical care is the use of an educational model based on training and professional growth to foster the necessary skills required for the development of experts of the translational medicine process (i.e. TMPs). TMPs are expert in the translational medicine itinerary and are equipped to act as interfaces supporting efficient communication among different parties of the process, e.g. clinicians, regulatory affairs, companies, basic researchers.

NEED FOR AN EDUCATIONAL SHIFT IN THE TRAINING OF YOUNG PROFESSIONALS

Dynamic change within biomedical research and healthcare requires a synchronous development of learning environments and opportunities to enhance the knowledge and skills of young professionals. Just like the acknowledged need for a shift in the general educational system gave rise to the P21 (8), a collaborative partnership among education, business, community and government leaders, the accelerated rate of changes in Translational Medicine requires a similar collaborative effort. The aims should be to train and support a community of TMPs to deal with the need of producing tangible products for the treatment of human diseases. Despite there being many excellent scientists and clinicians as well as experts of regulatory affairs and industry, professionals that can oversee the entire translational process as well as shared platforms where those experts can easily connect and access each other's information and skills are still scarce.

What educational innovations are required to positively impact on the translational process? First, there is the need to set up educational programs fostering the development of TMPs with multidimensional skills (ranging from specific competences on the translational process to communication, coaching, creative thinking, problem solving, management). Secondly, TMPs should avoid the "silo" mentality, a narrow outlook that has contributed to low efficiency and failure in science, but rather break down barriers and cross boundaries by sharing information and knowledge with other scientists, healthcare providers and industry (9).

EXISTING EDUCATIONAL PROGRAMS IN TRANSLATIONAL MEDICINE

Educational programs providing training, at different levels, related to stages of the therapeutic development pipeline, and addressing the needs of individuals with distinct roles in translational research (i.e. government, industry and academia) are available. Examples are the Clinical Translational Science (CTS) programs developed in the USA for graduate and postgraduate students (10), and the Canadian Child Health Clinician Scientist Program (CCHCSP), providing training for clinical researchers to develop knowledge and skills for a career as an independent scientist in child health research (11). These programs support the development of the translational scientist identity by offering a comprehensive training on both 'foundational' skills, such as research methodologies and data management, and 'functional' skills, such as communication, ethics, leadership and teamwork (12). However, these programs have a limited degree of diffusion and are not structured to provide peer support and mentorship in the long-term. The latter point, in our opinion, is an essential requirement to sustain group identity, and provide professional guidance as well as concrete help to access information or other types of resources related to Translational Medicine.

Another approach to cross the valley of death is to develop a community of TMPs with different skills and expertise that can be shared and utilized by all members. The Eureka Institute (13) founded in 2007, is an example of such a community. Currently, it comprises a worldwide network of over 200 fellows trained in interdisciplinary teamwork, open-minded thinking and principles of Translational Medicine (e.g. biology, intellectual property, funding, regulation and trial design) during a week-long course held in Siracuse (Sicily, Italy). Teaching comprises interactive sessions led by experts in the process of translational research, including scientific directors and deans of academic institutes, editors of high-impact journals, industry, and representatives of patient organizations. Fellows are trained in leadership, teamwork and communication to different types of audience as well as ethical and technical principles of the Translational Medicine machinery. To maximize the learning experience, the training technique requires students to work together to design strategies and solutions to real-life issues previously encountered by experienced TMPs. This network of translational researchers is growing, meetings and seminars are routinely scheduled, news in the translational medicine field are shared on social

networks and a database is currently under development including participant expertise and tools as well as a section to share ideas for the development of joint projects. and the The challenge now lies in its long-term sustainability eventually providing TMPs access to continuously updated knowledge and tools.

THE MARIE SKŁODOWSKA-CURIE ITN MODEL

European Union Marie Skłodowska-Curie Actions, named after the double Nobel Prize winning scientist for her work on radioactivity, support researchers working across all disciplines, including industrial and academic research studies, giving the researcher the possibility to gain experience abroad and develop competences useful to enhance future careers. This program provides a remarkable tool, the Innovative Training Network (ITN) program, allowing early-stage researchers to receive doctoral training and develop transferable skills by working on joint research projects within a worldwide network. One such ITN, EUtrain (EUropean Translational tRaining for Autoimmunity & Immune manipulation Network, http://www.eutrain-network.eu), was funded in 2011 and aimed at training a pool of young investigators in Translational Medicine providing (i) education on professional and personal skills, (ii) mentoring, and (iii) long-lasting network of TMPs.

EUtrain focused on patient-centred research with respect to diagnosis, prognosis and/or treatment of immune-mediated diseases. Participating fellows had diverse backgrounds (i.e. basic scientists, biotechnologists, medical doctors, chemists and engineers) and conducted 3-year research training in one of the 10 participating academic institutes based in the Netherlands, Italy, Germany, United Kingdom and France, or companies (Esaote Spa, Italy; Cavadis BV, The Netherlands; Proteros Biostructures GmbH, Germany). Identification of clinical needs provided the basis to motivate generation of translational solutions. EUtrain partners performed joint research projects combining basic immune biology studies on the regulation of inflammation with the development of novel high throughput and imaging technologies to detect biological markers of disease, and integrate this with clinical studies and systems biology. As an example, Work Package (WP) 2 aimed to identify both conventional and innovative biological markers for monitoring disease activity, progression, response to therapy and prognosis. WP2 included 4 joint research projects held by 4 early-stage researchers located in academia and industry, consisting of 1) develop and validate commercial assays for quantification of biomarkers of inflammation, 2) perform a global proteomic screening for novel protein biomarkers in blood of children with inflammatory diseases, 3) analyze the bias towards tolerance or inflammation based on functional and molecular analysis of the relevant pathways, and 4) develop a biomarker set that may predict the long-term risk of cardiovascular complications.

Besides the Eureka experience, trainees received additional training in specific aspects of the translational process from the conception of an idea to clinical testing and, ultimately, the development of a treatment for patients. Additionally, EUtrain fellows

were coached on professional skills, such as communication to a scientific and layman audience, collaboration, conflict solving, teamwork, and mentor/mentee relationships. EUtrain fellows experienced network-wide training events, such as the grant writing weekend, workshops on presentation skills, effective communication, moderation of scientific meetings, valorisation of results and intellectual property (IP).

Core of the training was mentorship on career and personal development, which was performed on a regular basis by experienced TMPs. Each career step requires the identification of an experienced and knowledgeable figure providing guidance and advice on work, career, or professional development (i.e. mentor); on several occasions during the training it was raised awareness on the importance of the identification of a mentor to fulfill the needs of one's life and career goals. Fellows met regularly in informal settings and were repeatedly exposed to these sessions and principles at different occasions during the 3-year training.

Here we provide a summary of the themes that emerged from the survey proposed to EUtrain fellows to determine whether and how EUtrain helped to achieve their professional goals. All survey respondents (13/14) agreed that the program enhanced their basic and translational knowledge, and enjoyed the integrated experience where network and training merged, allowing both scientific and personal growth. In particular, they all agreed that EUtrain helped creating a solid and long-lasting European-wide professional network as well as developing multidimensional skills. Thanks to EUtrain, fellows improved their technical competences and used them to answer clinical questions, got insights into the clinical practice, understood dynamics and objectives of academic and industrial research. Despite the overall achievement of goals, one fellow would have liked to have more time for training in personal and professional development, which was instead incompatible with lab duties. Notably, most of the fellows would not consider themselves independent researchers or TMPs yet at the end of the training, which might be due to their early career stage. It would be of interest to follow-up the experiences of these individuals, and to explore the effects of a similar type of training on more senior investigators. It is impossible to evaluate at this stage whether this training program will significantly improve the future development of clinically useful products. However, all EUtrain fellows have acquired the essential tools to be able to contribute to this goal. Future studies need to examine career outcomes to identity program components that effectively equip trainees with the skills needed to thrive in a translational environment.

CONCLUSIONS

Fragmented expertise in the translational research process and lack of platforms designed to share knowledge and tools among professionals are some of the barriers to the development of tangible products with clinical impact. Only specifically trained professionals such as TMPs can facilitate research to cross the valley of death. The proposed approach to fill the existing gap in Translational Medicine consists in the integration of

education within a network of professionals, allowing the sharing of skills and promoting collaborative efforts. This would also allow TMPs to access more or newer research tools and keep up to date on cutting-edge technologies and information provided by a large network. EUtrain fellows are now part of an international network (i.e. Eureka) from which they can obtain interdisciplinary and intersectoral support as well as identify a mentor for career guidance to ultimately attain their translational purposes. To close the translational gap there is urgent need of multidimensional TMPs endowed with creative and open-minded thinking, able to work in cross-functional teams, and embedded in a solid community of peers providing career guidance, theoretical framework and technical support.

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AUTHORS' CONTRIBUTIONS

AP, BJP and NDR equally contributed to the conception, design and writing of the manuscript. EUtrain fellows are listed in alphabetical order in the acknowledgement section and equally contributed in revising the manuscript.

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Chapter 8

GENERAL DISCUSSION

MECHANISMS UNDERLYING CHRONIC INFLAMMATION: FOCUS ON THE LOCAL MICROENVIRONMENT

T cell abundance, phenotype and mechanisms employed to exert their functions are influenced by the nature of the antigen (specificity) as well as the location (environment). During inflammation, T cells adapt to the environment, develop specific features and employ distinct mechanisms of action depending on the context of inflammatory response. Distinct sets of cytokines displayed by T cells in turn promote matched effector functions of other cells of the innate and adaptive arms of the immune system. Thus, T cell subsets serve not only as effectors, but also as *amplifiers* of an appropriate inflammatory response, which ultimately leads to antigen(s) clearance or sequestration (1). This general principle is now well established in the context of infectious diseases and based on a large body of experimental evidence indicating that naive CD4 T cells differentiate into different T helper effector subsets depending on the location and the nature of the trigger. For instance, T cells differentiate into IFN-γ– and TNF-α–producing effector TH1 cells during infection with a virus or intracellular bacteria; similarly, IL-4–, IL-5–, and IL-13– producing TH2 cells and IL-17–producing TH17 cells are elicited during infection with helminths and with extracellular bacteria and yeast respectively (2). With a similar mechanism, also Tregs use different suppressive mechanisms under homeostatic and inflammatory conditions (3). Indeed, while in steady state Treg only need to deprive responder T cells from activation signals, in highly inflammatory environments, activated Tregs need to adopt alternative mechanisms to suppress ongoing inflammation by acquiring cytotoxic properties and actively secreting suppressive molecules to inactivate effector T cells and antigen-presenting cells (3). For instance, in preclinical models, expression of T-bet in Tregs enables them to migrate and proliferate as well as accumulate at the sites of TH1 responses, and Tregs lacking T-bet expression were selectively impaired in regulation of TH1, but not TH2 or TH17, responses (4). This process is called *functional specialization*.

Human chronic inflammatory diseases display a-specific signs of inflammation in the periphery (5, 6). However, despite the relevance that these signs might have for diagnosis, follow up and prognosis, assessment of the immunological profile in the peripheral blood may provide only a partial picture of the mechanisms engaged and sustaining chronic inflammation. Such crucial information can only be obtained by investigating the target site of inflammation. However, these sites are extremely difficult to study in humans due to the hurdles to collect human tissues in physiology and disease.

In this thesis, as a model to investigate tissue-instructed T cell functional specialization we used the target site of inflammation of Juvenile Idiopathic Arthritis – JIA - (i.e. the synovium –SF-), a disease characterized by an aberrant immune response towards self-antigens leading to recurrence and severe tissue damage in the long term (7). In Chapter 3 we describe how CD4 and CD8 T cells can be affected in their functional properties once, from the periphery, they reach the target site of inflammation of autoimmune arthritis. It is now consolidated that FoxP3-Treg show an altered phenotype

in SF (i.e. elevated FoxP3 and other markers of functional Treg such as CTLA-4 and GITR) but, similarly to the periphery of the same patients, still retain suppressive properties (8). Effector T cells dramatically change their phenotype, showing a clear and consistent skewing towards the TH1 profile (9), which was confirmed by the open gene array shown in **Chapter 5**. Importantly, effector T cells display altered functional properties, such as inability to be regulated by Treg-dependent and independent mechanisms, rendering this subset highly harmful in this context (8). Still unclear was whether CD4 and CD8 T cells, once reached the synovium, are imprinted to this resistance or rather this is induced by the interplay with other cell subsets. In Chapter 3 we showed that CD8 T cells are intrinsically resistant to suppression (i.e. locally imprinted to resistance and maintaining this phenotype regardless the presence of other cell populations). Interestingly, CD8 T cells only in part share the mechanisms with CD4 T cells. Indeed, while TNF-α blockade was able to rescue both CD8 and CD4 T cell resistance, autocrine release of IFN-γ selectively sustained CD8 T cell resistance, which could be relieved by IFN-γ blockade. This data indicate that different mechanisms are adopted by different cell subsets to escape regulation, which appears extremely relevant for the proper design of therapeutic strategies in this context.

To date, most of the research focus is on CD4 T cells. However, CD8 T cells have all the features to be crucial players at the target site of inflammation, showing a prevalent effector memory phenotype and a mixed pro- and anti-inflammatory profile (reviewed in **Chapter 2**). In **Chapter 4** we showed that, in apparent contrast with the activated/effector phenotype of CD8 T cells expected in SF, this cell subset displays elevated expression of negative co-stimulatory markers, such as PD-1. PD-1, together with the expression of other inhibitory receptors, is described as a marker of 'exhausted' T cells meaning T cells that, upon chronic exposure to inflammation, have lost their functional properties (10, 11). In Chapter 4 we in depth describe the PD-1-expressing CD8 T cell subset, which appeared highly pro-inflammatory/cytotoxic at a transcriptome and protein level, metabolically active, and showed high clonality, suggesting its enrichment in antigen-specific cells. This is in line with recently developed evidences showing that PD-1-expressing CD8 T cells are a specific subset induced in chronic environments and, more than pure loss of function, PD-1 expression is a sign of functional adaptation to the chronically inflamed milieu (10). Indeed, PD-1 identifies a subset of antigen-experienced cells, generated upon stimulation by specific triggers and well adapted to the local environment. Additionally, growing data indicate that low-level antigen exposure promotes the formation of T cells with an acute phenotype in chronic infections (12) and that PD-1$^+$ T cells are enriched with high affinity antigen-specific cells (13, 14). In support of this hypothesis, we provided evidence that this subset is also present in inflamed sites of different human chronic inflammatory diseases such as in the lesional skin of patients with atopic dermatitis and the inflamed gut of patients with inflammatory bowel diseases (IBD). Data from PD1 conditional knock out mice suggest that PD-1 attenuate activation signals and thereby prevent terminal

differentiation and apoptosis (15). This may indeed occur in SF, where PD-1 (and other inhibitory receptors) could contribute to prolonged survival by preventing from "over-activation". PD-1 elevated levels at the target sites of inflammation may be an attempt of the immune system to shut down inflammation. PD-1 ligation prevents phosphorylation of AKT/PKB signaling pathway (16); however, we have shown previously, that T cells from SF show increased pAKT/PKB (8) suggesting that PD-1 is not a sufficient signal to restrain the immune response. A possible explanation for PD-1 impaired signaling might be the absence of available ligands (i.e. PDL-1 and PDL-2) in SF. However, the expression of both ligands is increased on SF-derived antigen presenting cells (personal communication, Boltjes). Therefore, as recently shown by Liu et al. (17), functional antagonism of PD-1 molecules mediated by soluble factors (e.g. soluble PD-1, sPD-1) may occur in SF.

Interestingly, we showed that PD-1-expressing CD8 T cells are enriched with the signature of the so-called tissue resident memory cells (TRM). Growing preclinical data show that TRM cells are responsible for the immunosurvellaince of tissues, being quiescent memory cells highly potent in protecting from re-exposure to the antigen they are specific for (18, 19). However, scant data are available in humans and, in particular, in the setting of chronic inflammatory diseases. In cadaveric donors, tissue-intrinsic compartmentalization of naive, effector and memory subsets are conserved between diverse individuals and CD69 allows the identification of tissue-resident and circulating T cells (20). In mice, Hobit has been identified as regulator of the TRM cell program shared by different tissues after viral infection (21). However, in humans, Hobit was found to be also elevated in peripheral CD8 T cells and NK cells of healthy donors (22). Our preliminary data show that Hobit is not exclusively expressed by PD-1$^+$ CD8 T cells from SF and is overall much lower in SF compared to PB. This suggests that Hobit is not a TRM-specific transcription factor in humans and that its expression is likely affected by the environment as well as antigen-specific stimulation. Interestingly, quite a large proportion of PD-1$^-$ cells express markers of tissue residency (i.e. CD69), while the largest proportion of PD1$^+$ cells were CD69$^+$CD103$^+$, with extremely few CD69$^+$CD103$^-$ cells observed within this subset. It's unknown what the functional differences of these two populations are. In the skin of patients undergoing anti-CD52 treatment for the depletion of circulating T cells, CD69$^+$CD103$^+$ cells showed less proliferative capacity but higher effector properties compared to CD69$^+$CD103$^-$ cells (23).

To conclude our data suggest that PD-1-expressing CD8 T cells are (auto)antigen-specific cells imprinted to stably reside at the target site of inflammation. Of note, PD-1$^+$CD69$^+$ CD8 T cells were found enriched in the inflamed gut mucosa from IBD patients compared to non-inflamed mucosa, suggesting that this subset has the potential to bear the local inflammatory response in chronic inflamed tissues thus playing a role in disease pathology. Despite further research should be made to dissect the transcriptional profile and the functional properties of TRM cells in human physiological and pathological conditions, our data clearly indicate that PD1$^+$ CD8 T cells are not exhausted but rather

show functional adaptation to the local milieu. However, it's still unclear why PD-1^{+} positive are enriched for tissue residency compared to PD-1^{-}. It might be that chronic antigen stimulation and inflammation play a role in driving the tissue-residency program, or at least upregulation of retention molecules.

In **Chapter 5** we attempted to elucidate the mechanisms of induction and the relative contribution of cytokines to the development of the TH1 signature observed in SF (24). IFN-γ is known to play a role in the pathology of autoimmune diseases by promoting development of TH1 cells via a positive feedback, stimulating B cell maturation and isotype switch, increasing MHC-I and MHC-II expression on APC, increasing expression of adhesion molecules on endothelial cells (25). Despite IFN-γ levels in SF of patients affected by autoimmune arthritis are low as compared to other cytokines (26, 27), the TH1 axis is known to be well represented in this context (24). Therefore, we wondered what cytokine(s) contribute to the skewing of CD4 T cells into TH1 cells at this site. IL-12 is an interleukin that is naturally produced by dendritic cells, macrophages and neutrophils in response to antigenic stimulation. This cytokine is well known as inducer of the TH1 cell phenotype (28). The relatively newly discovered cytokine IL-27 was shown to activate STAT1 and T-bet to promote responsiveness to IL-12 and production of IFN-γ (29). However, when Il27ra–/– mice were used in models of tissue-specific autoimmunity (30, 31), IL-27 displayed regulatory properties by antagonizing TH1, TH2, and TH17 responses. In preclinical models of autoimmune arthritis, IL-27 was found to be either protective or accelerating disease onset based on the model used (32, 33). Therefore, it is difficult to reconcile inhibitory activities of IL-27 with its ability to promote T cell growth, survival, and effector T cell functions (34). In patients with Rheumatoid Arthritis (RA), contradictory data are reported on the levels of IL-27 in SF (35, 36). In JIA patients, we found 3-fold increase of IL-27 levels in SF plasma compared to PB. Of note, no differences were evident in IL-12 levels, which instead appeared overall very low at both sites. Our data show that a clear TH1 but not TH17 signature is evident in SF. IL-17-producing cells are known to be enriched in SF (37); however, they represent quite a small fraction of the TH cell compartment. In this context of elevated pro-inflammatory responses, IL-27 was expected to mitigate the TH1 effector functions in favor of prominent inhibitory activities. Instead, we found that IL-27 synergized with IL-12 to induce IFN-γ production in naïve CD4 T cells and contributed to the maintenance and stabilization of the TH1 signature observed in SF via the engagement of the STAT1 signaling pathway. In our setting, no IL-27-mediated increase of IL-10 was observed, which is probably due to the pre-existing commitment of CD4 T cells towards the TH1 lineage. Interestingly, IL-27 induces PD-L1 and PD-1 (38, 39) as well as an IFN-signature (40) in T cells implying a potential role of this cytokine in controlling PD-1-expressing T cell subsets and the regulation of local effectors of the immune response. Apparently, IL-27 effects are context-dependent and differences in the temporal and cellular commitment may dictate IL-27 biological relevance during immune responses. These effects may be shaped by the individual microenvironment and by the degree of on-going inflammation.

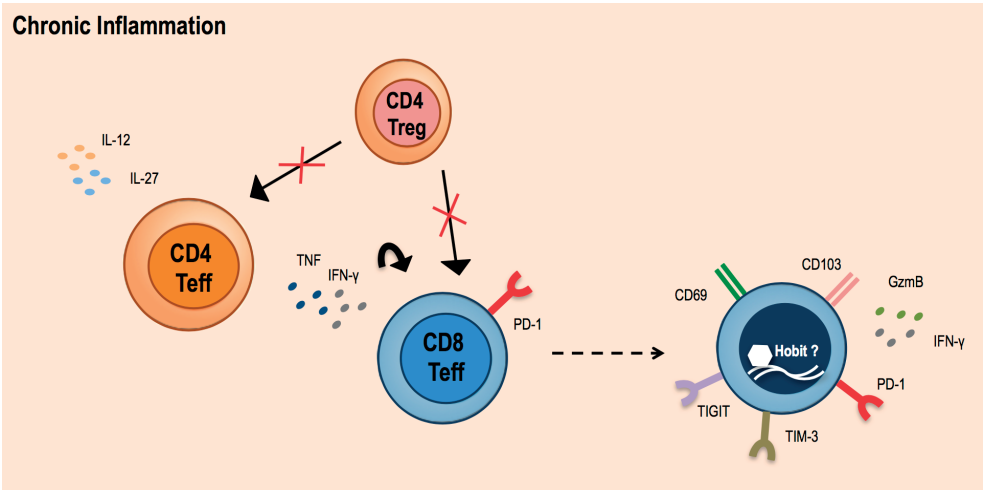

Figure 1. Interplay of different subsets of the adaptive immune system at the target site of inflammation. IL-12 and IL-27 (released by antigen presenting cells) induce an IFN-γ signature as well as the up-regulation of negative co-stimulatory markers such as PD-1. At the site of inflammation, CD8 T cells are imprinted to be resistant to Treg-mediated suppression and this is mediated by TNF and IFN-γ. PD-1-expressing CD8 T cells are enriched at this site and appear to be locally-adapted, antigen-experienced, and clonally expanding, sharing the profile of TRM cells (i.e. expression of CD69, CD103, GzmB, negative co-stimulatory markers; Hobit, transcription factor of mouse-derived TRM cells (21), is apparently not specific for human cells (22)).

In Figure 1 it is shown how different subsets of the adaptive immune system develop and interact at the target site of inflammation. Cytokines such as IL-12 and IL-27 (released by the innate compartment of the immune system) induce an IFN-γ signature in T cells as well as the up-regulation of negative co-stimulatory markers such as PD-1. At the site of inflammation, T cells are imprinted to be resistant to Treg-mediated suppression and, this lack of susceptibility to suppression, is constitutive and intrinsic, being unaffected by the presence of other cell subsets. Moreover, PD-1-expressing CD8 T cells are enriched and appear to be locally-adapted, antigen-experienced, and clonally expanding cells, sharing the profile with quiescent resident cells, which might be potentially reactivated during the flares. The features and mechanisms here described reflect the effects that microenvironment, together with specific triggers, specifically induce at the target site of inflammation, and do not apply to the peripheral blood. To identify targets and develop therapeutic strategies to halt disease pathology, a better understanding of the mechanisms underlying chronic inflammation at the site where the process is on-going are needed.

TREATMENT OF CHRONIC INFLAMMATORY DISEASES: TARGET COMMON MECHANISMS OR NEED FOR DISEASE-TAILORED APPROACHES?

Chronic inflammatory diseases share common immunological features at the target organ, which frequently are not in line with what is found in the peripheral blood. Elevated Treg cell frequency is evident in SF of autoimmune arthritis (8, 41), in the inflamed lamina propria as well as in basal lymphoid aggregates of patients with IBD (42-44), and in psoriatic lesional skin biopsies (45, 46). At the same time, TH1-related cytokines [e.g., tumor necrosis factor (TNF), interferon (IFN)-γ, interleukin (IL)-12] (47, 48) as well as TH17-associated cytokines (e.g., IL-17A, IL-21, IL-23) (49, 50) are markedly increased in inflamed mucosa of Crohn's disease (CD) patients. TH1 and TH17 cells are also elevated at the site of inflammation of autoimmune arthritis (9, 37), and TH17 cells are described as key master regulators of skin inflammatory diseases including psoriasis (51). These findings have been translated into the development of cytokine-targeting clinically available drugs. Indeed, antibodies anti-TNF are effective in autoimmune arthritis (52) and IBD (53), and antibodies targeting IL-17A or IL-17 receptor are remarkably effective in psoriasis trials (54), leading to the recommendation of secukinumab (anti-IL17A) as first-line therapy for patients with psoriasis. However, in surprising contrast, clinical trials of anti-IL-17A or anti-IL-17R antibodies in IBD show no improvement or even exacerbation of the disease (55). This is thought to be due to an alternative function of IL-17 in the gut mucosa, i.e. preserve the intestinal epithelial barrier (56). Moreover, secukinumab (57) and brodalumab (an anti-IL17R antibody), showed tiny or no evidence of a clinical effect in patients with RA. IL-23 is composed of an IL-12p40 subunit (that is shared with IL-12) and the IL-23p19 subunit. IL-23 promotes the expansion and maturation of inflammatory TH17 cells (58). Inhibitors for the p40 subunit have been considered as an important therapeutic target in chronic inflammatory diseases, because blocking IL-12 and IL-23 would prevent accumulation of both TH1 and TH17 cells. Ustekinumab is a p40 inhibitor, which has been successfully used in the treatment of IBD when anti-TNF therapy failed (59, 60). The effectiveness of anti-p40 compared to anti-IL17A treatment in IBD may be due to the fact the former still maintains local acceptable levels of IL-17, thus preserving mucosal integrity. In Chapter 3 we described that, in SF, CD4 and CD8 T cells have different mechanisms of induction of resistance to Treg-mediated suppression, showing that CD8 T cells are susceptible to both anti-IFN-γ and anti-TNF-α. This suggests that combinational treatments based on the inhibition of both cytokines may be beneficial in this disease. Anti-IFN-γ treatment has been tested in RA in a small clinical trial showing safety as well as similar effectiveness compared to anti-TNF-α (61). Even though inhibition of IFN-γ seems a logical interventional strategy in human chronic inflammatory diseases, little has been done to develop therapies targeting IFN-γ. This may stem from the fact that human deficiency of IFN-γ is associated with severe infection (62). Even so several efforts have and are being made to determine the effectiveness of anti-IFN-γ therapy. Fontolizumab,

a humanized monoclonal antibody against IFN-γ, was well tolerated and showed some efficacy in patients with Crohn's Disease (63). However, such non-specific targeting of IFN-γ is likely to impact both innate and adaptive immunity. Given the important role that IFN-γ plays in adaptive autoimmune responses, therapy targeting intracellular signaling regulating IFN-γ expression in T cells (e.g. Interferon Regulatory Factors – IRFs – or long non coding RNAs) may provide greater therapeutic benefit without adverse effect on innate responses to infection. As discussed in **Chapter 5**, an alternative strategy to inhibit TH1 differentiation and survival at the target site of inflammation of autoimmune arthritis may be the blockage of IL-27. In this specific context, indeed, IL-27 was found to be endowed with pro-inflammatory properties by specifically sustaining the maintenance of the TH1 phenotype. However, preclinical data seem to be controversial. IL-27 was reported to induce a TH1 immune response as well as susceptibility to proteoglycan-induced arthritis (33). Consistent with these findings, anti–IL-27 antibody treatment or IL-27 receptor knockout has been found to suppress ongoing severe inflammation and pro-inflammatory cytokine production in adjuvant-induced arthritis (64). However, in vivo injection of IL-27 into the ankles of mice with collagen-induced arthritis (CIA) attenuated joint inflammation, synovial lining thickness, bone erosion, and leukocyte migration (65). Similarly, another study, demonstrated that administration of IL-27 could attenuate CIA development at the onset of disease, by suppression of IL-17 and IL-6 synthesis (32). Overall, based on immunologic evaluations in preclinical models of autoimmune arthritis, it appears that IL-27 exerts both anti- and pro-inflammatory effects. In order to ascertain the exact mechanisms of action of IL-27 in rheumatic diseases and determine whether it is truly a friend or foe, further studies with large sample sizes and precise methodology are needed, especially in human systems.

Besides cytokine-directed strategies, identification and targeting of locally aggressive T cell subsets may represent an appealing therapeutic scenario for the treatment of chronic inflammatory diseases. In **Chapter 4** we describe that PD-1-expressing CD8 T cells are enriched at the target site of different chronic inflammatory diseases such as autoimmune arthritis, IBD and atopic dermatitis, and have the profile of antigen-specific and locally adapted effectors. PD-1 is a negative co-stimulatory receptor having a function of suppressing T cells activation signals (66). Analysis of PD-1 knock-out mice demonstrates that PD-1 signals play important roles in suppression of autoimmune diseases such as autoimmune dilated cardiomyopathy, and type I diabetes mellitus (67, 68). PD-1 agonist may be a potential prophylactic or therapeutic agent for chronic inflammatory diseases (69-71). However, immune checkpoint inhibitors, currently largely used in cancer therapy, may facilitate the development of autoimmune-like phenotypes (72-74), rendering systemic targeting a quite risky approach. In our study, we show that PD-1-expressing CD8 T cells in SF share the profile of TRM cells. Identity and functional specialization of TRM cells in human chronic inflammatory environments are yet to be explored, and further knowledge in this field might open new scenarios for therapeutic applications.

TRM specific subsets might be endowed with detrimental effects at this site and may be involved in chronic inflammatory disease relapses. In support of this idea, we showed that PD-1^{+}CD69^{+} CD8 T cells are enriched in the inflamed vs. non inflamed gut mucosa in IBD. However, specific features of this cell subset should be elucidated in preclinical models. Still an open question is the role of PD-1^{+}CD69^{+} CD8 T cells in non-inflamed mucosa: are they resting cells with the potential of effector functions or are they harmless bystander?

We have discussed how mechanisms underlying different diseases characterized by local inflammation can be of critical use to understand the pathology of other diseases and design therapeutic treatments. However, as discussed for IBD, where targeting IL-17A (relevant for disease-associated inflammation) leads to clinical exacerbation, disease-specific immunological features need to be explored and, therefore, therapeutic approaches should be specifically tailored on diseases and sometimes even on patients. In **Chapter 6** we discuss the issue we faced when we tested whether the protocol to generate donor-specific Tr1-cell–enriched medicinal product, previously set up in a different setting (75), was also suitable for cell therapy in patients with kidney failure. Likely due to a pro-inflammatory peripheral status (76-78), anergic Tr1-cell–enriched medicinal product could only be generated when the myeloid compartment was removed prior to the *in vitro* culture. In this study we emphasize how important is the careful assessment of the immune system before patient inclusion in clinical trials. We believe there is still need to sensitize translational scientists on the relevance of setting up disease or even patient-tailored protocols aiming at minimizing possible negative outcomes.

In line with this need, in **Chapter 7** we propose to reconsider the figure of translational scientist. We call for a shift in the educational system aiming to develop experts of the translational medicine continuum, able to bring together different and already existing competences relevant to translational medicine. We propose to develop a specific training system to generate figures of Translational Medicine Professionals (TMPs) endowed with specific skills required to overcome fragmentation of the whole itinerary of translational medicine, from basic discoveries to clinical application of a product or technology. In our commentary, we aim to stress on the importance for translational scientists to be part of a network, a platform where clinicians, basic scientists, scientific journals, academy, patient organizations, regulatory agencies and industry, can freely interact, exchange ideas, projects and dilemmas with the common end goal of developing a well-framed system leading to the improvement of the global healthcare system.

In Figure 2, by using The ONE Study as a model for translation of a product into the clinics, we describe how TPMs should act as interface with other experts of the translational medicine machinery, such as (*i*) the basic scientist, who attempt to answer questions regarding unmet clinical needs, (*ii*) the Good Manufacturing Practice (GMP) facility for the development of a cell product or the industry for the development of a compound, (*iii*) the regulatory agency for the ethical assessment of the clinical trial, (*iv*) the clinician for patient management.

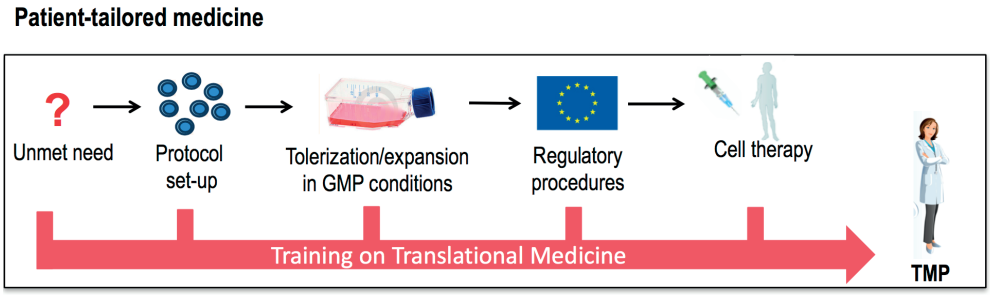

Figure 2. Role of TPMs in the translational medicine itinerary. TPMs should act as interface with other experts such as basic scientists (to answer clinical dilemmas), companies (for the development of medicinal products), regulatory agencies (for the ethical assessment), and clinicians (for patient management, treatment and follow-up).

To conclude, shared mechanisms underlying different diseases characterized by chronic inflammation are of extreme use to develop pharmacological treatments with multiple clinical applications. However, disease-specific mechanisms may halt or counteract the efficacy of these treatments and deserve likewise in-depth investigation for the development of suitable therapeutic interventions. Therefore, a greater understanding of the immunological mechanisms underlying chronic inflammatory diseases will provide the tools to elucidate observed differences in outcome and develop adequate disease-tailored therapeutic solutions to ultimately improve patient health.

FUTURE PERSPECTIVES

What therapeutic avenues might be envisaged in the future of chronic inflammatory diseases? A mandatory approach, in my opinion, would be prevention of disease onset. Therefore, identification of "at risk" subjects would be essential and only possible by expanding epidemiological studies, creating publicly available databases with patient and sibling data, and biobanking biological samples from these subjects. This is quite a challenge, requiring collaboration of a wide network of scientists for the development of international consortia (such as TrialNet for type 1 diabetes and UCAN for JIA). A more feasible approach though, would be the adoption of an early aggressive therapy in new onset patients, for instance by preventing long-term expansion of pathogenic clones or inhibiting local migration and accumulation of immune cells in tissues. I understand and support the effort of the scientific society to rapidly translate basic discoveries into widely available therapies for patients; however, I believe that the first essential step is a better understanding of the local immunological mechanisms underlying chronic inflammatory diseases.

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Chapter &

APPENDIX

ENGLISH SUMMARY

Chronic inflammatory diseases include a variety of organ-specific immune-mediated diseases characterized by a chronic and recurrent state of local inflammation. T cells are essential component of the adaptive immunity, whose imbalanced actions contribute to determine the pathology of chronic inflammatory diseases. Aim of this thesis is to dissect how the local inflammatory milieu influences T cell development and dynamics in chronic inflammatory diseases, with a particular focus on Juvenile Idiopathic Arthritis (JIA). In **Chapter 1** different subsets of the adaptive immune response known to take part in the pathogenesis and maintenance of chronic inflammatory diseases are described, as well as recently identified T cell subsets, such as the so called 'exhausted' T cells and tissue resident memory (TRM) cells, still unexplored in this context. In **Chapter 2** the current knowledge on the role of CD8 T cells in autoimmune inflammation is extensively reviewed, highlighting their crucial contribution to tissue damage and local inflammation. **Chapter 3** demonstrates that the resistance to suppression occurring at the site of chronic inflammation is an intrinsic feature of T cells rather than induced by other cell subsets. Of note, a different mechanism underlying the resistant phenotype was shown for the two major T cell subsets: indeed, unlike CD4 T cells, resistance of CD8 T cells to suppression is maintained by autocrine release of IFN-γ, indicating a potential therapeutic value of blocking IFN-γ to restore immune regulation in JIA. In **Chapter 4** a specific subset of CD8 T cells found in the synovial fluid of JIA patients is extensively characterized and we show that this overrepresented PD-1^{+}CD8 T cell subset has the phenotype of metabolically active, clonally expanding effectors, with no sign of *exhaustion*. This unique subset was enriched with the TRM cell transcriptional profile, and PD-1-expressing TRM cells were found increased also in other human inflamed tissues, such as the gut of patients with inflammatory bowel diseases (IBD) and the skin of patients with atopic dermatitis (AT). These data indicate that local chronic inflammation drives the induction of a unique subset of CD8 T cells endowed with potential detrimental properties in the context of chronic inflammatory diseases and might be an interesting target for therapy. **Chapter 5** elucidates the mechanisms underlying the TH1 cell enrichment observed in the synovial fluid of JIA patients. Despite the previously described contrasting, i.e. pro and anti-inflammatory, effects of IL-27, it was found that the elevated levels of IL-27 observed in the synovial fluid of JIA patients sustain TH1 cell maintenance and survival at this site, suggesting that this cytokine may be a promising therapeutic target for JIA. In **Chapter 6** a patient-tailored protocol for the generation of donor-specific type 1 regulatory T cells (Tr1) is developed to be infused in living donor kidney transplanted patients –characterized by a state of chronic peripheral inflammation- to obtain long-term acceptance of the graft. Here, it is discussed that, for clinical translation of basic discoveries, it is crucial to carefully screen the immune system of patients before enrollment in cell therapy clinical trials, in order to minimize possible negative outcomes and ultimately safeguard patient health. In line with this need, in **Chapter 7** we propose to reconsider the figure of translational

scientist by developing experts of the translational medicine continuum, i.e. Translational Medicine Professional (TMPs), starting from education and training. Such TMPs should be endowed with specific skills and be integral part of a worldwide network of experts, a platform designed to connect, interface and share ideas, data and tools, with the final goal to develop clinically applicable products and technologies. In **Chapter 8** I discuss how different subsets of the adaptive immune system develop and interact at the target site of inflammation of human chronic inflammatory diseases. Candidate checkpoints of the chronic inflammatory cascade were identified and proposed as potential therapeutic targets in human chronic inflammatory diseases. Also the importance of investigating shared as well as disease-specific mechanisms in order to develop suitable therapeutic interventions and ultimately improve patient health is highlighted.

NEDERLANDSE SAMENVATTING

Chronische inflammatoire aandoeningen zijn een verzameling orgaan-specifieke immuun-gemedieerde ziekten die gekarakteriseerd worden door een chronische en terugkerende lokale inflammatie. T cellen zijn een essentieel onderdeel van het verworven immuunsysteem, die, door soms te ontsporen, bijdragen aan de ernst van chronische inflammatoire aandoeningen. Het doel van dit proefschrift is nauwkeurig te onderzoeken hoe de lokale inflammatoire omgeving de ontwikkeling en plooibaarheid van T cellen in chronische inflammatoire aandoeningen beïnvloedt, met bijzondere aandacht voor Juveniele Idiopathische Artritis (JIA). In **Hoofdstuk 1** worden verschillende cel types, die een rol spelen in de pathogenese en het in stand houden van chronische inflammatoire aandoeningen, beschreven. Bovendien worden recent geïdentificeerde T cel subsets, zoals de zogenaamde 'uitgeputte' ('exhausted') T cellen, en T-geheugencellen die in weefsel voorkomen ('tissue-resident memory' cellen, of TRM), besproken. Beide subsets waren nog niet in een dergelijke context behandeld. **Hoofdstuk 2** bevat een uitgebreid literatuuroverzicht van de huidige kennis van de rol van CD8+ T cellen in auto-immuuninflammatie, waarbij extra aandacht wordt besteed aan hun cruciale bijdrage aan weefselschade en lokale ontsteking. **Hoofdstuk 3** laat zien dat de ongevoeligheid voor suppressie op de plaats van chronische ontsteking een intrinsieke eigenschap is van T cellen, in plaats van dat deze geïnduceerd wordt door andere cel types. Hierbij is van belang dat voor de twee belangrijkste T cel subsets is aangetoond dat er verschillende mechanismen ten grondslag liggen aan het resistente fenotype: in tegenstelling tot de ongevoeligheid van CD4+ T cellen wordt de resistentie voor suppressie van CD8+ T cellen in stand gehouden door autocriene uitscheiding van IFNγ, wat duidt op een potentieel therapeutisch toegevoegde waarde van het blokkeren van IFNγ om de immuunregulatie in JIA te herstellen. In **Hoofdstuk 4** wordt een specifieke CD8+ T cel subset, gevonden in het synoviaal vocht van JIA patiënten, uitgebreid gekarakteriseerd, en laten we zien dat deze overvloedig aanwezige PD-1+ CD8+ T cel subset het fenotype van metabool actieve, klonaal-geëxpandeerde effector T cellen heeft, zonder enig teken van *uitputting* (of *'exhaustion'*). Het transcriptieprofiel van deze unieke subset was verrijkt voor TRM-cel genen. Bovendien troffen we tevens PD-1+ TRM cellen aan in andere humane ontstoken weefsels, zoals de darmen van patiënten met inflammatoire darmziekten (IBD) en de huid van patiënten met constitutioneel of atopisch eczeem. Deze data laten zien dat de lokale chronische ontsteking de drijfveer is achter het ontstaan van een unieke subset CD8+ T cellen, die behept is met potentieel schadelijke eigenschappen in de context van chronisch inflammatoire aandoeningen en mogelijk een interessant therapeutisch doelwit is. **Hoofdstuk 5** belicht de mechanismen die ten grondslag liggen aan het toegenomen aandeel TH1 cellen in synoviaal vloeistof. Ondanks de eerder beschreven tegenstrijdige, i.e. pro- en anti-inflammatoire, effecten van IL-27, vonden wij dat de verhoogde waarden IL-27, gemeten in het synoviaal vocht van JIA patiënten, leiden tot handhaving en overleving van TH1 cellen in deze locatie. Dit suggereert dat dit

cytokine een veelbelovend therapeutisch doelwit is voor JIA. In **Hoofdstuk 6** is een op de patiënt-toegesneden protocol ontwikkeld om donor-specifieke type 1 regulatoire T cellen (Tr1) te genereren. Het is de bedoeling dat deze cellen vervolgens worden toegediend aan patiënten die een niertransplantaat hebben ontvangen van een levende donor waarbij chronische perifere ontsteking optreedt. Infusie van Tr1 cellen moet er voor zorgen dat het transplantaat langdurig geaccepteerd wordt. In dit stuk beargumenteren we dat, in het geval van translatie van basaal onderzoek naar klinische toepassing, het essentieel is om het immuunsysteem van de patiënten door te lichten alvorens hen te includeren voor klinische trials met celtherapie. Dit zorgt er voor dat een mogelijke slechte afloop voorkomen kan worden en de veiligheid van de patiënt gewaarborgd wordt. In overeenkomst met deze behoefte stellen we in **Hoofdstuk 7** voor de translationele wetenschapper te herdefiniëren, door experts te ontwikkelen die het hele spectrum van translationele geneeskunde bestrijken – de zogenoemde Translationele Medische Professional (TMP) – beginnende bij opleiding en training. Dergelijke TMP zouden toegerust moeten worden met specifieke vaardigheden en integraal deel moeten zijn van een wereldwijd netwerk van experts; een platform ontwikkeld om te verbinden, interacties aan te gaan, en ideeën, data en instrumenten te delen, met als uiteindelijk doel klinisch toepasbare producten en technieken te ontwikkelen. In **Hoofdstuk 8** bespreek ik hoe verschillende cel types van het verworven immuunsysteem tot ontwikkeling komen en interacties aangaan op de plaats van ontsteking in chronische inflammatoire aandoeningen bij de mens. Controlepunten, zogenaamde "Checkpoints", in de chronische inflammatoire cascade zijn geïdentificeerd en naar voren geschoven als potentieel therapeutische doelwitten in deze aandoeningen. Daarnaast is het belang van het exploreren van zowel mechanismen overeenkomstig tussen ziekten als mechanismen specifiek voor één ziekte belicht, dat moet leiden tot de ontwikkeling van gepaste therapeutische strategieën en uiteindelijk tot het verbeteren van de gezondheid van de patiënt.

CURRICULUM VITAE

Alessandra Petrelli was born in Bari, Italy. She moved to Milan for her medical studies and in 2007 she graduated as Medical Doctor at Vita-Salute San Raffaele University. In 2008 she was enrolled in the Internal Medicine residency program at San Raffaele Hospital in Milan, where she specialized on the management of diabetes and beta cell transplantation.

During the residency she was appointed at the Children's Hospital in Boston, MA (USA) for a 1-year Research Fellowship in Nephrology, where she focused on elucidating the mechanisms underlying the immunobiology of allograft rejection in animal models of type 1 diabetes.

Once completed the residency training, in 2013 she was selected as Early Stage Researcher within the Initial Training Network Marie Skłodowska-Curie EUTRAIN (www.eutrain-network.eu) and carried out her work under the supervision of Prof. Berent Prakken and Dr. Femke van Wijk at the University Medical Center of Utrecht (NL). Her project focused on understanding the mechanisms of differentiation and regulation of T cells at the target sites of chronic inflammatory diseases. The results of her PhD researches are the subject of the present thesis.

In 2016 she was awarded the AXA postdoctoral fellowship and the Marie Skłodowska-Curie Individual Fellowship. She returned to her own country to start a research line on Immunometabolism aiming to elucidate the immunological mechanisms underlying chronic inflammation in obesity and type 2 diabetes.

LIST OF PUBLICATIONS

1. Developing Translational Medicine Professionals: the Marie Skłodowska-Curie Action Model. **A. Petrelli**, BJ Prakken, N.D. Rosenblum, and EUtrain fellows. *Journal of Translational Medicine, 2016*

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The end of a melody is not its goal: but nonetheless, had the melody not reached its end it would not have reached its goal either. A parable.
Friedrich Nietzsche

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