T cell regulation and adaptation in chronic inflammation

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locatie is belangrijk
(met een samenvatting in het Nederlands)

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Chapter 1

General Introduction

CHRONIC INFLAMMATION

Inflammation is a physiological process aimed at restoring homeostasis by removal of the triggering agent, often a pathogen that is attempting to survive and multiply in the human body. This process generally starts in tissues, where the first encounter of pathogen with immune cells is taking place. Innate immune cells recognize and react to the pathogen, ultimately resulting in the recruitment of specialized cells of the adaptive immune system like T cells. T cells are a central cell subset in this process of tissue inflammation, by specifically targeting the causing agent and setting up a robust immune response towards it as well as by regulating this response to prevent inflammation-induced tissue damage. In acute inflammation the causing agent is contained and eliminated and inflammation resolves, whereas in chronic inflammation the process continues and T cells remain present in high numbers in the affected tissue. Moreover, abnormal T cell responses are associated with chronic inflammation that can occur either locally or systemically and is a pathological condition. Persistent presence of the causing agent, such as a virus that escaped the immune attack, can lead to chronicity of inflammation. Besides pathogens, continuous exposure to allergens or self proteins recognized as foreign can as well induce chronic inflammation. In the latter case it is also referred to as an autoimmune disease. Although it is well-established that T cells play a key role in chronic inflammatory conditions, in-depth knowledge about the regulation and function of different T cell subset in local human inflammation is scarce. Moreover, inflammation in itself is a normal physiological response but due to a limited availability of data on human inflamed tissues there is a gap in our knowledge of what is a normal inflammatory response or what is pathogenic and thus how to properly interfere with local inflammatory processes. Insight into the local process during chronic inflammation in humans will help to understand the general immuno-pathophysiology as well as to better monitor and treat affected patients.

JUVENILE IDIOPATHIC ARTHRITIS

Juvenile Idiopathic arthritis (JIA) is a disorder characterized by chronic inflammation due to an autoimmune reaction. It is the most common autoimmune disease in children[1]. Similar to adult Rheumatoid Arthritis (RA), the tissue restricted inflammation localizes primarily to the joints. JIA encompasses all forms of arthritis arising before the age of 16 years that are stratified based on laboratory and clinical features. Although some forms are difficult to classify and classification is currently being revisited[2], the patients that have been investigated in this thesis are diagnosed with either oligo arthritis, extended oligo arthritis or Rheumatoid Factor negative poly arthritis, all with a typical relapsing-remitting course of disease. Oligo arthritis refers to an involvement of 4 joints or fewer, whereas extended oligo arthritis applies to patients that have more than 4 joints affected after the first 6 months after

disease onset. Poly arthritis is also based on the number of joints involved, with more than 4 joints affected at or around disease onset, and is further stratified based on the presence or absence of the autoantibody Rheumatoid Factor (RF). RF positive poly arthritis is the childhood equivalent of adult RF positive RA and usually has a progressive course of disease[2]. Like it is the case for most human autoimmune diseases, the cause of JIA is multifactorial with a combination of genetic susceptibility and unknown environmental triggers[3]. As the disease can have a severe impact on the growing skeleton, current treatment strategies are aimed at preventing joint damage and controlling disease by general immunosuppression and/or targeted therapy interfering with inflammatory mediators (so-called biologicals). Treatment also often involves intra-articular injections of corticosteroids following aspiration of the inflammatory exudate in the affected joints that allows researchers to study human immune cells that are actually taking part in the inflammatory process. Genomic research underscores the important role of T cells in JIA[4], which is in line with the abundant presence of T cells in the inflammatory exudate termed synovial fluid (SF).

T CELL DEVELOPMENT

T cell receptor

T cells comprise a whole array of lymphocytes with diverse phenotypes and functions, but all are characterized by their maturation in the thymus and the expression of a T cell receptor (TCR). The majority of circulating T cells have a TCR composed of an α and a β chain and a minority have a γδ TCR. The function of TCRs is to recognize a large set of antigens that are presented on a Major Histocompatibility Complex (MHC) molecule on the surface of an antigen presenting cell[5]. MHC molecules are encoded by Human Leukocyte Antigen (HLA) genes that are highly polymorphic, and determine the peptide binding presented as antigen for the TCR. Also on the side of the TCR there is a high degree of variability. TCRs are formed during T cell maturation in the thymus through the unique process of somatic recombination of V, D and J gene segments. The α chain is generated by VJ recombination, whereas the β chain is formed by VDJ recombination. The resulting α and β chain combine to unique TCR heterodimers. Somatic recombination, the number of different V, D and J genes, and the combination of different α and β chains allow a large number of different TCRs to be produced. The variety of the TCR repertoire is further increased through junctional diversity – that is the addition or removal of small numbers of nucleotides at the junctions of V, D and J genes[6]. This part forms the complementary-determining region 3 (CDR3) which is the region involved in peptide/MHC binding and as such is the main determinant of antigen specificity. As a result, the TCR of each T cell leaving the thymus is highly unique and can be considered as a natural "barcode" for a T cell clone. Analysis of the TCR repertoire therefore promises to yield important insights in T cell biology and dynamics in different conditions.

Immune tolerance mechanisms

To ensure that T cells leaving the thymus recognize the appropriate antigens, the randomly generated TCRs are selected by positive and negative selection[7]. In the thymic cortex T cells are positively selected based on their ability to engage a loaded MHC molecule with peptide. Although crucial for generating diversity to allow protection against an unpredictable amount of pathogens, the TCR rearrangement inevitably generates T cells that can recognize self-antigens. Negative selection will result in the elimination of such autoreactive T cells with high affinity for autoantigens to avoid autoimmunity and to ensure central immune tolerance. The targeted elimination is mediated by antigen presenting cells (APCs) in the medulla that specifically present tissue-restricted antigen. Some T cells with high affinity for self-antigens escape this elimination and develop into regulatory T cells (Treg), determined by the strength of TCR and co-stimulation as well as the availability of IL-2, that together induce a specific transcriptional and epigenetic program[8–10]. Treg are characterized by FOXP3 expression and are dedicated to maintain peripheral immune tolerance. All lymphocytes that have passed selection enter the periphery as naïve T cells, characterized by the expression of CD45RA as well as chemokine receptor CCR7 and lymph node homing receptor CD62L. This allows them to recirculate through peripheral blood and lymphatic system to search for their cognate antigen presented by an APC. Binding of antigen with high affinity to the TCR will induce a signaling cascade initiated by the TCR complex that includes CD3 as a co-receptor. Once encountered their cognate antigen, T cells will further differentiate to become specialized effector cells. This process is guided by transcriptional and epigenetic changes that translate the signals provided by the APC and the local environment into a specific cell phenotype.

T cell subsets

T cells leaving the thymus with an αβ TCR can be divided in two major subsets based on their co-receptor expression besides CD3, namely $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells can interact with MHC II on the surface of APCs that presents peptides derived from ingested proteins. Subsequently, they orchestrate the immune response by producing cytokines and through cell-cell contact, and provide help to B cells to induce antibody production. As a consequence , $CD4^+$ T cell subsets are termed T helper (Th) cells. Examples are Th1, Th2 and Th17 cells, that each can be defined by expression of a specific master transcription factor (T-BET, GATA-3 and RORγt, respectively) and the production of specific cytokines, such as IFNγ and TNFα for Th1, IL-4, IL-5 and IL-13 for Th2 and IL-17 and IL-21 for Th17 cells[11]. The induction of a Th subset ensures a tailored immune response that targets specific microbial pathogens. $CD8^+$ T cells are endowed with receptors and cellular mechanisms to kill target cells, for example virus-infected cells, and hence are called cytotoxic T cells[12]. Target cells are recognized by a specific antigen loaded on MHC I molecules which are expressed on nearly all nucleated cells of the body and presents intracellular content, possibly including viral proteins.

Treg are a specific subset of the $CD4^+$ T cells, and, as mentioned before, are important for maintaining immune homeostasis. Next to thymus-derived $CD4^+$ Treg, several less abundant subtypes of $CD4^+$ Treg have been identified. These cells can be induced in the periphery under specific circumstances and differentiate from naïve T cells upon antigen encounter. They can be either $FOXP3^+$, referred to as peripherally induced Treg, or $FOXP3^-$, including T regulatory 1 (Tr1) and T helper 3 (Th3) cells producing IL-10 or TGFβ, respectively[13,14]. Mutations in the gene encoding FOXP3 result in the lack of Treg cells, and leads to the development of severe systemic inflammatory diseases manifested by autoimmunity, colitis, and allergies[15–17]. This thesis focuses on $CD4^+FOXP3^+$and will be referred to as "Treg". Whereas thymus-derived Treg, also known as natural Treg, have a TCR repertoire directed against self-antigens, peripheral induced Treg are thought to recognize non-self-antigens as they arise from naïve $CD4^+$ T cells[18]. Moreover, in mice they are particularly found in mucosal tissues and maternal placenta where they seem to establish tolerance towards commensals, food, allergens and the developing fetus[19,20]. To date, there are no discriminative markers regarding thymus-derived and peripherally induced Treg and therefore the induction of Treg *in vivo* in humans is yet not conclusively established. For Treg identification from human samples, the best approach till now is high expression of the IL-2 receptor α chain, CD25, and low expression of CD127 (IL-7 receptor α chain), combined with FOXP3 expression[21,22]. The suppressive mechanisms that Treg use *in vivo* are still not completely understood, but most likely include (but are not restricted to) the production of immunosuppressive cytokines (IL-10, TGFβ), expression of inhibitory receptors (CTLA-4, PD-1, TIGIT), competition for IL-2, release of cytotoxic enzymes and conversion of extracellular ATP to adenosine[23,24].

T CELLS IN AUTOIMMUNE INFLAMMATION

Autoreactive $CD4^+$ and $CD8^+$ T cells are abundantly present in the SF of JIA and RA patients. The local (auto)antigens that contribute to the sustained inflammation have not been identified thus far. $CD4^+$ T cells found in SF of JIA and RA patients are predominantly polarized towards an Th1 phenotype, characterized by the upregulated expression of the chemokine receptors CCR5 and CXCR3 and production of pro-inflammatory cytokines like IFNγ, and mixed Th1/Th17 phenotypes are detected as well[25–27]. $CD8^+$ T cells have recently gained more interest in the context of autoimmune arthritis. Their killing capacities and ability to produce large amounts of pro-inflammatory cytokines such as IFNγ and TNFα make them very plausible pathogenic candidates. However, their exact role in autoimmune arthritis remains to be resolved. A more elaborated overview of our current knowledge of T cells at the site of inflammation in human rheumatic diseases can be found in chapter 7. Given the development of overt autoimmune disease when *FOXP3* is mutated and the dysregulated inflammatory response in the joints of JIA and RA patients, defects in the

Treg compartment have long been suspected to be involved in disease. As a consequence, administration or targeting of Treg are currently being explored as therapeutic options in several human diseases including autoimmune diseases[28]. Impaired regulation can be attributed to deficiencies in Treg number or function, or resistance of T cell to Treg suppression[29]. Since Treg are shown to be abundantly present in SF, express FOXP3, and can suppress cells derived from peripheral blood[30,31], in this thesis we have focused on the responsiveness of local T cells to suppression. Therefore, studying local cells as well as the local inflammatory environment is crucial for the identification of the underlying mechanisms of impaired regulation.

T CELL ADAPTATION

In recent years is has become clear that T cell differentiation is far more complex and sophisticated as was assumed before. This is illustrated by the discovery of tissue resident memory T (Trm) cells and an enormous diversity of Treg subsets discovered in different tissues that reveal a large degree of adaptability. It is well accepted now that in steady state, tissues harbor a pool of T cells that maintain local homeostasis. Trm stably reside in tissues and continuously scan the tissue to prevent re-infection[32]. A hallmark of Trm is their ability to rapidly respond with secretion of cytotoxic mediators and cytokines, as well as the expression of inhibitory receptors[33,34]. This dual phenotype prevents an unnecessary inflammatory response, and thus damage, but allow for accelerated action if needed. Trm throughout the body share a specific transcriptional profile, confirming that this is a separate lineage, but their transcriptomes also exhibit tissue-specific adaptations[35,36]. This is very much in line with the recently discovered tissue Treg (reviewed in[37,38]). The adaptation of tissue Treg stretches even further than Trm, since tissue Treg have been shown to exert tissue specific functions that go beyond regulating immune homeostasis. For example, Treg found in adipose tissue co-express PPAR-γ next to FOXP3 and are needed to regulate insulin sensitivity[39,40], whereas in skin Treg co-express JAGGED-1 and regulate hair follicle stem cell proliferation and differentiation[41]. This shows a large degree of environment-instructed differentiation, but how this is achieved and regulated on a transcriptional and epigenetic level remains to be explored. Moreover, this 'functional specialization' is now demonstrated repeatedly in mice, but to what extend this happens in humans is an under-studied research area.

During inflammation, local conditions change dramatically including antigen availability, cytokine exposure as well as changes in available nutrients and metabolites. Next to that, inflammation results in the activation of local cell types like epithelial and stromal cells. This impacts immune cell differentiation, including Treg[42]. Especially during an inflammatory response regulation of immune cells is needed to limit tissue damage. In peripheral

blood, activated Treg that have a memory phenotype and heightened FOXP3 expression are characterized[43]. In mice, these cells are also detected in tissues and are characterized by maintained Treg function as well as the upregulation of functional markers such as ICOS and CTLA4. This, together with their expression of markers similar to activated Th cells resulted in their name effector (e)Treg[44–46]. Moreover, Treg are able to control diverse types on inflammation by the acquisition of Th cell characteristics that are dominating the response[47]. For example, in a Th1 polarized inflammation, Treg are detected that co-express T-BET next to FOXP3 and also upregulate the chemokine receptor CXCR3[48]. This allows their migration to this specific site of inflammation and to efficiently control the inflammatory response. Thus, Treg can adapt to their environment while maintaining their lineage identity. The exact transcriptional and epigenetic mechanisms that mediate this flexibility on one hand and perseverance on the other hand are not completely understood. Next to that, most of the information about tissue T cells is based on mouse data. Data from human tissues are scarce, and especially for therapeutic usage as an ultimate goal, it is important to study human samples from inflamed sites.

SCOPE AND OUTLINE OF THE THESIS

The scope of this thesis is to explore human T cell programming and behavior in local inflammation, with a focus on immune regulation and T cell adaptation.

In **Chapter 2** the effects of standard JIA therapy on Treg biology is reviewed, with the aim to gain knowledge about the role of Treg in disease pathogenesis. In **Chapter 3** the suppressive function of local SF derived Treg is dissected using both local and peripheral blood derived target cells to study the role of the inflammatory environment on T cell regulation. In **Chapter 4** the focus is on the phenotype and functional characteristics of PD-1 expressing $CD8^+$ T cells in SF as well as other sites of chronic inflammation. In **Chapter 5** the molecular and epigenetic programming of SF Treg is unraveled and compared to tumor-infiltrating Treg to gain in-depth knowledge about Treg programming in a human inflammatory environment. In **Chapter 6** the immune architecture and T cell receptor profile of SF $CD4^+$ T cells and Treg is determined in patient samples that are collected at the same time from both affected knees and over time during the relapsing-remitting course of disease, with the aim to provide fundamental insight into the antigen-specific T cell response associated with local disease. In **Chapter 7**, we review and discuss recent literature on local adaptation of $CD4^+$ T cell, $CD8^+$ T cell and Treg to inflammation in pediatric rheumatic disorders, including published findings from this thesis. Finally, in **Chapter 8**, unpublished data presented in this thesis are discussed in the context of recent literature and put in a broader perspective.

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Chapter 2

The effect of autoimmune arthritis treatment strategies on regulatory T cell dynamics

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ABSTRACT

Since their discovery over 15 years ago, intensive research has focused on the presence, phenotype and function of FOXP3^{+} regulatory T cells (Treg) in autoimmune diseases such as rheumatoid arthritis (RA). The questions of whether Treg deficiencies underlie autoimmune pathology and whether or how Treg-related therapeutic approaches might be successful are still subject of a vivid debate. In this review we give an overview of how current therapies influence Treg numbers and function in RA and juvenile idiopathic arthritis (JIA) and discuss these findings in the light of new Treg-based intervention strategies for autoimmune arthritis. The attempt to relate rheumatic diseases like rheumatoid arthritis and juvenile idiopathic arthritis to Treg has led to somewhat heterogeneous observations. So far, no clear defects in Treg numbers or function have been identified in autoimmune arthritis. The current standard therapies, that is methotrexate and biologicals, are generally effective but the exact mechanism of action and their effect on Treg is not fully known. Nevertheless, the majority of *in vitro* and *ex vivo* data point towards a positive influence of these treatments on Treg number and function. These observations are not all consistent, however, and it is not known whether the observed effects on Treg are primary or secondary effects. To safely conduct targeted regulatory T cell therapy in rheumatic diseases more knowledge about regulatory T cell function in an inflammatory environment is needed that coincides with the initiative to elucidate the exact mechanism of current therapies.

INTRODUCTION

It is well established that FOXP3 expressing regulatory T cells (Treg) play an indispensable role in maintaining self-tolerance and suppressing inflammation. The importance of self-tolerance controlled by Treg is reflected in clinical manifestations of severe generalized autoimmune disease in mice and men lacking functional Treg, due to mutations in the Foxp3 gene[1–3]. Because of this evident relation between Treg and autoimmunity, there has been an extensive search for linking autoimmune rheumatic disorders (like rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA)) to Treg (dys)function. RA and JIA are characterized by chronic inflammation of the joints[4,5]. Nowadays, the majority of RA and JIA patients are treated with disease modifying anti-inflammatory drugs (DMARDS) or a combination of DMARDS with biologicals with the aim to reach life-long disease remission. Although major progress has been made, there are still hurdles to overcome including patients that do not respond to therapy, the occurrence of side effects after long-term use and patients who relapse[6]. Importantly, the mechanism of action and the effect on Treg of these approved drugs is not fully known. On the contrary, the concept of targeting Treg in autoimmune disease has recently gained a lot of attention. Indeed, experimental therapies are being developed that directly or indirectly target Treg.

In this review we will focus on the mechanism of current and experimental therapies to treat RA, JIA in light of their quantitative and/or qualitative effect on Treg.

REGULATORY T CELL FUNCTION IN RHEUMATIC DISEASE

In arthritis patients, Treg frequency and function can be measured in peripheral blood as well as at the site of inflammation. Studies investigating circulating Treg in RA and JIA show variable results especially regarding Treg inhibitory function[7–11], but at the local site of inflammation enriched Treg levels are consistently reported[10–18]. The overall consensus is that these are active Treg that have an increased suppressive capacity[11–15,18,19]. In line with this it has been shown in synovium of RA patients that there is an enhanced commitment towards Treg lineage based on Foxp3 DNA methylation status[20]. In the inflamed synovium of RA and JIA patients local tissue and various immune cells interact with each other via cytokines and/or cell-cell contact[21]. Pro-inflammatory cytokines, like TNF-α and IL-6, and antigen presenting cells can also interfere with Treg function. So, although present in large amount and suppressive *in vitro*, the suppressive *in vivo* capacity of Treg could be counteracted by the highly inflammatory environment or hampered by the resistance of Teff to Treg-mediated suppression[9,15,18,22–24].

EFFECTS OF STANDARD THERAPY ON REGULATORY T CELLS

Although there is no clarity about the exact contribution of Treg defects (either intrinsic and/or extrinsic) to disease pathology in arthritis, several studies have shown that current therapies for RA may (indirectly) affect Treg numbers and/or function. The current standard therapy for treating rheumatic diseases includes DMARDS, of which methotrexate (MTX) is the most commonly used, and biologicals.

Methotrexate

Methotrexate is a very effective drug in treating RA and JIA[25–28]. MTX is an anti-folate drug which suppresses purine and pyrimidine synthesis thus inhibiting DNA replication. MTX therefore has an anti-proliferative effect, particularly if used in high dosages for the treatment of malignant diseases. In rheumatic diseases, MTX is used in up to 50 times lower dosages where it exerts anti-inflammatory effects[29,30]. Mechanisms accounting for MTX's anti-inflammatory properties have not been fully elucidated, however *in vitro* and *in vivo* evidence shows that MTX-mediated release of anti-inflammatory adenosine may contribute to its effects in rheumatic diseases[31–33]. A putative link between MTX's mechanism of action and Treg function is provided by the fact that Treg, through high expression of CD39 and CD73 ectoenzymes, can convert the 'danger signal' ATP to AMP, and AMP to adenosine[34–38]. In turn, adenosine can bind to the adenosine receptor A2a expressed on T cells; subsequent signaling through this receptor results in an increase of intracellular cAMP[36–38], which leads to suppression of proliferation and cytokine production in effector T cells[39]. Moreover, triggering of the A2a receptor by adenosine may also have a direct effect on Treg resulting in their expansion and heightened immune regulatory activity[40]. However, there is no direct evidence that MTX or MTX-mediated adenosine release impacts the number and/or function of Treg.

Biologicals

Biologicals are a relatively new subset of therapeutics that are directed at specific biological targets that play a role in the inflammatory cascade. Here we will review the biologicals that target TNFα and IL-6, the IL-1 receptor antagonist and soluble CTLA-4 and their impact on Treg dynamics as summarized in Figure 1.

Anti-TNFα

Three biological agents that target TNFα (infliximab, adalimumab and etanercept) are approved worldwide and currently used to treat both RA and JIA. In addition, golimumab and certolizumab pegol have been recently approved for use in RA. A relevant difference between the therapeutics is their structure; etanercept is composed of the extracellular part of two human TNF receptor 2 linked to the Fc portion of human IgG1, whereas

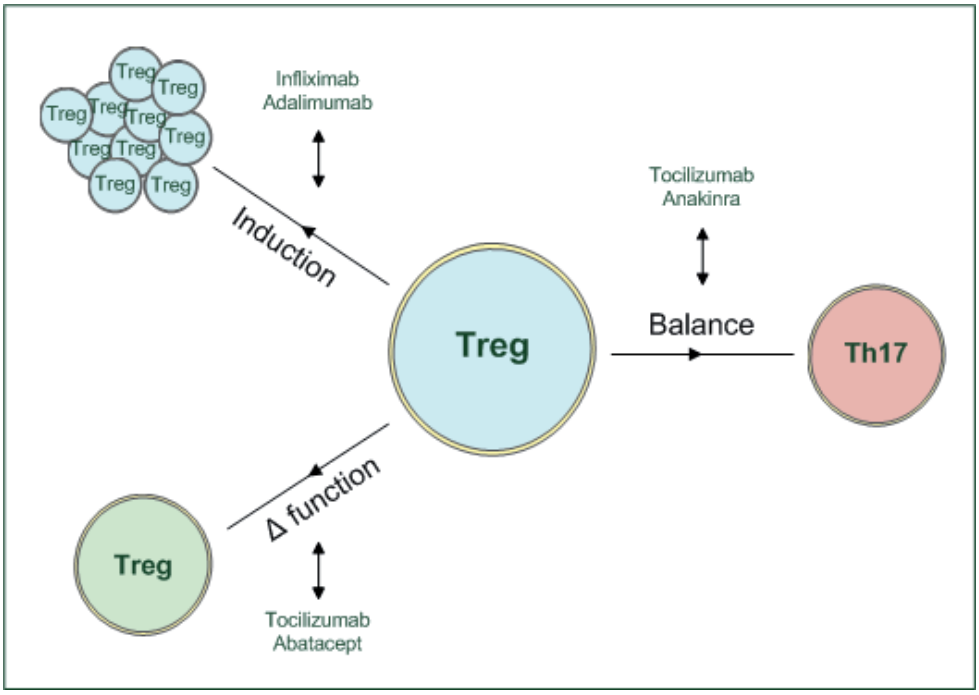

Figure 1. Biologicals in rheumatic disease may target Treg at different levels.

Biological therapy can affect Treg by causing induction of a new Treg population as described for infliximab and adalimumab. Another possibility is enhancement of Treg functionality, as tocilizumab and abatacept may do. Tocilizumab and anakinra are reported to influence the balance between Treg and Th17 cells. Cells from Servier Medical Art.

infliximab, adalimumab and golimumab are monoclonal antibodies against human TNFα. Certolizumab pegol is a Fab fragment of an anti-TNFα IgG1 mAb[41]. The therapeutic strategy of targeting TNFα has proven to be very successful in both RA and JIA patients[42,43].

Soluble TNFα (sTNFα) exerts multiple effects on various cell types by binding TNF receptors type 1 and 2 (TNFR1 and TNFR2, respectively). In addition, transmembrane TNFα (tmTNFα), the precursor of soluble TNFα, is also capable of binding these receptors as well as inducing reverse signalling[44,45]. TNFR1, that carries an intracellular death domain and can induce apoptosis, is ubiquitously expressed whereas TNFR2 expression is limited to lymphocytes amongst others. Signalling via TNFR2 activates NF-kB, AP1 and MAPKs resulting in T cell activation and proliferation[46]. All anti-TNF agents interfere at the interaction between both soluble and transmembrane TNF with its receptors.

Targeting TNFα in chronic inflammatory disease is a rational approach because of the prominent role of this cytokine in inflammation[47]. Furthermore, high levels of TNFα, which are found at the site of inflammation in RA and JIA patients, are able to interfere with Treg function[9,48,49]. Interestingly, recent *in vitro* studies[50–53] in murine cells show that

TNFα can enhance Treg suppressive capabilities, in particular of natural Treg, and this is amplified via upregulation of the TNF superfamiliy members 4-1BB and OX40. Moreover, this results in an additional boost of Treg expansion. It is suggested that TNFα serves as a feedback loop in which the Teff that are suppressed by Treg promote Treg expansion and functionality[54]; a role earlier proposed for IL-2[55,56]. This contradicts with human data which show that TNFα decreases the suppressive function of both peripheral blood Treg and *in vitro* induced Treg, and, in addition, neutralizing TNFα results in Treg expansion[9,48,57–59]. The miscellaneous role of TNFα on Treg function and expansion (and hence on inflammation) and the opposing results in mice and human studies[54,60] warrants further research, especially in the arthritis setting where anti-TNFα agents are commonly used.

In RA the efficacy of the anti-TNF agents appear to be very similar to one another[61]. Their influence on Treg, however, separates the agents from each other. In 2004 it was shown that patients who respond to infliximab have an increase in circulating Treg compared with nonresponders and MTX-treated patients[7]. This increase in Treg does not result from expansion of existing Treg, but is rather due to the induction of a new Treg population capable of producing TGFβ and IL-10 (Figure 1)[62]. Apart from this induced Treg population, infliximab can also target Treg function directly via TNF-R2 signaling which results in restoration of suppressive function[9]. In line with this, recent reports discussing other autoimmune diseases like inflammatory bowel disease (IBD) and Behcet's disease also show a positive influence on infliximab on Treg number and function[63–65].

The fully humanized anti-TNFα antibodies adalimumab and golimumab are increasingly being used, because of similar efficacy and better administration characteristics than the more classical anti-TNFα drugs like infliximab and etanercept. *In vitro* experiments have shown that adding adalimumab to a co-culture of synovial fluid $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells derived from RA patients enhances suppression[49]. The outcomes of *ex vivo* studies[66–69], to analyze the effects of adalimumab on Treg number and phenotype in RA patients, are inconsistent and indicate that the time point of analysis is a major determinant. This suggests that Treg do not significantly contribute to the therapeutic effects of adalimumab. However, a study[70] performed in a small group of RA patients observed expansion of Treg and a decrease of Th17 cells. Moreover, a recent study that compares adalimumab with etanercept treatment shows that RA patients responding to adalimumab have increased peripheral blood Treg that are potent and stable (Figure 1). Furthermore, these induced Treg are resistant to conversion to Th17 cells that is most likely due to IL-6 modulation[71].

The effect of etanercept, the soluble decoy receptor, has been recently investigated in combined administration with MTX in RA patients and mice. In mice, etanercept normalizes the Treg / Teff balance both in peripheral as well as central organs, whereas in a small population of RA patients the imbalance between Th17 and Treg can be reversed[72,73]. However, etanercept does not seem to be capable of inducing Treg as reported for infliximab and adalimumab[62,71].

In conclusion, current literature points towards a positive effect of anti-TNFα therapy on both Treg number and function. However, to firmly conclude this and to elucidate whether this is a primary or secondary effect more extensive research is needed, especially regarding sequential *ex vivo* experiments.

Anti IL-6

Next to TNFα, IL-6 is significantly increased in synovial fluid of both RA and JIA patients[18,49]. Targeting IL-6 will have dual effects, since IL-6 itself functions as a pro-inflammatory cytokine and as such can directly weaken Treg suppressive function[22]. In addition, IL-6 is one of the key cytokines that influences the Th17/Treg balance by switching from the induction of Foxp3$^+$ Treg to Th17 cells[74,75]. Th17 cells have been shown to play a central role in the disease pathology of RA and JIA by secreting IL-17. This cytokine can activate multiple cells, like synovial fibroblasts and monocytes, which are involved in causing joint damage[21,76].

Tocilizumab, a humanized antibody that targets the IL-6 receptor, is successfully administered to RA patients and systemic onset (so)JIA patients[77–79]. As hypothesized based on the IL-6 mediated effects, the amelioration of disease of RA patients receiving tocilizumab is associated with an decrease of Th17 cells and an increase in Treg numbers, indicating that tocilizumab is indeed able to correct the Th17/Treg balance[80] (Figure 1). Additionally, blocking IL-6 in co-cultures of synovial effector T cells and Treg increases the suppressive capacity of Treg[49], perhaps by abrogating IL-6 induced methylation of the *FOXP3* gene[81]. In line with these observations, combination therapy of tocilizumab with MTX can profoundly retard joint damage progression in RA patients[82]. Overall, these data point to a role for tocilizumab in positively affecting Treg number and functionality in rheumatic patients (Figure 1).

IL-1 receptor antagonist

IL-1 is, together with IL-6 and others, an important cytokine in skewing the induction of pathogenic Th17 cells[73,74,83,84]. IL-1 is a central pro-inflammatory cytokine able to induce multiple cell types and consequently cause bone and cartilage destruction. Next to TNFα and IL-6, IL-1 plays an important role in disease pathology of RA and JIA, albeit arguably less so. However, soJIA patients are characterized by an IL-1 signature[85].

Blocking the IL-1 mediated effects by administration of the human recombinant IL-1 receptor antagonist (IL-1ra; anakinra) on top of endogenous IL-1ra will dampen the immune response and might restore the balance of Th17/Treg. The use of anakinra as a therapy in RA and JIA is now approved, and although the exact mechanisms remain largely unknown its use has proven to be effective and safe[86,87]. A recent study[88] strongly indicates that anakinra indeed affects the Th17/Treg balance, as combination therapy of MTX with anakinra decreases Th17 and Th1 cells but increases Treg in RA patients and this is correlated to the clinical improvement (Figure 1).

CTLA4-IG

Cytotoxic T lymphocyte associated Ag-4 (CTLA-4) is highly expressed on Tregs and is decisive in their suppressive function[89]. Activated T cells also express CTLA-4, but binding to its ligands will induce different effects in effector T cells and Treg; that is co-stimulation via CTLA-4 binding to CD80 and CD86 will inhibit effector T cell function whereas it augments Treg suppressive capacity[90]. The importance of CTLA-4 in rheumatic diseases is reflected in the implication of gene polymorphisms and the observation that CTLA-4 expression is diminished in Treg of RA patients[8,91].

CTLA-4-Ig (abatacept) is a fully human fusion protein consisting of the extra-cellular domain of CTLA-4 with the Fc portion of immunoglobulin-G1 and as such functions as an agonist to reduce immune activity. It is now being used in the clinic for RA and soJIA patients[92]. Two different modes of action have been proposed for abatacept. The first and most obvious way of immune modulation by CTLA-4-Ig is by blocking the second signal, that is ligation of CD80/CD86 to CD28 on the surface of the T cell that is needed for proper T cell activation[93]. Secondly, CTLA-4-Ig is thought to promote Treg cell generation by inducing tolerogenic dendritic cells trough reverse signaling via CD80/86 on the surface of dendritic cells[94–98]. Interestingly, the CD80/86-CD28 interaction is also important in Treg generation and suppressive function, which suggests that abatacept may have a negative effect on Tregs[99,100]. The bifurcation of function and the fact that CTLA-4-Ig can target both effector T cells as well as Treg make it difficult to pinpoint its exact mechanism of action. This is also reflected in literature on the effect of CTLA-4-Ig on Treg numbers[95,101–104]. In RA patients, however, abatacept induces a decrease of Treg numbers in peripheral blood but enhances their suppressive function[105] (Figure 1). A possible explanation for the conflicting results could be that on one hand CTLA-4-Ig inhibits Tregs by blocking the CD80/86-CD28 interaction and on the other hand induces or expands Treg in the periphery via tolerogenic dendritic cells. Further studies are needed to understand the complexity of CTLA-4-Ig on both effector T cells and Treg.

Treg-based therapies

The successes of the biological therapies in RA and JIA have had a stimulating effect on the search for new therapeutic opportunities with the goal to eventually reach complete clinical remission. Nowadays, much research is performed on cell based therapy in which Treg are targeted to reestablish tolerance. This can be reached by *ex vivo* expansion of Treg or *in vivo* induction of Treg[106–108]. However, caution is needed to translate *in vitro* results by induced Treg to *in vivo* application[109]. To ensure stable and functional Treg that are able to suppress effector T cells at the site of inflammation several strategies are proposed, like ectopic FOXP3 expression[110] or modulation of the acetylation level of FOXP3 in order to regulate Treg numbers and functionality[111,112]. However, for cell-based therapies to be successful, targeting Treg may not be enough as is evident from recent literature that underscores the role of the balance between Treg and Teff in rheumatic diseases[15,18].

Therefore, the optimal approach might be to combine Treg enhancing strategies with specific targeting of Teff. Candidate targets to restore responsiveness of effector cells as well as enhancing Treg suppression are protein kinase B/c-akt and protein kinase C-θ, amongst others[18,113]. Another approach is to target Treg in an antigen specific way. Currently in rheumatic diseases various options are being explored using stress proteins such as binding immunoglobulin protein (BiP), heat shock proteins and dnaJP[114–116].

Moreover, there are some important unresolved questions regarding Treg function in rheumatic disease that limit the application of Treg-based therapies. For example, it is still unknown whether the observed effects of current therapy on Treg reflect the underlying mechanism that contributes to clinical improvement. Instead, the differences in the Treg compartment might not be primarily involved in the amelioration of disease but could also reflect a secondary 'side' effect. On top of that, there are various reasons that hamper a proper readout of Treg function. First of all, up till now there is no unique Treg marker in humans as highly activated T cells also transiently express Foxp3. Second, in the human setting the site of inflammation is not or hardly accessible so the majority of studies is based on observations in the periphery and this may not reflect what is happening at the site of inflammation. Recent new insights into how Treg suppress reveals that Treg are able to adapt to their surroundings and display a certain multiplicity of Treg subsets[117,118]. This setting, where Treg are instructed by the environment, argues against Treg based therapy without targeting the inflammatory environment, in which they will act, as well.

Taken together, more knowledge is needed about the role of Treg in human rheumatic diseases prior to targeted Treg-based therapies may be successful.

CONCLUSION

The attempts to relate Treg deficiencies to the pathogenesis of rheumatic diseases have as yet not provided a clear outcome. It is most presumptive that Treg are not necessarily defective but are hampered by the ongoing inflammation. The current knowledge indicates that there is no single cell type that dictates the pathogenesis, but rather the interplay of cell subsets and cytokines that are present at the site of inflammation[119]. In order to gain more insight into the role of Treg in RA and JIA pathogenesis, it is necessary to investigate the effects of standard therapy on Treg. In case of MTX there are tentative indications that Treg are affected by MTX treatment. As regards targeting the pro-inflammatory cytokines TNFα, IL-6 and IL-1 and the co-stimulatory molecule CTLA-4 the majority of studies confirm a stimulating effect of blocking therapy on Treg. However, it remains to be clarified whether this is a direct or indirect effect.

All together, current therapies in RA and JIA are able to obtain a remission on medication in a majority of patients. The next challenge will be how to obtain a lasting disease remission, without medication. Though exact contribution of Treg to RA pathogenesis is

not known, it seems clear that Treg are important to establish this goal. This underscores the importance of further studies on the effects of current therapies on Treg function, and ultimately the application of targeted Treg based therapies.

KEY POINTS

- In rheumatic diseases like rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) there does not seem to be an intrinsic defect in Treg function but rather a resistance of effector T cells to suppression due to the pro-inflammatory environment
- The majority of studies conclude that methotrexate and biological therapies do affect the Treg population, but to what extent and how this mechanistically is achieved is not known
- The biologicals tocilizumab (anti-IL-6) and anakinra (IL-1 receptor antagonist) may alter the balance between Treg and pathogenic Th17 cells
- Therapies that directly or indirectly target Treg are being developed but their clinical application warrants further research

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Chapter 3

Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells

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ABSTRACT

During the last decade research has focused on the application of FOXP3^{+} regulatory T cells (Treg) in the treatment of autoimmune disease. However, thorough functional characterization of these cells in patients with chronic autoimmune disease, especially at the site of inflammation, is still missing. Here we studied Treg function in patients with juvenile idiopathic arthritis (JIA) and observed that Treg from the peripheral blood as well as the inflamed joints are fully functional. Nevertheless, Treg-mediated suppression of cell proliferation and cytokine production by effector cells from the site of inflammation was severely impaired, because of resistance to suppression. This resistance to suppression was not caused by a memory phenotype of effector T cells (Teff) or activation status of antigen presenting cells (APCs). Instead, activation of protein kinase B (PKB)/c-akt was enhanced in inflammatory effector cells, at least partially in response to TNFa and IL-6, and inhibition of this kinase restored responsiveness to suppression. We are the first to show that PKB/c-akt hyperactivation causes resistance of effector cells to suppression in human autoimmune disease. Furthermore, these findings suggest that for a Treg enhancing strategy to be successful in the treatment of autoimmune inflammation, resistance because of PKB/c-akt hyperactivation should be targeted as well.

INTRODUCTION

Since their discovery 15 years ago[1], it is now well established that $CD25^+$ regulatory T cells (Treg) are indispensable for immune homeostasis and self-tolerance. Treg suppress the activation, proliferation, and effector functions of a wide range of immune cells via multiple mechanisms[2]. FOXP3 has been identified as a master transcription factor, controlling both Treg development and functionality[3,4]. In addition, human Treg can be identified by high CD25 and low IL-7 receptor (CD127) expression[5,6]. A critical role of Treg in controlling autoimmune responses is demonstrated in various animal models of autoimmune disease[7]. Furthermore, lack of functional Treg leads to severe, systemic autoimmunity in humans[8,9].

Because of their unique function, Treg are considered important for the treatment of autoimmune disease, and several strategies are now being explored to target these cells for therapeutic purposes[10]. However, there is still an ongoing debate whether the numbers and/or function of Treg are changed in patients suffering from chronic autoimmune inflammation[11]. In rheumatoid arthritis (RA) and multiple sclerosis, similar Treg numbers[12,13,] or even enhanced numbers in RA[14], were observed in peripheral blood (PB) of patients compared with healthy controls (HC). Thus, it appears that Treg numbers are not reduced in patients suffering from autoimmune inflammation. In addition, it remains unclear whether Treg function is impaired; some studies report reduced functioning of Treg in PB of patients[12,13,15], whereas others have found no difference[14,16].

In addition to these discrepancies concerning Treg numbers and function in the periphery, characterization of Treg functionality at the site of autoimmune inflammation in humans is missing. High levels of Treg have been found at the inflammatory sites in patients with arthritis and inflammatory bowel disease and these cells can suppress $CD4^+CD25^-$ effector cells *in vitro*[17]. Also at the site of inflammation in juvenile idiopathic arthritis (JIA), one of the most common childhood autoimmune diseases, we have previously shown that Treg are present in high numbers and suppress proliferation of $CD4^+CD25^-$ effector cells *in vitro*[18]. However, *in vivo* inflammation persists despite the large numbers of Treg present, suggesting that these cells are defective in their ability to control the ongoing autoimmune response. This may result from the local proinflammatory environment, because *in vitro* experiments have shown that proinflammatory cytokines can affect both Treg function[15,19-21] as well as effector T cell (Teff) responses[22,23]. These data suggest that increasing Treg numbers or enhancing their function for therapeutic purposes might be less effective in a chronic inflammatory environment. However, *ex vivo* data from patients with autoimmune disease are required to clarify the role of Treg at the site of inflammation in humans.

Here, we studied Treg function at the site of inflammation in patients with JIA and compared their inhibitory potential to Treg from PB of both patients and HC. With this approach, we show that Treg from inflamed joints demonstrate efficient suppressive capacity similar

to Treg from HC, but control of effector cell proliferation and cytokine production is severely impaired, because of resistance of Teff to suppression. This unresponsiveness to suppression is, at least partially, caused by hyperactivation of protein kinase B (PKB)/c-akt and can be restored by selectively inhibiting PKB/c-akt activation. Taken together, these findings identify resistance of effector cells to suppression and, more specifically, enhanced PKB/c-akt activation of effector cells as a potential new target in the treatment of autoimmune inflammation.

METHODS

Patients and healthy controls

34 patients with oligoarticular and 3 with extended oligoarticular JIA, according to the revised criteria for JIA[24], were included in this study. All patients had active disease and underwent therapeutic joint aspiration at the time of sampling. Patients were between 5 and 18 years of age and were either untreated or treated with nonsteroidal anti-inflammatory drugs (NSAIDs), methotrexate (MTX), or both at the time of inclusion. Informed consent was received from parents/guardians or from participants directly when they were over 12 years of age. 27 volunteers from the laboratory with no history of autoimmune disease were included as HC. The study procedures were approved by the Institutional Review Board of the University Medical Center Utrecht (UMCU) and performed according to the principles expressed in the Helsinki Declaration.

Cell isolation

From JIA patients, synovial fluid (SF) was collected during therapeutic joint aspiration, and, at the same time, blood was drawn via veni puncture or intravenous drip. Blood was collected from HC via veni puncture. SF mononuclear cells (SFMC) and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and were used either directly, or frozen in FCS (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation.

Cell culture conditions

Cells were cultured in RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10% human AB serum or 10% FCS (all obtained from Invitrogen) in round-bottom 96-well plates (Nunc). Cells were stimulated with 1.5 mg/ml plate-bound anti-CD3 (clone OKT3; eBioscience) and cultured at 37°C and 5% CO2.

Suppression assays

Total SFMC or PBMC were used as effector cells and cultured at 200.000 or 100.000 cells per well in 200 or 100 ml culture volume. $CD4^+$ cells were isolated by magnetic cell

sorting, using a CD4 T Lymphocyte Enrichment Set (BD Biosciences), according to the manufacturer's instructions. Subsequently, $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry on FACS Aria (BD Biosciences; Supplemental Figure 1 A). Treg were co-cultured with effector cells at a 1:8 and 1:4 ratio. To control for cell density, effector cells instead of Treg were added at a 1:4 ratio. In some experiments, PKB/c-akt inhibitor VIII (0.1 mM; Calbiochem) was added from the start of culture. At day 4, proliferation of effector cells was analyzed or supernatant was collected to measure cytokine production.

Analysis of cell proliferation

To measure proliferation, effector cells were labeled with 2 mM CFSE (Invitrogen) for 10 minutes at 37°C and extensively washed before use in suppression assays. At day 4, proliferation of effector cells was analyzed by flow cytometry by gating on $CFSE^+$ cells. Proliferation of $CD4^+$ and $CD8^+$ T cells was measured by subsequently gating on $CD3^+$ cells, followed by gating on $CD4^+$ and $CD8^+$ cells, respectively.

Detection of cytokines in culture supernatant, PB, and SF

Supernatant was collected from suppression assays, stored at -80°C and processed within 1 month. Plasma was obtained by centrifugation of PB at 150g and SF at 980g for 10 minutes and stored at -80°C. Cytokine concentrations were measured with the Bio-Plex system in combination with the Bio-Plex Manager Version 4.0 software (Bio-Rad Laboratories), which employs the Luminex technology as previously described[25].

Suppression assay with sorted memory and naive Teff

To study suppression of naive and memory Teff, $CD4^+$ T cells were isolated by magnetic cell sorting, using a CD4 T Lymphocyte Enrichment Set (BD Biosciences, according to the manufacturer's instructions), and subsequently $CD4^+CD25^+CD127^{low}$ Treg, $CD4^+CD25^-$ total Teff, $CD4^+CD25^-CD45RA^+CD45RO^-$ naive Teff, and $CD4^+CD25^+CD45RA^-CD45RO^+$ memory Teff were sorted by flow cytometry on FACS Aria (BD Biosciences). Teff (25.000 cells) were cultured in 75 ml culture volume. Irradiated (3500 rad), autologous PBMC (30.000 cells per well) depleted of $CD3^+$ cells by magnetic cell sorting with anti-human CD3 particles (BD Biosciences, according to the manufacturer's instructions) were used as APCs. Treg were added at a 1:2 ratio to Teff and at day 5 proliferation of Teff was analyzed and supernatant collected to measure cytokine production.

Suppression assay with sorted APCs and T cells

To study suppression of synovial T cells in the presence of PB- or SF-derived APCs, $CD4^+CD25^+CD127^{low}$ Treg, $CD3^+$ T cells, and $CD3^-$ APCs were sorted by flow cytometry on FACS Aria (BD Biosciences). CFSE labeled $CD3^+$ T cells (100.000 cells per well) were cultured together with 100.000 unlabeled $CD3^-$ APCs in the presence of Treg at a 1:4 and 1:8 ratio. At day 4, proliferation of T cells was analyzed.

TGFβ suppression assay

To investigate suppression of SFMC and PBMC by TGFβ, CFSE-labeled SFMC and PBMC were cultured in the presence or absence of 40 ng/ml recombinant human TGFβ1 (Koma Biotech). In some experiments, PKB/c-akt inhibitor VIII (0, 0.01, 0.1, and 1μM; Calbiochem) was added from the start of culture. Cells were cultured for 4 or 5 days and proliferation of $CD4^+$ T cells was analyzed by gating on $CD3^+$ cells and subsequently on $CD4^+$ cells. To study the effect of TNFα and IL-6 on TGFβ-mediated suppression, cells were untreated or pre-treated overnight with TNFα (50 ng/ml), IL-6 (100 ng/ml) or both, CFSE labeled and cultured in the presence or absence TNFα and IL-6 with or without TGFβ.

Methylation of FOXP3 Treg-specific demethylated region (TSDR)

To determine methylation of FOXP3 TSDR, male HC and JIA patients were included. DNA was isolated from sorted $CD4^+CD25^+CD127^{low}$ Treg, using QiaAmp DNA Mini Kit (QIAGEN). Demethylation of the FOXP3 TSDR was determined as previously described[26].

Flow cytometry

To detect intracellular cytokine production, cells were stimulated for 4.5 hours with PMA (20 ng/ml; MP Biomedicals) and ionomycin (1 μg/ml; Calbiochem), with Golgistop (1/1500; BD Biosciences) added for the last 4 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. After surface staining, cells were washed twice in FACS buffer and acquired directly, or fixed, permeabilized, and intracellularly stained using antihuman FOXP3 staining set (eBioscience, according to the manufacturer's instructions). To stain for phosphorylated PKB/c-akt, cells were fixed, permeabilized and stained using BD Phosflow method according to the manufacturer's instructions. Cells were acquired on FACSCalibur or FACSCanto II and analyzed using CellQuest Version 3.3 or FACS Diva Version 6.13 software, respectively (all BD Biosciences). All antibodies used for flow cytometry are described in the Supplemental Information.

Statistical analysis

For statistical analysis of multiple groups, 1-way ANOVA, or in case of unequal variances, Kruskal-Wallis test was used. Bonferroni or Dunns posthoc test were used to compare between selected groups and Dunnet posthoc test to compare all groups versus a control group. To analyze paired patient samples, paired T test, or in case of unequal variances, Wilcoxon matched pairs test were used. P values below 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism Version 5.03 (Graphpad Software).

RESULTS

Incomplete suppression of T cell proliferation at the site of autoimmune inflammation

To study Treg function at the site of autoimmune inflammation, mononuclear cells were isolated from the inflamed synovium of patients with oligoarticular JIA. Consistent with previous reports[18,19] Treg numbers were enriched at the site of inflammation in these patients (Figures 1A and 1B), whereas Treg levels in PB did not differ between patients and HC (Figure 1B). To investigate suppressive capacity, $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry[5,6] (Supplemental Figure 1A) and functionally analyzed in *in vitro* suppression assays. FOXP3 analysis consistently revealed a high percentage of $FOXP3^+$ cells within the sorted $CD4^+CD25^+CD127^{low}$ population, which did not differ between SFMC (84% ± 9.1%) and PBMC of both patients (81% ± 3.9%) and HC (82% ± 7.6%). However, when synovial fluid (SF) derived Treg were co-cultured with effector cells and proliferation of $CD4^+$ and $CD8^+$ T cells was analyzed, only a minor reduction in the percentage of proliferating cells was observed (Figure 1C) and this suppression of both $CD4^+$ (Figure 1D) and $CD8^+$ T cell proliferation (Figure 1E) was significantly reduced in SFMC (white bars) compared with PBMC of patients (gray bars) and HC (black bars). In contrast, no difference in suppression was observed between PBMC from JIA patients and PBMC from HC. To control for cell density, effector cells instead of Treg were added, which did not result in suppression (Figure 1D-E; +eff). These data demonstrate that, locally, at the site of autoimmune inflammation, proliferation of both $CD4^+$ and $CD8^+$ T cells is poorly controlled by Treg.

Deficient inhibition of cell proliferation is caused by resistance of effector cells to suppression

Both reduced functioning of Treg as well as resistance of effector cells to suppression could play a role in the incomplete control of proliferation of effector cells from the site of inflammation[11]. Phenotypical analysis of paired patient samples revealed that FOXP3 content per cell (mean fluorescence intensity (MFI)) was increased in SF derived $CD4^+CD25^+CD127^{low}$ Treg compared with PB Treg (Supplemental Figures 1B and 1C). In addition, the percentage of cells with demethylated FOXP3 TSDR was not different for Treg from SF (Supplemental Figure 1D), suggesting that these cells do not display decreased stability. Other Treg markers, such as, the percentage of CTLA-4 expressing cells (Supplemental Figure 1E) and both the percentage of GITR expressing cells as well as GITR content per cell (MFI; Supplemental Figure 1F) were enhanced in SF $CD4^+FOXP3^+$ Treg compared with PB Treg. Thus, SF derived Treg are stable and show enhanced expression of functional and activation markers suggesting that these cells are not deficient in their suppressive capacity. To confirm this, cross-over experiments were performed, in which SF derived Treg (SF-Treg) were co-cultured with PB effector cells, and vice versa. When SF-

Treg were co-cultured with PBMC (light gray bars), inhibition of $CD4^+$ (Figure 2A) and $CD8^+$ T cell proliferation (Figure 2B) was completely comparable with suppression of PBMC by PB derived Treg (PB-Treg; black bars).

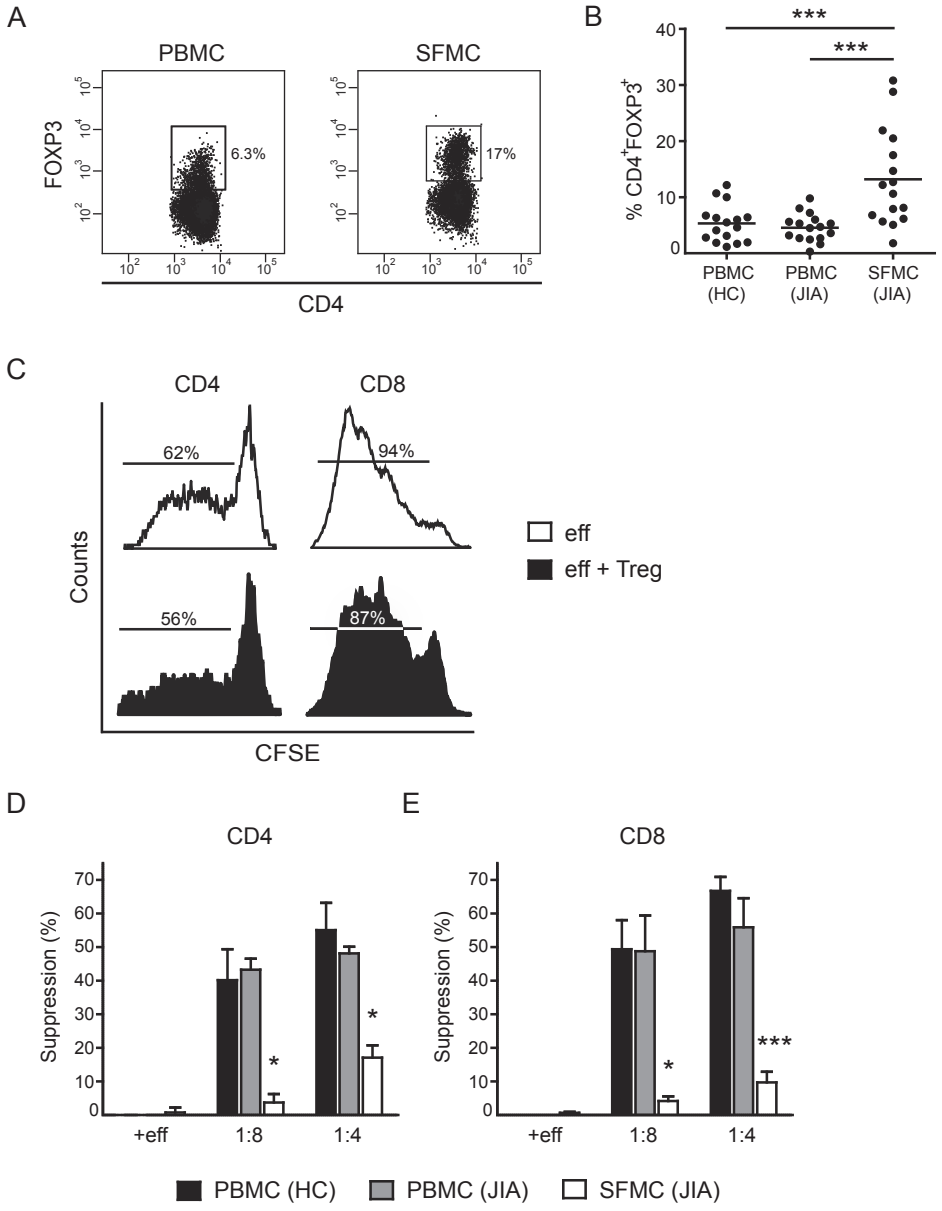

Figure 1. Treg-mediated suppression of T cell proliferation is impaired at the site of autoimmune inflammation.

A-B PBMC and SFMC were stained for CD4 and FOXP3 expression by flow cytometry. **A** Dotplots showing the percentage of $FOXP3^+$ cells within $CD4^+$ cells in paired PBMC (left panel) and SFMC (right panel) of JIA patients,

one representative of n=16. **B** Accumulative data of the percentage of $CD4^+FOXP3^+$ cells in PBMC of HC and paired PBMC and SFMC from JIA patients (n=16), ***p<0.001. **C-E** $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry and co-cultured with CFSE labeled effector cells. At day 4, proliferation of $CFSE^+$ effector cells was analyzed. **C** Proliferation of $CD4^+$ (let panel) and $CD8^+$ (right panel) SFMC in the absence (open histograms) or presence of Treg at 1 to 4 ratio (closed histograms). Percentages indicate the percentage of proliferating cells, one representative of n=3. **D-E** Suppression of $CD4^+$ (**D**) and $CD8^+$ T cell proliferation (**E**) in the presence of Treg at a 1 to 8 ratio (1:8) or Treg (1:4) or additional effector cells (+eff) at a 1 to 4 ratio for PBMC from HC (black bars), PBMC from JIA patients (grey bars) and SFMC from JIA patients (white bars). The results show percentage of suppression in the presence of Treg or additional effector cells relative to effector cells cultured alone. Bars represent mean ± SEM of n=6 PBMC HC, n=2 PBMC JIA and n=3 SFMC JIA, *p<0.05, ***p<0.001 compared to PBMC HC.

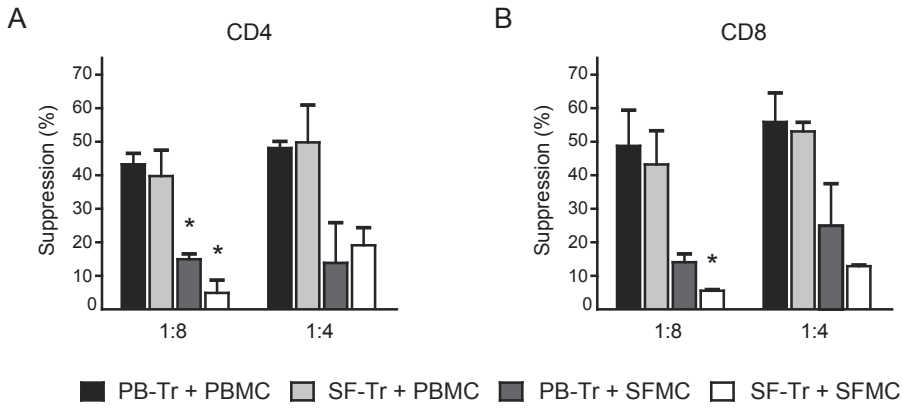

Figure 2. Normal Treg function at the site of inflammation, but resistance of effector cells to suppression of cell proliferation.

A-B PBMC and SFMC were isolated from paired PB and SF samples from JIA patients. $CD4^+CD25^+CD127^{low}$ Treg were sorted from PBMC by flow cytometry and co-cultured with CFSE labeled PBMC (black bars) or SFMC (dark grey bars) at a 1 to 8 (1:8) and 1 to 4 (1:4) ratio. Conversely, $CD4^+CD25^+CD127^{low}$ Treg were sorted from SFMC and co-cultured with CFSE labeled PBMC (light grey bars) or SFMC (white bars). At day 4, suppression of CD4+ (**A**) and CD8+ T cell proliferation (**B**) was measured. The results show percentage of suppression in the presence of Treg relative to effector cells alone. Bars represent mean ± SEM of n=2, *p<0.05 compared to PB-Tr + PBMC.

Thus, Treg from the site of inflammation show similar suppressive capacity to Treg from PB and, in line with their phenotype, are not impaired in their suppressive function. However, when PB-Treg were co-cultured with SFMC (dark gray bars), the level of suppression of both $CD4^+$ (Figure 2A) and $CD8^+$ T cell proliferation (Figure 2B) was markedly reduced compared with PB-Treg cultured with PBMC (black bars). Thus, in the presence of the same PB-Treg, SFMC are less responsive to suppression compared with PBMC. Furthermore, this decrease in suppression was comparable with the decrease in suppression in cultures with both Treg and effector cells from the site of inflammation (SF-Treg + SFMC; white bars). Therefore, the reduced suppression of cell proliferation observed in suppression assays with cells from the site of inflammation is attributable to unresponsiveness of effector cells to suppression.

Impaired control of cytokine production because of resistance of effector cells to suppression

Because we observed impaired Treg-mediated suppression of proliferation of Teff from the site of inflammation, we wondered whether suppression of other Teff functions, such as cytokine production, was also impaired. After 4.5 hours PMA and ionomycin stimulation, $CD4^+$ (upper panel) and $CD8^+$ (lower panel) SFMC (gated as shown in Supplemental Figure 2A) not only express proinflammatory cytokines associated with autoimmune pathology, such as IL-17, TNFα, and IFNγ, but also IL-10 (Figure 3A) and expression of these cytokines was enhanced compared with paired PBMC (Supplemental Figures 2B-E). When suppression assays were performed using PBMC from either HC (Figure 3B) or JIA patients (Figure 3C), the amount of IL-5, IL-13, IL-10, TNFα, and IFNγ in the culture supernatant generally declined when Treg were added at a 1:8 ratio and further decreased with higher numbers of Treg present (1:4). No clear reduction in IL-17 levels was observed, consistent with previous reports[27,28]. Adding additional effector cells instead of Treg (+eff) did not result in a decrease in cytokine levels and Treg cultured alone did not produce significant amounts of cytokines (data not shown). In contrast to these results with PBMC from patients and HC, cytokine levels in SFMC cultures (Figure 3D), did not decrease when Treg were added at a 1:8 ratio and only modestly in the presence of Treg at a 1:4 ratio. When the level of suppression at a 1:4 ratio was calculated for each cytokine, suppression was significantly lower in SFMC (white bars) compared with PBMC of both patients (gray bars) and HC (black bars), whereas, again, there was no clear difference between PBMC of JIA patients and PBMC from HC (Figure 3E). These data demonstrate that a broad range of cytokines produced by effector cells from the site of inflammation are insufficiently controlled by Treg.

To study the role of Treg malfunctioning versus resistance of effector cells to suppression in the incomplete restriction of cytokine production, cross-over experiments were again performed (Figure 3F). In these assays, levels of suppression were clearly reduced when PB-Treg were co-cultured with SFMC (white bars) compared with PB-Treg and PBMC co-cultures (black bars), demonstrating that effector cells from the site of inflammation show resistance to suppression of cytokine production. In contrast, the level of suppression was not affected in SF-Treg and PBMC cultures (gray bars) compared with PB-Treg and PBMC co-cultures, showing that SF-Treg are not impaired in their cytokine suppressive capacity. Together our data show that, despite normal Treg function, suppression of proliferation and cytokine production by cells from the site of autoimmune inflammation is impaired, because of resistance of effector cells to suppression.

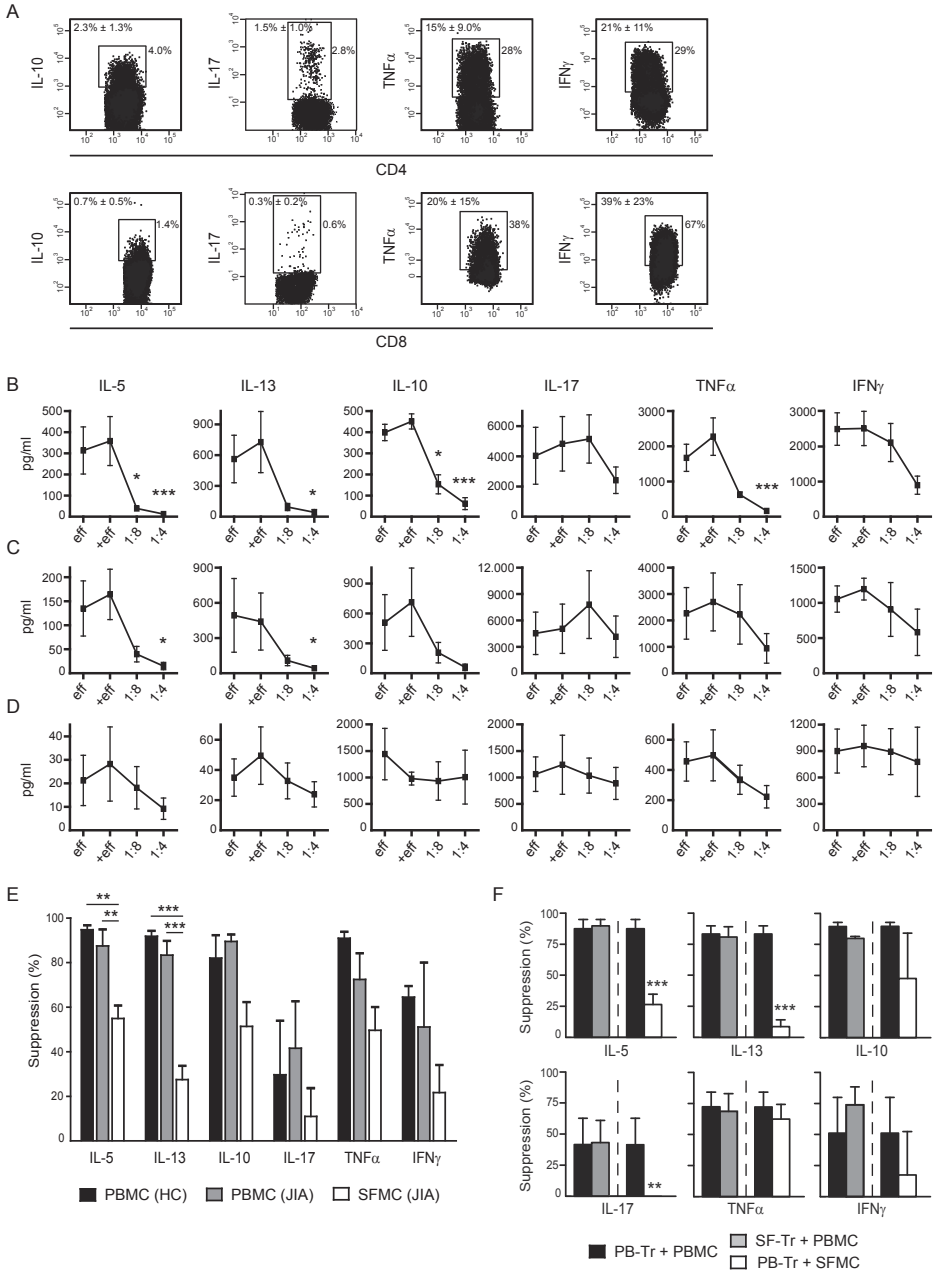

Figure 3. Effector cytokine production at the site of inflammation is insufficiently controlled, because of resistance of effector cells to suppression.

A SFMC were stained for cytokine expression by flow cytometry after 4.5 hrs PMA and Ionomycin stimulation. Dotplots showing the percentage of IL-10, IL-17, TNFα, and IFNγ-positive cells in $CD4^+$ (upper panel) and $CD8^+$ cells (lower panel), 1 representative of n=4. Percentages indicate the percentage of positive cells from representative data, or, in upper line, average percentage ± SD from accumulative data of n=4. **B-F** $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry and co-cultured with effector cells. At day 4, cytokine production in the culture supernatant was analyzed. **B-D** IL-5, IL-13, IL-10, IL-17, TNFα, and IFNγ levels in the absence (eff) or presence of Treg at a 1 to 8 ratio (1:8) or Treg (1:4) or additional effector cells (+eff) at a 1 to 4 ratio for PBMC from HC (**B**),

PBMC from JIA patients (**C**) and SFMC from JIA patients (**D**). Data represent mean cytokine levels in pg/ml ± SEM of n=4 PBMC HC, n=4 PBMC JIA and n=8 SFMC JIA, *p<0.05, ***p<0.001 compared to effector cells (eff). **E-F** Percentage suppression of IL-5, IL-13, IL-10, IL-17, TNFα, and IFNγ production in the presence of Treg at a 1 to 4 ratio relative to effector cells alone. **E** Percentage suppression in co-cultures of Treg and effector cells from PBMC of HC (black bars), PBMC of JIA patients (grey bars) and SFMC of JIA patients (white bars). Bars represent mean ± SEM of n=4 PBMC HC, n=4 PBMC JIA and n=8 SFMC JIA, **p<0.01, ***p<0.001. **F** Percentage suppression in co-cultures of PB derived Treg and PBMC (black bars), PB Treg and SFMC (white bars), or SF derived Treg and PBMC (grey bars). Bars represent mean ± SEM of n=4, **p<0.01, ***p<0.001 compared to PB-Tr + PBMC.

Resistance of autoimmune effector cells to suppression is not caused by a memory phenotype

To mimic the *in vivo* situation as closely as possible, total mononuclear cells from the site of inflammation were used as effector cells in our assays. These SFMC differ in cellular composition from PBMC, which could contribute to the reduced responsiveness of these cells to suppression. Therefore, we carefully phenotyped paired SFMC and PBMC *ex vivo* by flow cytometry to gain insight into these differences in cellular constitution. We observed a significant increase in $CD45RA^{-}CD45RO^{+}$ memory cells in SFMC compared with PBMC in both $CD4^{+}$ ($p < .01$) and $CD8^{+}$ T cells ($p < .05$; Figures 4A-C), which was accompanied by a decrease in the presence of $CD45RA^{+}CD45RO^{-}$ naive cells. In mice, it has been shown that memory effector cells are more resistant to Treg-mediated suppression compared with naive cells[29]. Therefore the high numbers of memory T cells present among effector cells from the site of inflammation might influence the responsiveness of these cells to suppression. To investigate this, we determined whether in our assays memory $CD4^{+}$ T cells sorted from PBMC of HC are less responsive to suppression compared with naive cells (Figures 4D and 4E). We found that in the absence of Treg (open histograms) $CD25^{-}CD45RA^{-}CD45RO^{+}$ memory $CD4^{+}$ effector cells (Figure 4D; right panel) showed enhanced proliferation compared with $CD25^{-}CD45RA^{+}CD45RO^{-}$ naive effector cells (Figure 4D; middle panel). However, on addition of Treg (filled histograms), T cell proliferation was significantly reduced in both memory and naive cells (Figure 4D). Similarly, IL-5, IL-13, TNFα, and IFNγ production was suppressed in both memory (white bars) and naive cells (gray bars), albeit to a slightly lower extend in memory cells compared with naive and total cells (Figure 4E; black bars). In conclusion, consistent with previous results[29] memory cells show reduced responsiveness to suppression, but, the difference in suppression is minimal and not comparable with the highly diminished suppression observed in SFMC. Therefore, the general memory phenotype of cells at the site of autoimmune inflammation cannot explain their resistance to suppression.

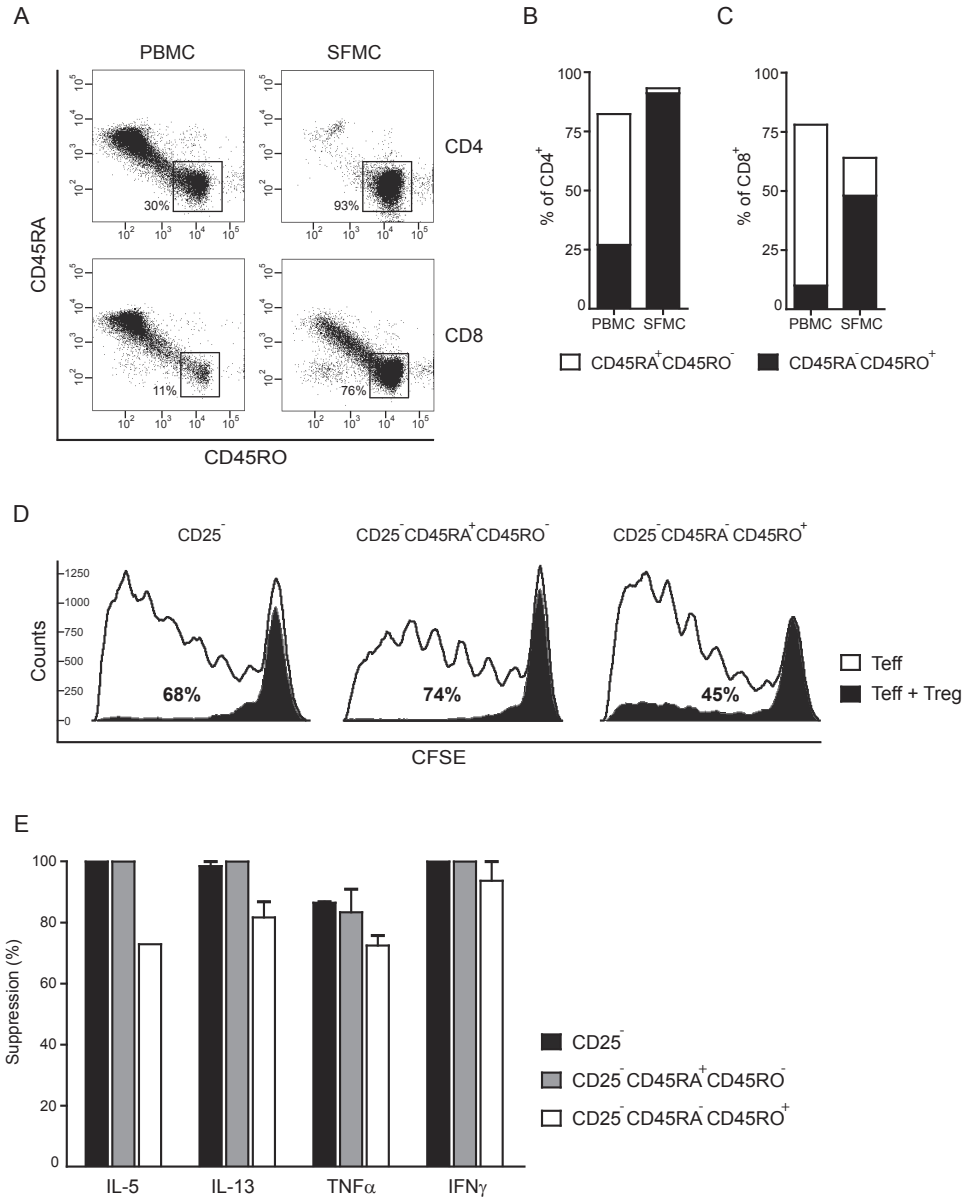

Figure 4. Effector cells from the site of inflammation have a general memory phenotype, which is not the cause of their resistance to suppression.

A-C Paired PBMC and SFMC were stained for CD45RA and RO expression by flow cytometry. **A** Dotplots showing the percentage of $CD45RA^{-}CD45RO^{+}$ memory cells in PBMC (left panel) and SFMC (right panel) $CD4^{+}$ (upper panel) and $CD8^{+}$ T cells (lower panel), 1 representative of n=4. **B-C** Percentage of $CD45RA^{-}CD45RO^{+}$ memory (black bars) and $CD45RA^{+}CD45RO^{-}$ naive cells (white bars) in $CD4^{+}$ T cells (**B**) and $CD8^{+}$ T cells (**C**) of paired SFMC and PBMC of n=4. **D-E** $CD4^{+}CD25^{+}CD127^{low}$ Treg were sorted by flow cytometry and co-cultured with CFSE labeled total, naïve, and memory effector T cells at a 1:2 ratio. At day 5, proliferation of $CFSE^{+}$ effector cells (**D**) and cytokine production in the culture supernatant (**E**) was analyzed. **D** CFSE profile of $CD4^{+}CD25^{-}$ total effector

T cells (left panel), $CD4^+CD25^-CD45RA^+CD45RO^-$ naïve effector T cells (middle panel) and $CD4^+CD25^-CD45RA^-CD45RO^+$ memory effector T cells (right panel) cultured in the absence (open histograms) or presence of Treg (closed histograms). Percentages indicate the percentage suppression of cell proliferation in the presence Treg relative to effector cells alone, one representative of n=2. **E** Percentage suppression of IL-5, IL-13, TNFα, and IFNγ production by $CD4^+CD25^-$ total effector T cells (black bars), $CD4^+CD25^-CD45RA^+CD45RO^-$ naïve effector T cells (grey bars) and $CD4^+CD25^-CD45RA^-CD45RO^+$ memory effector T cells (white bars) in the presence of Treg relative to effector cells alone. Bars represent mean ± SEM of n=2.

Resistance to suppression not caused by activation status of APCs

In RA patients it was demonstrated that monocytes from the site of inflammation displayed a more activated phenotype that interfered with Treg-mediated suppression[20]. Because the effector cell population in our experiments contained monocytes, we investigated whether changes in monocyte number and/or activation could explain the resistance of SFMC to suppression. Monocytes were gated on forward side scatter (Supplemental Figure 3) and phenotypically analyzed. No difference in percentage of monocytes between SFMC (24% ± 12%) and PBMC (23% ± 12%) was observed. However, in line with previous reports in RA[20] and JIA[30], monocytes from SF displayed higher expression of CD80, CD86 and HLA-DR (Figures 5A and 5B), which could interfere with Treg inhibition. Therefore, we investigated whether in our *in vitro* suppression assays $CD3^-$ APCs contribute to resistance of SFMC to suppression or whether this resistance solely resides within the $CD3^+$ T cell population (Figures 5C and 5D). When SF $CD3^-$ APCs were co-cultured with SF $CD3^+$ T cells in the presence of Treg (gray bars), suppression of both $CD4^+$ (Figure 5C) and $CD8^+$ T cell proliferation (Figure 5D) was again reduced compared with cultures containing PB-derived APCs and T cells (black bars). However, this reduction in suppression was similar when SF T cells were co-cultured with PB APCs (white bars), clearly demonstrating that the resistance of SFMC to suppression resides within $CD3^+$ T cells and is not caused by enhanced activation of SF APCs.

Hyperactivation of PKB/c-akt leads to resistance of effector cells to suppression

In addition to a general memory phenotype, $CD4^+$ cells from the site of inflammation expressed higher levels of proliferation and activation markers compared with cells from PB (Supplemental Figure 4), indicating that these cells are in a highly activated state. Because the PI3K-PKB/c-akt module is an important intracellular signaling pathway involved in T cell activation[31] and in mice hyperactivation of this pathway has been shown to induce resistance to suppression[32], we investigated whether hyperactivation of this pathway may be responsible for the resistance of SFMC to suppression. First, we analyzed PKB/c-akt activation in paired SFMC and PBMC *ex vivo* by measuring the level of phosphorylated PKB/c-akt, a measure of activation status, by flow cytometry (Figure 6A). We found that compared with HC (black bars), the level of phosphorylated PKB/c-akt was unchanged in $CD4^+$ T cells from the PB of JIA patients (gray bars), however, cells from the site of inflammation (white bars) clearly showed enhanced phosphorylated PKB/c-akt levels (Figure 6B). Thus, Teff at the site of autoimmune inflammation show increased levels of PKB/c-akt activation.

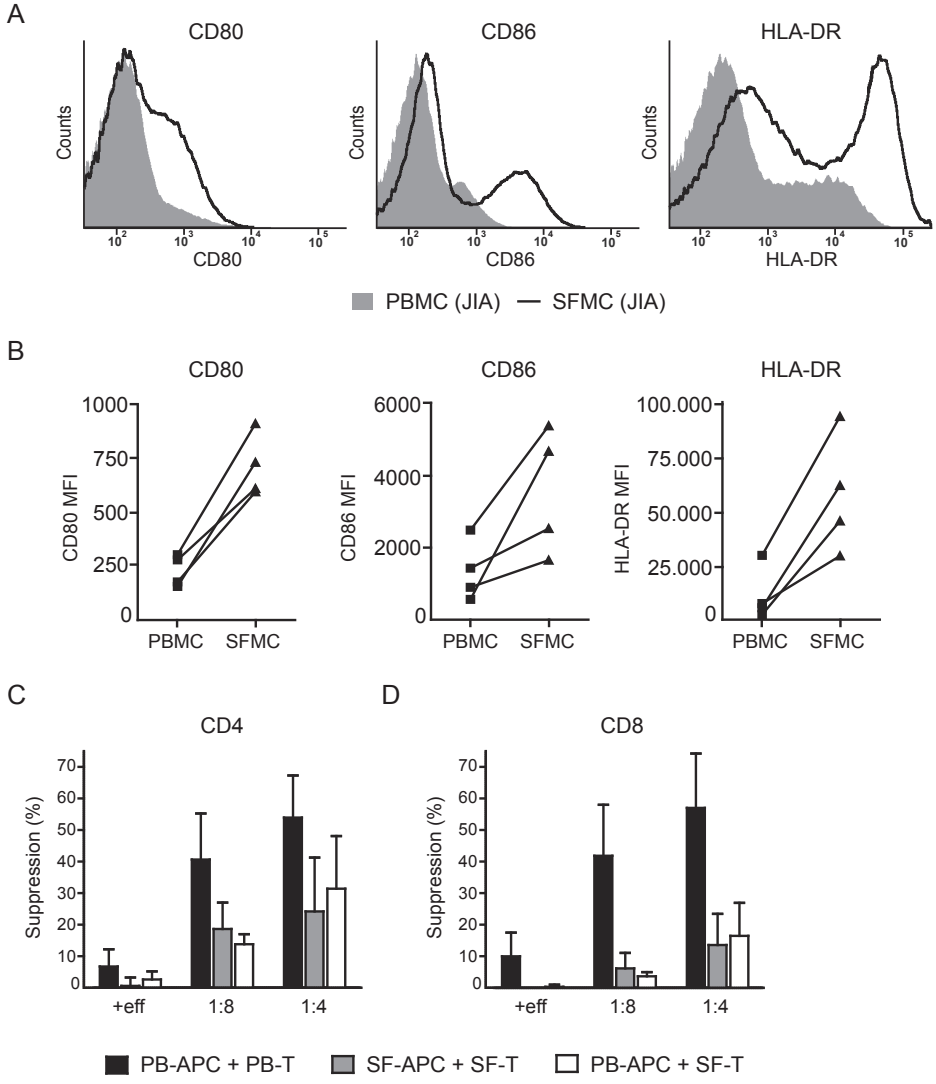

Figure 5. Resistance of SFMC to suppression is not caused by activation status of APCs.

A-B Monocytes from paired PBMC and SFMC of JIA patients were analyzed for CD80, CD86 and HLA-DR expression by flow cytometry. **A** Histograms showing CD80 (left panel), CD86 (middle panel) and HLA-DR (right panel) fluorescence intensity in paired PBMC (solid grey) and SFMC (black line), one representative of n=4. **B** MFI of CD80 (left panel), CD86 (middle panel) and HLA-DR (right panel) in monocytes from paired PBMC and SFMC of n=4. **C-D** $CD3^+$ T cells, $CD3^-$ APC and $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry. PB T cells were co-cultured with PB APC (black bars) and SF T cells were co-cultured with SF APC (grey bars) or PB APC (white bars) in the absence or presence of SF Treg at a 1 to 8 ratio (1:8) or Treg (1:4) or additional effector cells (+eff) at a 1 to 4 ratio. Suppression of $CD4^+$ (**C**) and $CD8^+$ T cell proliferation (**D**) was measured. The results show percentage of suppression in the presence of Treg or additional effector cells relative to effector cells alone. Bars represent mean ± SEM of n=3.

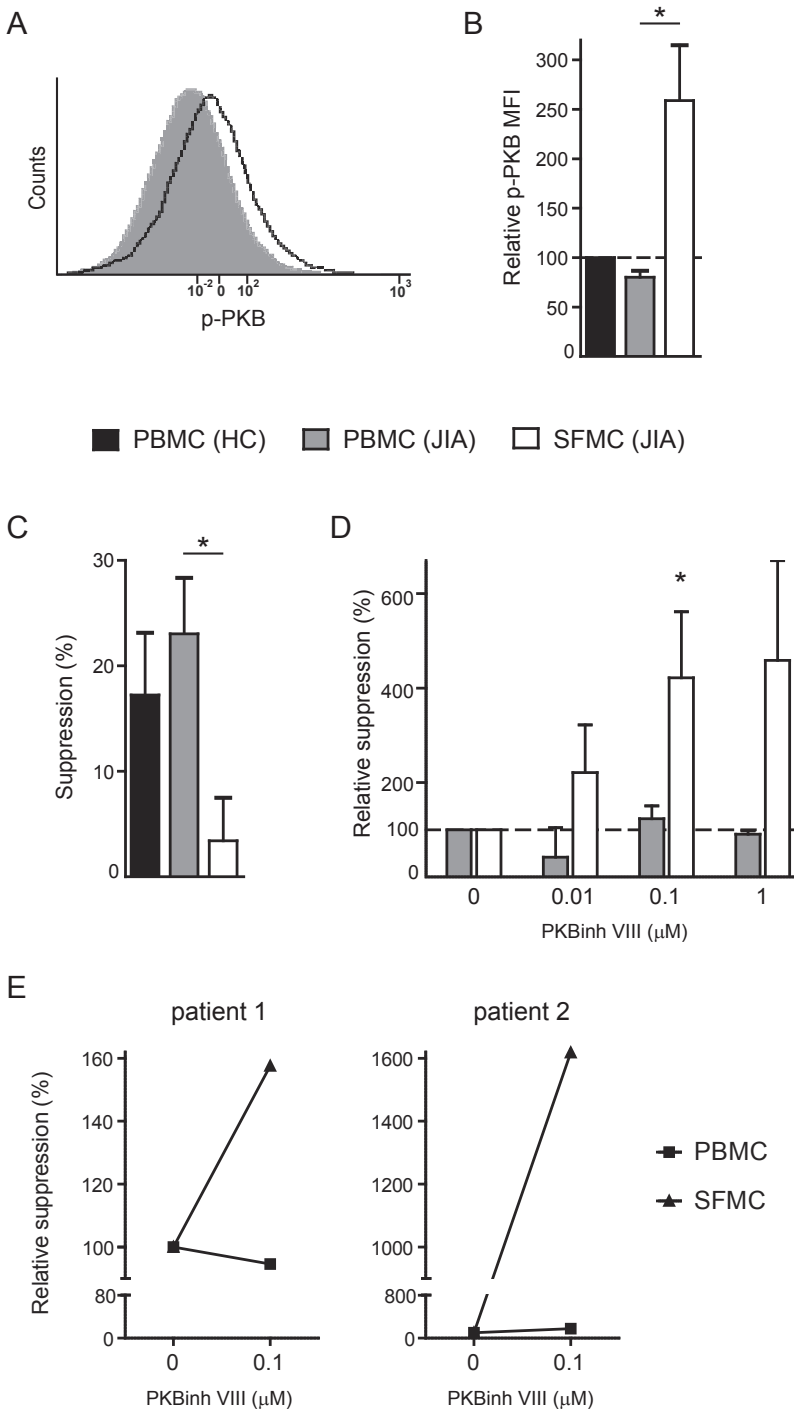

Figure 6. PKB/c-akt hyperactivation causes resistance of effector cells to suppression.

A-B PBMC and SFMC were stained for phosphorylated PKB/c-akt expression by flow cytometry. **A** Histogram showing phosphorylated PKB/c-akt (p-PKB) fluorescence intensity in $CD4^+$ T cells from paired PBMC (solid grey) and SFMC (black line), one representative of n=3. **B** MFI of phosphorylated PKB/c-akt (p-PKB) in $CD4^+$ T cells from paired PBMC (grey bar) and SFMC (white bar) of JIA patients relative to PBMC from HC (black bar). Bars represent mean ± SEM of n=3, *p<0.05. **C-D** CFSE labeled PBMC and SFMC were cultured in the presence or absence of recombinant human TGFβ1 (40 ng/ml) and increasing concentrations of PKB/c-akt inhibitor VIII (PKBinh VIII) (0, 0.01, 0.1, 1 μM). At day 5, proliferation of CD4+ T cells was analyzed. **C** TGFβ-mediated suppression of $CD4^+$ T cell proliferation for PBMC from HC (black bars) and PBMC (grey bars) and SFMC (white bars) from JIA patients. The results show percentage of suppression in the presence of TGFβ relative to cells cultured without TGFβ. Bars represent mean ± SEM of n=3 PBMC HC, n=4 PBMC JIA, and n=5 SFMC JIA, *p<0.05. **D** TGFβ-mediated suppression of $CD4^+$ T cell proliferation for paired PBMC (grey bars) and SFMC (white bars) in the presence of increasing concentrations of PKB/c-akt inhibitor VIII. The data show the change in TGFβ-mediated suppression for each concentration of PKB/c-akt inhibitor relative to cultures without PKB/c-akt inhibitor. Bars represent mean ± SEM of n=3, *p<0.05 compared to 0 μM PKB/c-akt inhibitor VIII. **E** $CD4^+CD25^+CD127^{low}$ Treg were sorted from SFMC by flow cytometry and co-cultured with CFSE labeled PBMC (squares) or SFMC (triangles) at a 1 to 4 ratio in the presence or absence of PKB/c-akt inhibitor VIII (PKBinh VIII) (0.1 μM). At day 4, Treg mediated suppression of $CD4^+$ T cell proliferation was analyzed. The data show the change in Treg mediated suppression in the presence PKB/c-akt inhibitor relative to cultures without PKB/c-akt inhibitor.

In mice, expression of constitutively activated PKB/c-akt renders effector cells resistant to both Treg- as well as TGFβ-mediated suppression[33], therefore, we investigated whether SFMC, showing enhanced PKB/c-akt activation, are also refractory to TGFβ-mediated suppression. Proliferation of $CD4^+$ T cells from PBMC of HC (black bars) and JIA patients (gray bars) was clearly suppressed by the presence of TGFβ, whereas, only very low levels of suppression were detected in SF $CD4^+$ T cells (white bars; Figure 6C), showing that these cells are resistant to TGFβ-mediated suppression. To investigate whether increased PKB/c-act activation directly leads to resistance of these cells to suppression, we determined whether inhibiting this kinase restored responsiveness to TGFβ. Culture of SFMC in the presence of a specific PKB/c-akt inhibitor dose-dependently decreased PKB activation in these cells as measured by flow cytometry (Supplemental Figure 5A). As a result, TGFβ-mediated suppression of SFMC (white bars), but not already responsive PBMC (gray bars), was enhanced in the presence of this inhibitor (Figure 6D).

In addition, PKB/c-akt inhibition restored responsiveness of SFMC (triangles) to Treg-mediated suppression, without affecting suppression of PBMC (squares; Figure 6E). Importantly, PKB/c-akt inhibitor treatment did not affect proliferation of SFMC in the absence of TGFβ and Treg (Supplemental Figure 5B), showing that it specifically targets the unresponsiveness of these cells to suppression, and does not inhibit proliferation in general. Together these data clearly demonstrate that the resistance of effector cells to suppression at the site of inflammation is caused by PKB/c-akt hyperactivation. Furthermore, by pharmacologically targeting this pathway responsiveness of the cells to suppression can be restored.

TNFα and IL-6 present at the site of inflammation induce PKB/c-akt activation and resistance to suppression

We are the first to show that PKB/c-akt hyperactivation occurs under physiologic conditions at the site of inflammation in human autoimmune disease. It is therefore intriguing to identify the cause of this enhanced PKB/c-akt activation. To investigate whether soluble factors present in the inflammatory environment lead to enhanced PKB/c-akt activation, we first measured the expression of proinflammatory cytokines in PB plasma of HC and paired PB plasma and SF of JIA patients (Figure 7A). In line with previous reports[25] and regarding effectiveness of TNFα- and IL-6–blocking strategies in arthritis[34], we observed elevated levels of TNFα and IL-6 in SF of JIA patients. Incubation of PBMC from HC with these cytokines resulted in an up-regulation of p-PKB (Figure 7B) and reduced responsiveness to TGFβ-mediated suppression (Figure 7C), which was significant when both TNFα and IL-6 were added ($p < .05$). Together, these data identify TNFα and IL-6 as proinflammatory factors that contribute to enhanced PKB/c-akt activation and subsequent resistance to suppression at the site of human autoimmune inflammation.

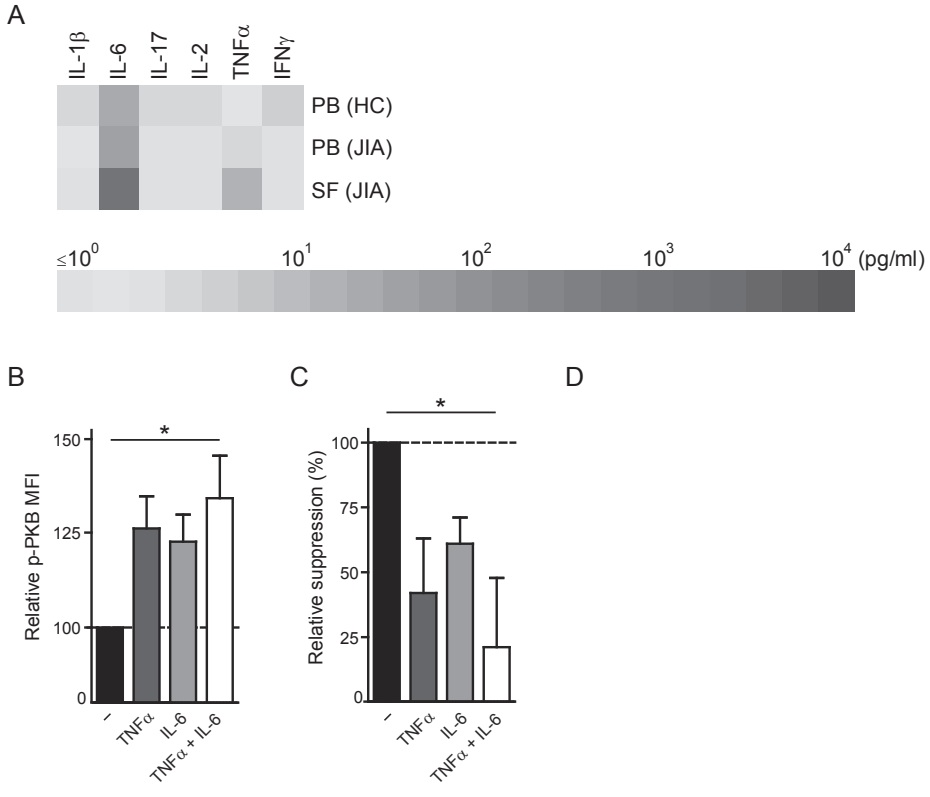

Figure 7. TNFα and IL-6 present at the site of inflammation induce PKB/c-akt activation resulting in resistance to suppression.

A IL-1β, IL-6, IL-17, IL-2, TNFα and IFNγ expression in n=4 PB plasma of HC and n=8 paired PB plasma and SF of JIA patients measured by Luminex. **B-C** PBMC from HC were untreated or incubated overnight with TNFα (50 ng/ml), IL-6 (100 ng/ml) or both TNFα and IL-6. After incubation period, cells were stained for phosphorylated PKB/c-akt expression by flow cytometry (**B**) or CFSE labeled and cultured in the presence or absence of recombinant human TGFβ1 (40 ng/ml) and TNFα and IL-6 to measure TGFβ mediated suppression (**C**). **B** MFI of phosphorylated PKB/c-akt (p-PKB) in $CD4^+$ T cells in the presence of TNFα (dark grey bars), IL-6 (light grey bars) or both (white bars) relative to cultures without cytokines added (black bars). Bars represent mean ± SEM of n=5, *$p<0.05$. **C** TGFβ-mediated suppression of $CD4^+$ T cell proliferation in the absence (black bars) or presence of TNFα (dark grey bars), IL-6 (light grey bars) or both (white bars). The data show the change in TGFβ-mediated suppression in the presence of cytokines compared to cultures without cytokines added. Bars represent mean ± SEM of n=5, *$p<0.05$.

DISCUSSION

After 15 years of research into Treg biology the main question is whether these cells can be used in treatment of autoimmune disease. To resolve this issue more information on Treg function at the site of autoimmune inflammation in humans is required. Here we studied Treg function in patients with JIA and show that both in the periphery and at the site of inflammation, Treg are not deficient in number and function. Nevertheless, effector cells from the site of inflammation are poorly controlled, because these cells are resistant to suppression. We further demonstrate that this unresponsiveness to suppression is, at least partially, caused by PKB/c-akt hyperactivation and can be restored by specific PKB/c-akt inhibition. These data therefore identify PKB/c-akt as a potential novel target for the treatment of autoimmune disease.

In PB of JIA patients, no change in the numbers of Treg was observed and these cells suppressed both cell proliferation and cytokine production similar to Treg from HC. In contrast, defective Treg function has previously been described in peripheral blood of RA patients[12,15]. Perhaps differences in Treg function in the periphery are less pronounced in oligoarticular JIA, because of its relative mild and local pathology. Alternatively, because CD127 has become available as an additional marker to isolate Treg[5,6], the differences in results could also be explained by differences in Treg purity. Other studies are, however, in agreement with our data and report no difference in Treg function in patients with established arthritis[14,16,35].

At the site of inflammation in patients with JIA, increased numbers of Treg were present and these cells showed normal suppressive capacity. Thus, we and others[14,16,18,19,36] have observed that Treg from the site of inflammation are fully functional. However, by mimicking the *in vivo* situation as closely as possible and using total mononuclear cells as effector cells in our assays, we were able to demonstrate that these Treg still failed to control effector cells from the site of inflammation. This was caused by reduced responsiveness of these effector cells to suppression. In other experimental models of autoimmune disease and some patient studies, most profoundly systemic lupus erythematosus and type 1 diabetes, resistance of effector cells to suppression has also been described[22,37-42].

However, in oligoarticular JIA unresponsiveness of effector cells to suppression occurs locally, at the site of inflammation, and not systemically. In genetically prone mice effector cells become resistant to suppression before clinical overt disease[37,39], suggesting that this phenomenon acts early in disease pathology and is therefore an important target in controlling autoimmune inflammation. However, to target this resistance of effector cells effectively, the underlying mechanism needs to be clarified.

Here, we show that autoimmune inflammatory effector cells are resistant to both TGFβ- as well as Treg-mediated suppression and that this resistance does not result from a general memory phenotype of the cells or activation status of APCs. Instead, hyperactivation of PKB/c-akt is responsible for the unresponsiveness to suppression, as $CD4^+$ Teff from the site of inflammation showed increased PKB/c-akt phosphorylation and selective inhibition of this kinase restored responsiveness of cells to suppression. We are the first to show that PKB/c-akt is involved in resistance to suppression in human autoimmune disease, consistent with findings in mice[33,43].

Phosphorylation and activation of PKB/c-akt is regulated by the generation of lipid products by phosphoinositide 3 kinases (PI3Ks) and PI3Ks become activated in lymphocytes upon binding of antigens, costimulatory molecules, cytokines, and chemokines[32,44]. PI3K-PKB activation by chemokines is, however, both rapid and transient[44], therefore, enhanced PKB/c-akt activation in cells at the site of inflammation is likely to result from either TCR, CD28, or cytokine signaling. We show that TNFα and IL-6 are elevated in synovial fluid of JIA patients and induce PKB/c-akt activation and resistance to suppression, in line with previous reports showing that these cytokines can confer resistance to suppression in mice[22,23]. Other cytokines and CD28 signaling can activate the PI3K-PKB pathway as well[45], therefore it is likely that a combination of environmental factors contributes to resistance to suppression at the site of human autoimmune inflammation. However, given the effectiveness of TNFα and IL-6 blockade in the treatment of arthritis[34], it is intriguing to speculate that TNFα and IL-6 are critical in inducing resistance to suppression and that the effectiveness of these strategies can partially be explained by inhibiting PKB/c-akt activation.

Independent of the initial cause of PKB/c-akt activation, we show that selective PKB/c-akt inhibition is sufficient to restore responsiveness of effector cells to suppression, making this kinase an attractive target for therapeutic intervention. Clinical efficacy of PI3K inhibition, upstream of PKB/c-akt, has already been demonstrated in experimental models of arthritis[46] and systemic lupus erythematosus[47]. Targeting this pathway might therefore be beneficial in a wide range of autoimmune inflammatory conditions. However, for a PKB/c-akt targeted approach to be successful negative effects on Treg function must be prevented. In human PB-derived Treg PKB/c-akt was hypoactivated and this hypoactivation was essential for their suppressive function[48]. Therefore, PKB/c-akt inhibition might not negatively affect Treg function and could even enhance de novo generation of Treg[49,50]. As a result, selective PKB/c-akt inhibition might be especially effective when combined with

a Treg enhancing strategy, ensuring responsiveness of effector cells to suppression and simultaneously creating an environment suited for Treg induction.

In conclusion, the data presented in this study provide new insights in the pathology of autoimmune disease and raise important therapeutic implications. Our findings argue for a Teff instead of Treg-targeted approach to control autoimmune inflammation. More specifically, responsiveness of effector cells to suppression should be restored by selectively inhibiting PKB/c-akt activation.

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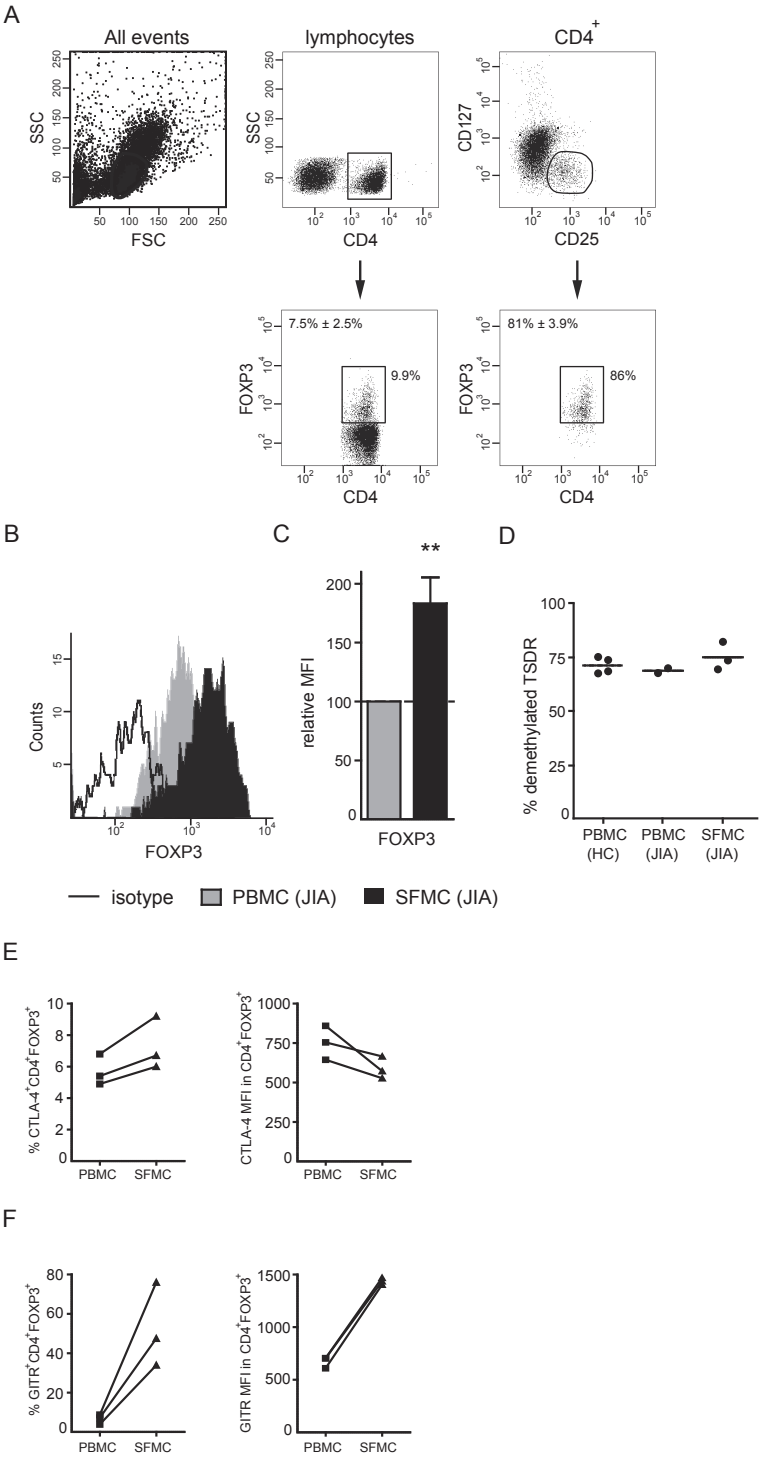
SUPPLEMENTAL INFORMATION

Antibodies used for flow cytometry.

To stain cells for sorting of Treg, Teff and APCs, the following monoclonal antibodies were used: anti-CD3 PerCp/Cy5.5 (UCHT1, Biolegend), anti-CD4-FITC, anti-CD4-APC or anti-CD4-PcBlue (all clone RPA-T4, eBioscience), anti-CD25-APC, anti-CD25-PE/Cy7 (both clone M-A251, BD Biosciences) or anti-CD25-PerCp/Cy5.5 (BD96, Biolegend), anti-CD45RA-FITC (JS-83, eBioscience), anti-CD45RO PcBlue (UCHL-1, Biolegend), and anti-CD127-PE (hIL-7R-M21, BD Biosciences). To check for FOXP3 expression in sorted $CD4^+CD25^+CD127^{low}$ Treg, anti-CD4-PercP (SK3, BD Biosciences) or anti-CD4 PerCP/Cy5.5 (RPA-T4, Biolegend), anti-CD25-FITC (MA251), anti-CD127-PE (hIL-7R-M21, both BD Biosciences) and anti-FOXP3-PcBlue or anti-FOXP3-eFluor450 (both clone PCH101, eBioscience) were used. Anti-CD3-PerCp (SK7, BD Biosciences) or anti-CD3 PerCp/Cy5.5 (UCHT1), anti-CD4-PcBlue (RPA-T4, both from Biolegend) and anti-CD8-APC (SK1, BD Biosciences) were used to distinguish between $CD4^+$ and $CD8^+$ T cells within CFSE-labeled effector cells. For phenotyping, paired SFMC and PBMC were stained with the following antibodies: anti-CD4-PE (RPA-T4), anti-CD4-PerCp (SK3, both from BD Biosciences) or anti-CD4-PcBlue (RPA-T4, Biolegend), anti-CD8-PerCp (SK1), anti-CD25-PE/Cy7 (M-A251), anti-CD62L-FITC (Dreg 56), anti-CD69-APC (FN50), anti-CD80-FITC or anti CD80-PE (both L307.4), anti-CD86-FITC or anti-CD86-PE (both IT2.2), anti HLA-DR-PE/Cy7 (L243), anti-Ki67-FITC (B56, all obtained from BD Biosciences), anti-CD45RA-FITC (JS-83, eBioscience), anti-CD45RO-PcBlue (UCHL-1, Biolegend), anti-FOXP3-APC or anti-FOXP3-PcBlue (both clone PCH101, eBioscience), anti-IL-10-PE (JES3-19F1, BD Biosciences), anti-IL-17-PE (eBio64DEC17, eBioscience), anti-TNFa-APC (Mab11, Biolegend), anti-IFNg-FITC (25723,11), anti-CD152(CTLA-4)-APC (BNI3, both BD Biosciences), and anti-GITR-FITC (110416, R&D Systems). To measure phosphorylated PKB/c-akt in $CD4^+$ T cells, cells were stained with anti-CD3-PE/Cy7 (UCHT1), anti-CD4-PcBlue (RPA-T4, both Biolegend), and anti-akt (PKB)-PE (pS473) (BD Biosciences).

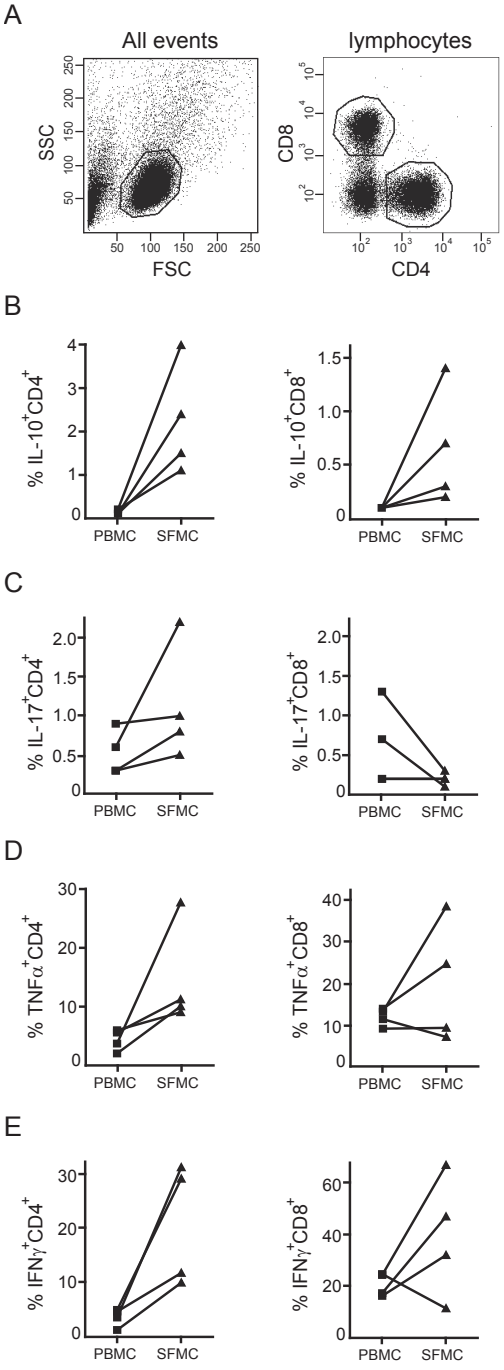
Supplemental figures

Supplemental Figure 1 shows the purity of sorted Treg and *ex vivo* phenotype of SF and PB derived Treg. Supplemental Figure 2 represents intracellular cytokine production in paired PBMC and SFMC. Supplemental Figure 3 depicts gating strategy applied to analyze monocytes by flow cytometry. Supplemental Figure 4 shows *ex vivo* expression of proliferation and activation markers in $CD4^+$ T cells of paired SFMC and PBMC. Supplemental Figure 5 represents p-PKB MFI and proliferation of $CD4^+$ T cells upon treatment with PKB/c-akt inhibitor.



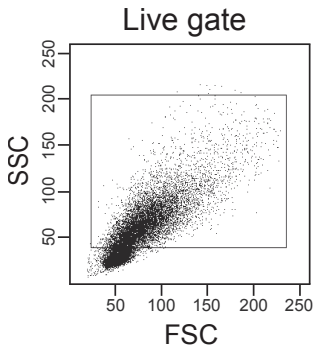
Supplemental Figure 1. Purity of sorted Treg and *ex vivo* phenotype of SF and PB derived Treg, showing enhanced FOXP3 content per cell in SF CD4$^+$CD25$^+$CD127^{low} Treg.

A Sorting strategy applied to isolate $CD4^+CD25^+CD127^{low}$ Treg by flow cytometry as described in Methods. The areas depicted represent the gates used to sort the cells or analyze the percentage of $FOXP3^+$ cells. **B** Histogram showing FOXP3 fluorescence intensity in sorted SF derived $CD4^+CD25^+CD127^{low}$ Treg (filled black histogram), paired PB derived $CD4^+CD25^+CD127^{low}$ Treg (filled gray histogram) and isotype control (open histogram), one representative of n=14. **C** FOXP3 MFI in sorted SF derived $CD4^+CD25^+CD127^{low}$ Treg (black bar) relative to paired PB derived $CD4^+CD25^+CD127^{low}$ Treg (gray bar). Bars represent means ± SEM of n=14, $^{**}p<0.01$. **D** Percentage of cells with demethylated FOXP3 TSDR in sorted $CD4^+CD25^+CD127^{low}$ Treg from n=4 PBMC of HC, n=3 PBMC of JIA patients (2 patients pooled) and n=3 SFMC of JIA patients. **E** Percentage of CTLA-4 content per cell (right panel) in paired $CD4^+FOXP3^+$ PBMC (squares) and SFMC (triangles) of n=3. **F** Percentage of GITR expressing cells (left panel) and GITR content per cell (right panel) in paired $CD4^+FOXP3^+$ PBMC (squares) and SFMC (triangles) of n=3.



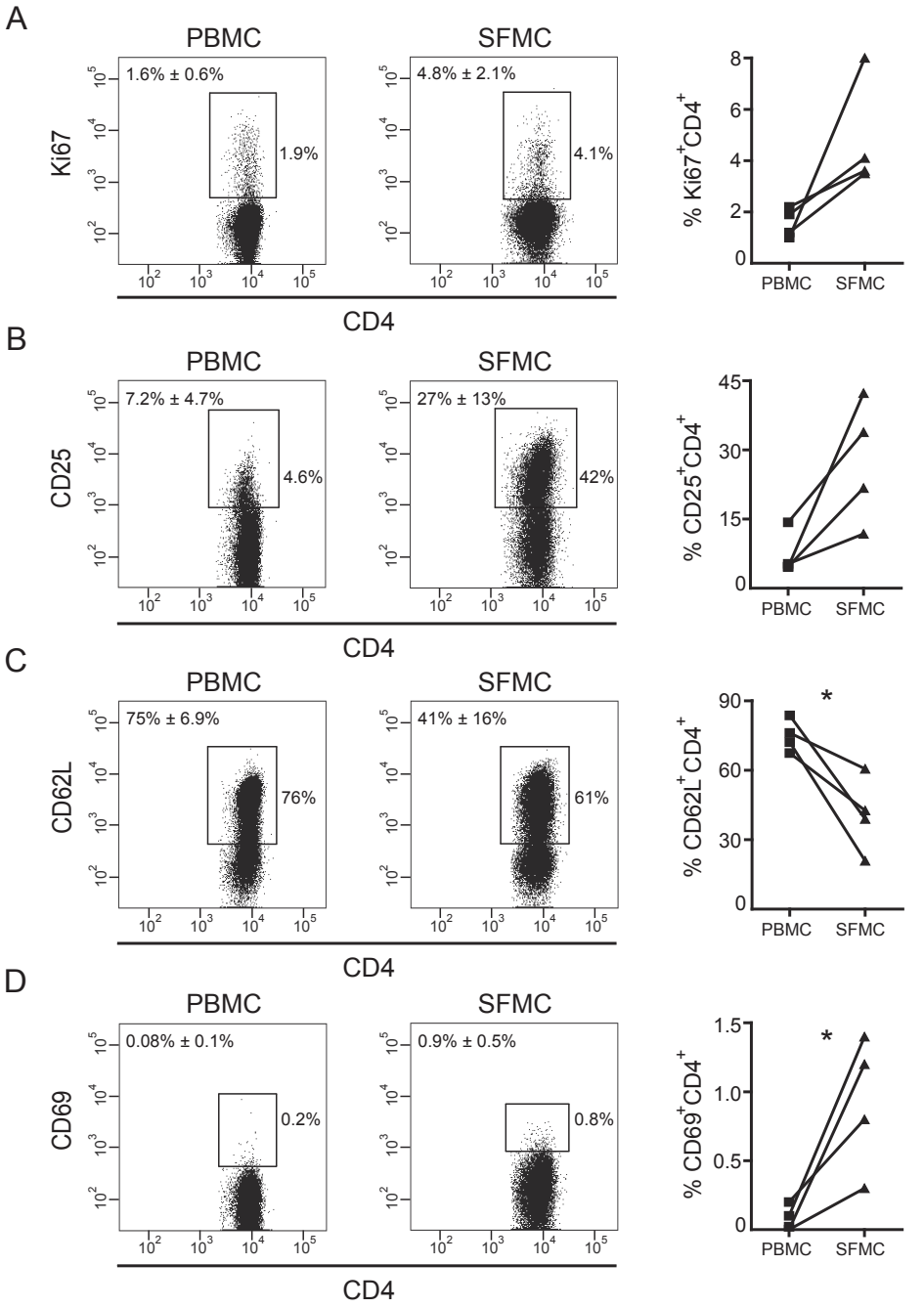
Supplemental Figure 2. ytokine production in paired PBMC and SFMC after short PMA and Ionomycin stimulation.

A Gating strategy applied to analyze intracellular cytokine production in $CD4^+$ and $CD8^+$ cells by flow cytometry. **B-E** Percentage of IL-10 (**B**), IL-17 (**C**), TNFα (**D**), and IFNγ (**E**) positive cells in $CD4^+$ cells (left panels) and $CD8^+$ cells (right panels) in paired PBMC (squares) and SFMC (traingles) of n=4 JIA patients.



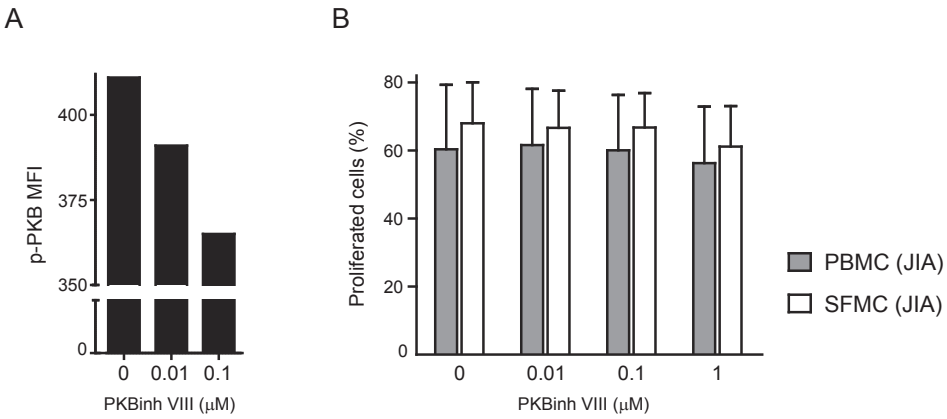
Supplemental Figure 3. Gating strategy applied to analyze phenotype of SF and PB derived monocytes by flow cytometry.

After exclusion of dead cells, monocytes were gated on forward side catter using the depicted area.



Supplemental Figure 4. CD4⁺ cells from SFMC show enhanced expression of proliferation and activation markers ex vivo compared to PBMC.

Percentage of Ki67 (**A**), CD25 (**B**), CD62L (**C**) and CD69 (**D**) positive cells in CD4⁺ cells from paired PBMC and SFMC from JIA patients measured *ex vivo* by flow cytometry. Representative dotplots showing the percentage of positive cells within CD4⁺ cells (left panels) and accumulative data (right panels) of n=4, *p<0.05.



Supplemental Figure 5. Treatment with PKB/c-akt inhibitor dose-dependently reduces PKB phosphorylation, but does not change cell proliferation.

A-B CFSE labeled PBMC and SFMC were cultured in the presence of increasing concentrations of PKB/c-akt inhibitor VIII (PKBinh VIII) (0, 0.01, 0.1, 1 μM). At day 5, expression of phosphorylated PKB/c-akt (**A**) and proliferation of $CD4^+$ T cells (**B**) was analyzed by flow cytometry. **A** MFI of phosphorylated PKB/c-akt (p-PKB) in $CD4^+$ SFMC, n=1 in duplicate. **B** Proliferation of $CD4^+$ T cells from paired PBMC (grey bars) and SFMC (white bars) of JIA patients, bars represent means ± SEM of n=3.

Chapter 4

PD-1$^+$ CD8 T cells are clonally-expanding effectors in human chronic inflammation

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ABSTRACT

Chronic inflammatory diseases are characterized by recurrent inflammatory attacks in the tissues mediated by auto-reactive T cells. Identity and functional programming of CD8 T cells at the target site of inflammation still remain elusive. One key question is whether, in these antigen-rich environments, chronic stimulation leads to CD8 T cell exhaustion comparable to what is observed in infectious disease contexts. 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, functional studies 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 were enriched for a tissue-resident memory (T_{RM}) cell transcriptional profile and demonstrated increased clonal expansion compared to the PD-1^{-} counterpart, suggesting antigen-driven expansion of locally adapted cells. Interestingly, this subset was found increased also in target tissues of other human chronic inflammatory diseases. These data indicate that local chronic inflammation drives the induction and expansion of CD8 T cells endowed with potential detrimental properties. Together these findings lay 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 inflammation. Local T cells, 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/PD-L1 pathway inhibition[13,14], and this is confirmed by the evidence that anti-PD-L1 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, by using several different techniques to evaluate transcriptional, functional and metabolic phenotype as well as the T cell receptor repertoire, we investigated whether PD-1-expressing CD8 T cells from chronically inflamed tissue sites show signs of exhaustion, terminal differentiation or rather effector function. 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 (T_{RM}) 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 may provide the rationale for investigation of therapeutic strategies targeting local PD1-expressing CD8 T cell subsets in chronic inflammation.

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). Description of clinical features of patients enrolled in this study are shown in Table 1. A total number of n=52 JIA patients were included in this study, n=40 with oligoarticular JIA and n=12 with polyarticular JIA, according to the revised criteria for JIA[59]. Biological samples were collected, isolated and stored in liquid nitrogen, then retrospectively selected based on inclusion criteria (age ≤ 18, affected by either oligo or polyarticular JIA) and exclusion criteria (presence of systemic and concurrent infectious diseases). 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 5.8 years (range 0.5-19.5 years). Synovial fluid (SF) was obtained from patients with active disease undergoing therapeutic joint aspiration. From n=16 patients paired blood and joint aspirate samples were collected. Peripheral blood from adult healthy volunteers (HC, n=33) 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 active Crohn's disease (n=3) or Ulcerative Colitis (n=1) underwent endoscopy for surveillance, and n=4 biopsies were taken from macroscopically inflamed colonic mucosa.

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 μm cell strainer (Costar), counted and used for flow cytometry experiments.

Table 1. Patient characteristics

	Age in years Median (IQR, 25th-75th percentile)	Sex (M/F)	Type of JIA (Oligoarticular/ Polyarticular)	ANA positivity	Presence of RF	Treatment
JIA patients (n=52)	11,6 (8-15,6)	26/26	40/12	N=26	N=2	N=19: MTX N=3 low dose prednisone N=6 anti-TNF monoclonal antibody N=1 MMF N=23 untreated

JIA: juvenile idiopathic arthritis, IQR, Interquartile range, ANA: antinuclear antibodies, RF: rheumatoid factor, MTX: methotrexate, MMF: mycophenolate mofetil

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 (clone OKT-3, Biolegend), CD4 Alexa488 (clone RPA-T4, BD), CD8 APC-Cy7 (clone SK1, BD), PD-1 PE (EH12.1, BD) and APC (clone MIH4, BD), CD45RA FITC (clone HI100, BD), CD45RO PB (clone UCHL1, Sony Biotechnology), CCR7 APC (clone G043H7, Sony Biotechnology), TIGIT Percp-eF710 (clone MBSA43, eBioscience), CD69 APC (clone FN50, BD), CD103 FITC (clone 2G5, Beckman Coulter). To determine cell dead the Fixable Viability Dye eFluor® 506 (eBioscience) was used. For intracellular staining, after surface staining, cells were fixed and permeabilized by using Fixation and Permeabilization Buffers (eBioscience), and stained with anti-human Ki-67 FITC (clone B56, BD), CTLA-4 APC (clone BNI3, BD) and GzmB FITC (clone GB11, BD). For intracellular cytokine production, cells were first stimulated for a total of 4 hours with PMA (20 ng/ml; MP Biomedicals) and ionomycin (1 μg/ml; Calbiochem); Golgistop (1/1500; BD Biosciences) was added for the last 3.5 hours of culture. Afterwards, cells were incubated with surface antibodies, and then fixed, permeabilized, and intracellularly stained with anti-human GzmB FITC, IFNγ PE-Cy7 (clone 4S.B3, BD) and TNF-α FITC (clone Mab11, Biolegend). In the gut of IBD patients, cells were stained with CD3 BV510, CD4 APC (clone RPA-T4, eBioscience), CD8α PB (RPA-T8, Sony Biotechnology), TCRγδ PE (Beckman Coulter), CD69 FITC (clone FN50, BD), PD-1 Percp-Cy5.5 (clone EH12.2H7, Sony Biotechnology) monoclonal antibodies. In some experiments, after surface staining, cells were sorted by flow cytometry with FACS Aria III (BD Biosciences) to perform additional assays.

Cell cultures

In some experiments, PD-1$^-$ CD8 T cells from SF-JIA were sorted by BD FACSARIA III and plated in the presence of anti-CD3/CD28 (Dynabeads® Human T-Activator CD3/CD28,

ThermoFisher Scientific) at 1:5 ratio (1 bead to 5 cell ratio). After 40-hour cells were incubated for 4h with Golgistop (1/1500; BD Biosciences) and stained for anti-human PD-1, IFNγ and GzmB antibodies after fixation and permeabilization. In other experiments, SF-derived PD-1$^+$ and PD-1$^-$ CD8 T cells were sorted, plated with Dynabeads at 1:50 ratio in the absence or presence of 10 µg/ml anti-PD-1 agonist (Recombinant Human PD-L1/B7-H1 Fc Chimera Protein, R&D). After 40-hour cells were incubated for 4 h with Golgistop (1/1500; BD Biosciences) and stained with anti-human PD-1, IFNγ and GzmB antibodies after fixation and permeabilization.

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 assessed 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)[60]. Data were analyzed using DEseq2 [61] 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 [62] 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.

Validation of whole transcriptome sequencing

Total RNA was extracted from PD-1+ and PD-1- CD8 T cells from SF-JIA and PB-HC using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. RNA was reverse-transcribed with iSCRIPT cDNA synthesis mix (Bio-Rad, Cat# 170-8891) using the C1000 Thermal Cycler (Bio-Rad). qRT-PCR was performed using SYBR SelectMaster Mix (Thermo Fisher Scientific, cat# 4472919) with the Quantstudio TM 12K Flex Real-time PCR system (Thermo Fisher Scientific). PCR cycling conditions were performed for all samples as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The primers used were the following: CCR1, 5'-AGTACCTGCGGCAGTTGTTC-3' (Fw), 5'- AAGGGGAGCCATTTAACCAG-3' (Rv); CCL4, 5'-CTGCTCTCCAGCGCTCTC-3' (Fw), 5'-ACCACAAAGTTGCGAGGAAG-3' (Rv); GzmB, 5'-GAGACGACTTCGTGCTGACA-3' (Fw), 5'-GTCGGCTCCTGTTCTTTGAT-3' (Rv); TOP2A, 5'-TTGTGGAAAGAAGACTTGGCTA-3' (Fw), 5'-TGTTCATCTTGTTTTTCCTTGG-3' (Rv). PCR reactions for each template were done in duplicates in a MicroAmp® Fast Optical 96-well reaction plate (0,1mL). The comparative CT method was used to determine gene expression in PD-1+ CD8 T cells relative to the value observed in PD-1- CD8 T cells, using B2M as normalization control.

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[63]. 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[64]. 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[65]. The MiTCR output file was used to calculated the Simpson's Index (D), $D = \frac{\sum n(n-1)}{N(N-1)}$, 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[66]. 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. Two-tailed 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. Biological samples were cross-sectionally collected through a general Biobank protocol (IRB approval: Pharmachild n.11-499/C). The study was conducted in accordance with local ethics committee approval and the Declaration of Helsinki.

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, but not to the PB of healthy control children (HC-Ch) or healthy control adults (HC-Ad) (Supplemental Figure 1A). 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) (Supplemental Figure 1B), similarly to what was previously described in RA patients[4]. Of note, markers that further characterize the differentiation status of CD8 T cells such as CD28, CD27 and CD127 were comparable between PB-HC-Ad and PB-JIA, and therefore are not age-dependent (Supplemental Figure 2).

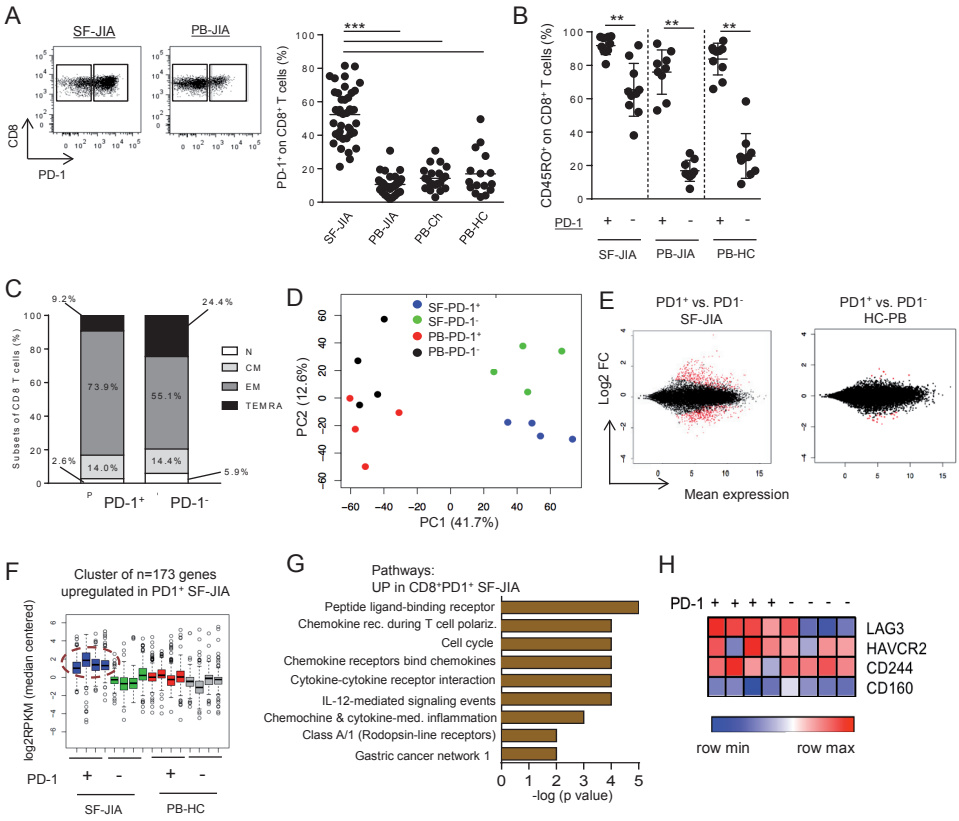

Figure 1. PD1-expressing CD8 T cells are highly activated at the target site of inflammatory arthritis.

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 cell differentiation is shown by using CD45RA and CCR7 markers. Data are means from n=6 SF-JIA samples. **D** 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. **E** Differentially expressed genes (red dots) between PD-1+ and PD-1- CD8 T cells in SF and PB are depicted in MA plots. **F** K-means analysis identifies a set of genes specifically up-regulated in PD-1+ CD8 T cells from SF. **G** Pathways specifically enriched in PD-1+ CD8 T cells from SF are listed. **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; N= naïve (CD45RA+CCR7+); CM: (CD45RA-CCR7+); EM= effector memory (CD45RA-CCR7-); TEMRA (CD45RA+CCR7-

PD-1$^+$ CD8 T cells were highly enriched in SF-JIA when compared to the PB of JIA patients, HC-Ch and HC-Ad (Figure 1A), and this was true also for PD-1 expression levels (MFI) on CD8 T cells (data not shown). Similarly to PB of both JIA patients and HC-Ad, PD-1$^+$ CD8 T cells from SF were almost exclusively memory cells (i.e. CD45RO$^+$), while the only CD45RO$^-$ CD8 T cells present in SF found within the PD-1$^-$ compartment (Figure 1B). However, a larger proportion of PD-1$^-$ cells were found to be CD8 T_{TEMRA} compared to the PD-1$^+$ fraction (24,4% versus 9,2%), meaning that, despite the different level of differentiation, the PD-1+ and PD-1- subset in SF are both predominantly made by memory cells (Figure 1C).

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 (Supplemental Figure 3). Principal Component Analysis (PCA) confirmed this data, additionally showing a better-defined segregation between PD-1$^+$ and PD-1$^-$ CD8 T cells in SF compared to PD1$^+$ and PD1$^-$ in PB (Figure 1D). 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 Figure 1E, left panel) compared to PB-HC (i.e. n=29, red dots in Figure 1E, right panel). Therefore, although these CD8 T cells are derived from the same inflammatory environment and have a memory phenotype in common, PD-1 expression seems to define a unique CD8 T cell subset in SF of JIA patients.

K-mean analysis revealed a cluster of n=173 genes that was 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 1F). 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 (Figure 1G). Selected genes up-regulated in the PD-1$^+$ subset from SF are shown in Table 2, and include chemokine receptors and ligands (e.g. *CCR1*, *CCR2*, *CCR5*, *CXCR6*, *CCL4* and *CCL5*), IL-12-induced effector molecules (i.e. *IFNG*, *GZMA*, *GZMB*) and proteins directly involved in the cell cycle (i.e. *TYMS*, *E2F2*, *TOP2A*). Differentially expressed genes between PD-1$^+$ and PD-1$^-$ cells in SF are shown in Supplemental Table 1. In Supplemental Figure 4 expression levels of individual genes are shown. Interestingly, gene expression of negative co-stimulatory markers typically elevated in exhausted T cells such as *LAG3*, *HAVCR2*, *CD160* and *CD244* was not consistently higher in PD-1$^+$ CD8 T cells from SF compared to the PD-1$^-$ subset (Figure 1H). A total of n=179 genes, instead, was found to be downregulated in PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF-JIA, including killer cell immunoglobulin-like receptors (KIRs) (Supplemental Figure 5A and 5B). Downregulation of molecules with inhibitory functions confirms the activated phenotype of the PD-1$^+$ CD8 T cell subset. Additionally, we ruled out that phenotypical and functional differences observed between PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF-JIA were driven by the memory phenotype; indeed, both proliferation (i.e.

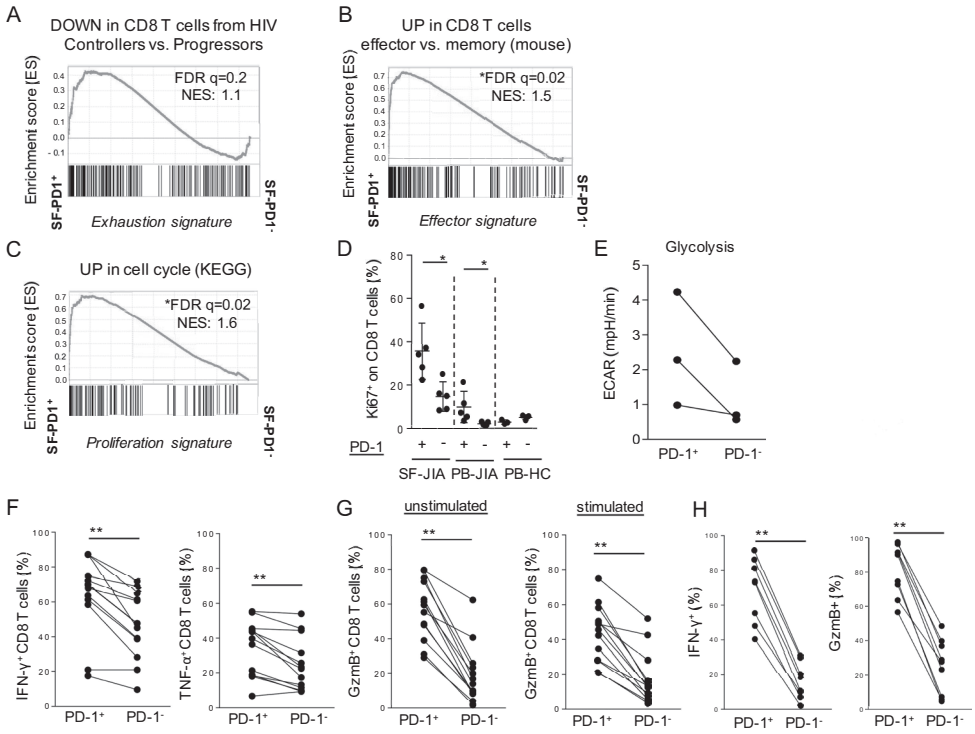

Figure 2. PD1-expressing CD8 T cells are effector, metabolically active and not exhausted cells at the target site of inflammatory arthritis.

A Enrichment of previously published gene signatures of CD8 T cell exhaustion (described in[18]) was tested on PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF by Gene Set Enrichment Analysis (GSEA). **B** Enrichment of previously published gene signatures of effector CD8 T cells (described in[19]) was tested on PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF by GSEA. **C** 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. **D** Assessment of cell proliferation was performed by Ki-67 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. **E** 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. **F** The frequency of IFNγ (left panel) and TNF-α (right panel) -producing PD-1$^+$ and PD-1$^-$ CD8 T cells was tested upon *in vitro* PMA/Ionomycin stimulation. **p<0.01, Paired Student's t-test. **G** The cytotoxic potential of PD-1$^+$ and PD-1$^-$ CD8 T cells was tested by assessing the frequency of GzmB-producing cells *ex vivo* (left panel) and upon *in vitro* PMA/Ionomycin stimulation (right panel). **p<0.01, Paired Student's t-test. **H** PD-1$^-$ CD8 T cells were sorted from SF-JIA and plated in the presence of anti-CD3/CD28 stimuli (1:5 ratio). After 40-hour stimulation, intracellular levels of IFNγ (left panel) and GzmB (right panel) on PD-1$^+$ and PD-1$^-$ CD8 T cells were measured. **p<0.01, 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.

Ki67) and cytotoxicity (i.e. GzmB production) were increased in CD45RO$^+$ PD-1$^+$ when compared to CD45RO$^+$ PD-1$^-$ cells (Supplemental Figure 6), showing that the effector profile observed in PD-1+ cells is not driven by the memory phenotype itself.

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.

Table 2. Selected genes up-regulated in PD-1$^+$ CD8 T cells from SF are shown.

Selected up-regulated genes			
Chemokines and receptors		**IL-12-med. Signaling**	**Cell cycle**
CCR1	*CCL4*	*IFNG*	*TYMS*
CCR2	*CCL5*	*GZMA*	*CENPE*
CCR5	*CCL23*	*GZMB*	*E2F2*
CXCR6	CCL3		CDCA5
			TOP2A

PD-1-expressing CD8 T cells from the target site of inflammatory arthritis are enriched for an "effector" and not an "exhaustion" 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 (Figure 2A). Instead, the signature of effector CD8 T cells[19] was found significantly enriched in PD-1$^+$ CD8 T cells (Figure 2B), as well as the set of genes featuring proliferating cells (Figure 2C), compared to SF-derived PD-1$^-$ CD8 T cells. Furthermore, T-bet and Blimp-1, transcription factors implicated in CD8 T cell exhaustion[20-22], showed a similar expression level in the PD-1+ and PD-1- subsets (data not shown), indicating that transcriptional regulators of exhausted cells are not relevant in this setting. These data, together with the elevated intracellular expression of the marker of cell proliferation Ki-67 (Figure 2D), strongly suggest that SF-derived PD-1$^+$ CD8 T cells are not exhausted and proliferate *in vivo*.

Complementary analysis confirmed these data showing that genes up-regulated in PD-1$^+$ vs. PD-1$^-$ CD8 T cells in SF (Supplemental Figure 7A) or shared between PD-1$^+$ SF-JIA vs. PD-1$^+$ PB-HC and PD-1$^+$ vs. PD-1$^-$ SF-JIA (Supplemental Figure 7B) were consistently enriched in pathways associated with effector cells.

Immune cell differentiation and function depends on the activation of specific metabolic pathways: quiescent cells generate energy (i.e. adenosine triphosphate, ATP)

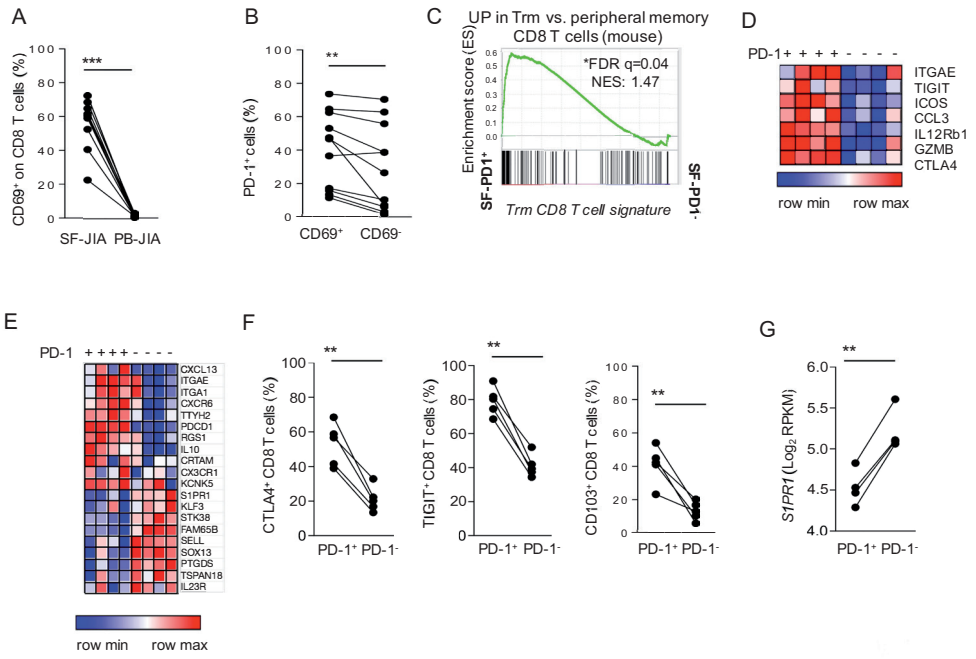

Figure 3. PD1-expressing CD8 T cells display a T_{RM} profile at the target sites of human inflammatory arthritis.

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 T_{RM} (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 T_{RM} CD8 T cells (described in[29]) 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 selected genes upregulated in mouse-derived T_{RM} cells[29] in PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. **E** The heatmap shows color-coded gene expression levels of selected signature genes of human-derived T_{RM} cells[32] in PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. **F** The frequency of CTLA-4$^+$, TIGIT$^+$ and CD103$^+$ cells was measured on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF. **p<0.01, Paired Student's t-test. **G** 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 *SF-PD1$^+$: synovial fluid-derived PD1$^+$CD8 T cells; SF-PD1$^-$: synovial fluid-derived PD1$^-$CD8 T cells; UP: up-regulated*

primarily in their mitochondria via oxidative phosphorylation, while effector cells engage anaerobic glycolysis, converting glucose into lactate[23,24]. 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 setting, PD-1 is known to regulate metabolism by inhibiting glycolysis[25]. 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 (Figure 2E).

We further assessed PD-1$^+$ CD8 T cell properties by performing a short non-specific stimulation with PMA/Ionomycin. This showed an increased expression of pro-inflammatory (i.e. IFNγ and TNF-α) cytokines by PD-1$^+$ compared to PD-1$^-$ CD8 T cells

(Figure 2F). Furthermore, PD-1$^+$ cells were found to produce elevated levels of the cytotoxic enzyme GzmB both *ex vivo* (Figure 2G, left panel) and upon *in vitro* stimulation (Figure 2G, right panel). Finally, PD-1$^-$ CD8 T cells from SF-JIA were sorted and plated in the presence of anti-CD3/CD28 stimuli. After 40-hour TCR stimulation, 42.8±25.7% PD-1$^-$ cells turned PD-1$^+$, while the leftover remained negative for PD-1 (data not shown). Interestingly, ex-PD-1$^-$ cells that turned PD-1$^+$ showed an effector phenotype (i.e. elevated production of IFNγ and GzmB) compared to cells that remained negative for PD-1 (Figure 2H). These data indicate that PD-1$^+$ CD8 T cells are induced by TCR stimulation and have a superior pro-inflammatory and cytotoxic potential compared to the PD-1$^-$ counterpart.

Furthermore, we attempted to define whether PD-1 signaling was functional in SF. The PD-1 pathway was triggered by co-culturing SF-derived PD-1$^+$ with anti-CD3/CD28 beads in the presence of anti-PD-1 agonist (Supplemental Figure 8). Both GzmB and IFNγ production were reduced upon PD-1 ligation, indicating that effector PD-1$^+$ CD8 T cells induced at the site of chronic inflammation of JIA can, at least in part, respond to PD-1 triggering *ex vivo*.

Taken together, these findings indicate that PD-1-expressing CD8 T cells are endowed with typical features of effector but not exhausted cells at the transcriptional, metabolic and functional level.

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

Tissue-resident memory T cells (T_{RM}) have been recently described as a resting memory cell subset expressing CD69, stably residing in tissues, with a critical role in local immunosurveillance[26-28]. Although the SF-derived cells by definition are not tissue resident - they are isolated from exudate and not from tissue -, we were wondering whether they may share some characteristics, especially since PD-1 and other negative co-stimulatory markers (such as CTLA-4, ICOS and TIGIT) have shown to be upregulated on T_{RM} in mice[29] and humans[30].

In our setting, CD69$^+$ CD8 T cells were present at a high frequency in SF compared to PB of the same patients (Figure 3A) and PD-1 expression was higher on total CD69$^+$ compared to CD69$^-$ CD8 T cells in SF (Figure 3B). Moreover, CD69$^+$CD103$^+$ and CD69$^+$CD103$^-$ CD8 T cells, associated with two distinct subsets of CD8 T_{RM} cell [31], were both present in SF, with a prevalence of the CD69$^+$CD103$^-$ fraction (Supplemental Figure 9A). Interestingly, barely any CD103$^+$ CD8 T cell was present in the PD-1$^-$ subset (Supplemental Figure 9B).

GSEA showed that PD-1$^+$ CD8 T cells from SF-JIA, but not PD-1$^-$, were enriched with the signature of T_{RM} cells[29] (Figure 3C). Moreover, increased expression levels of signature genes of T_{RM} cells in mice[29] (Figure 3D) and differential expression of the core transcriptional signature of human T_{RM} cells[32] (Figure 3E) were found in SF-derived PD-1$^+$ cells. This was confirmed by the increased protein expression of CTLA-4, TIGIT, and CD103 on PD-1$^+$ CD8 T cells (Figure 3F) as well as 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[33]) (Figure 3G). We also found that the signature of PD-1$^+$ cells from SF was partially shared with the one from human tumor-specific CD8 Tumor Infiltrating Lymphocytes (TIL)[34], with a trend towards enrichment with genes upregulated in CD8 TIL from melanoma patients, and no enrichment in either of the two subsets for downregulated genes (Supplemental Figure 10). These data demonstrate an overlapping profile between PD-1-expressing CD8 T cells in SF and T_{RM} cells, suggesting that inflammation may be one of the drivers of the T_{RM}-associated phenotype.

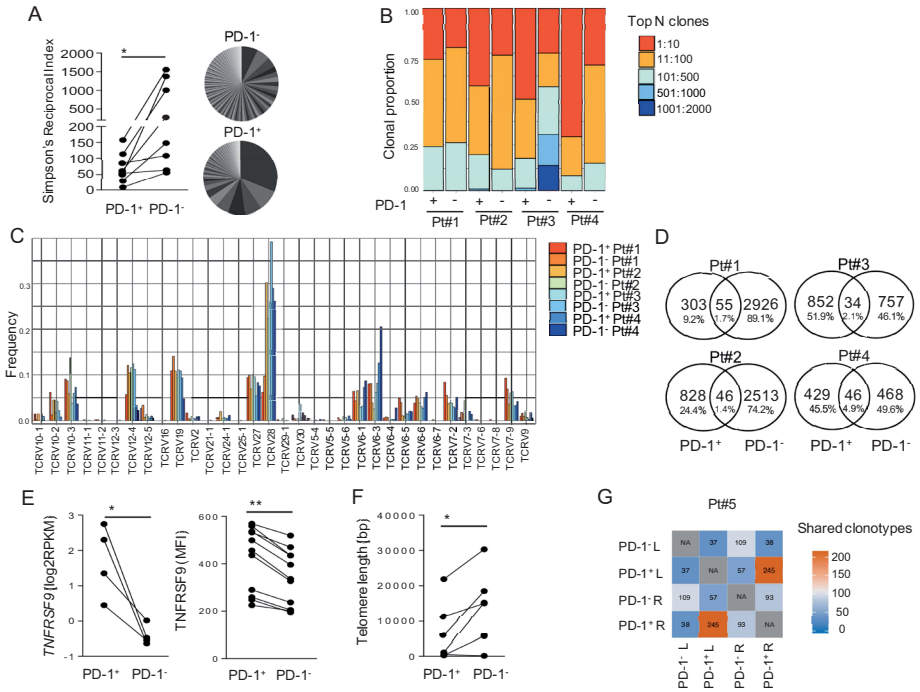

Figure 4. PD1-expressing CD8 T cells are a unique subset undergoing local clonal expansion at the target site of inflammatory arthritis.

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** Analysis of the clonal proportion showing prevalence of the top clones in PD-1+ and PD-1- CD8 T cell subsets from SF. **C** The usage of the TCR Vβ chain was assessed in both PD-1+ and PD-1- CD8 T cells from SF. **D** 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. **E** 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. **F** Telomere length was tested on PD-1$^+$ and PD-1$^-$ CD8 T cells from SF, *p<0.05, paired Student's t-test. **G** In n=1 patient PD-1$^+$ and PD-1$^-$ CD8 T cells from 2 joints were sorted and TCR sequencing was performed. The number of clones shared between PD-1$^+$ and PD-1$^-$ CD8 T cells coming from the two (right-R, left-L) joints are indicated in each square.

Furthermore, expression levels of Hobit, master regulator of T_{RM} cell development in mice[35], were similar between SF-derived PD-1$^+$ and PD-1$^-$ cells, and lower when compared to PB-derived CD8 T cells and NKT cells from HC (data not shown), indicating that Hobit is not a transcription factor selective for SF-derived PD-1$^+$ CD8 T cells in humans.

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 (Supplemental Figure 11, upper panel A). Serial sections from the same AD patients were stained for the T_{RM} cell marker CD69 showing its ubiquitous expression in lesional infiltrates (Supplemental Figure 11, lower panel A). Approximately half of T_{RM} cells present in the dermis co-expressed PD-1. Sections of non-lesional skin were stained as well, but very few CD8$^+$ cells were present (Supplemental Figure 12). Similar results were also obtained from the site of inflammation of IBD patients with active disease (n=3 Crohn's disease and n=1 Ulcerative Colitis), i.e. the colonic mucosa, where in the macroscopically inflamed gut mucosa, PD-1$^+$ CD8 T cells were elevated compared to PB of HC, and almost exclusively expressed by T_{RM} CD8α$^+$CD4$^-$ T cells (i.e. CD69$^+$ cells) and not by recirculating (i.e. CD69$^-$ cells) CD8 T cells (Supplemental Figure 11, panel B).

Overall, these data show that PD-1$^+$ CD8 T cells are present and enriched in both tissue and exudate of human chronic inflammatory diseases and display a T_{RM}-like profile, suggesting that inflammation might be a driver for the local development or expansion of these cells.

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 (Figure 4A, left panel). For instance, in patient #4 (Figure 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. This was confirmed by analysis of the clonal proportion, showing that the top 10 most expanded clones account for 25 to 70% of the repertoire of PD-1$^+$ cells and for 20 to 25% of the one of PD-1$^-$ cells (Figure 4B). Additionally, we found that the TCRV28 chain is preferentially used by all CD8 T cells from SF, with no specific skewing of the V repertoire in PD-1$^+$ CD8 T cells (Figure 4C). 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 Figure 4D),

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 and associated with recent TCR-triggered activation[36], as well as the length of telomeres, known to shorten along with cell division and differentiation[37]. PD-1$^+$ CD8 T cells showed higher mRNA expression level of *TNFRSF9* (Figure 4E, 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 (Figure 4E, 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 (Figure 4F). In n=1 patient the telomere had such a short length already, that no differences between the PD-1$^+$ and PD-1$^-$ subset could be detected (Figure 4F).

In n=1 JIA patient (i.e. #5) we could obtain PD-1$^+$ and PD-1$^-$ CD8 T cells from two different inflamed joints involved during active disease. A clear increased overlap of TCR sequences between joints was found in PD-1$^+$ but not PD-1$^-$ cells (245 vs. 109 respectively, Figure 4G), indicating that the expansion occurring at different sites of inflammation in the PD-1$^+$ subset may be 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 possibly 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 factors such as the tissue 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 can reshape or even resolve the inflammatory responses occurring at these sites. Elucidating these mechanisms might open new therapeutic avenues for clinical application.

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/PD-L1 pathway[13-15]. PD-1 is not the sole maker of exhausted cells, and doesn't *per se* mean 'exhaustion'. Indeed, two studies showed that the transcriptional signature reflecting CD8 (but not CD4) T cell exhaustion enables the prediction of a better prognosis in individuals with chronic inflammatory diseases[38,39]. However, recent studies suggest that it may not be a pure loss of function, but more a sign of functional adaptation to the

chronically inflamed milieu[11,40]. In this study, the role of PD-1-expressing CD8 T cells from the target site of human chronic inflammatory diseases was dissected to explore whether these cells show signs of exhaustion or rather undergo a site-specific adaptation.

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 did not upregulate the whole spectrum of negative co-stimulatory markers that are typically enriched in exhausted CD8 T cells[41]. Additionally, they 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[25]. This finding, together with the evidence of a pro-inflammatory and cytotoxic phenotype, 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]. Local expansion of PD-1+ CD8 T cells is most likely driven by T Cell Receptor (TCR) triggering, which promoted PD-1 expression in synovial fluid (SF)-derived PD1-negative CD8 T cells as well as induced their effector profile. Although local PD-1$^+$ CD8 T cell expansion may additionally be driven by cytokines promoting homeostatic proliferation (i.e. IL-7 or IL-15), we found only very low levels of these cytokines present in SF (data not shown).

PD-1 is a well-known marker of activation[42] and it has been shown to identify a population of oligoclonal CD8 T cells endowed with tumor-specific effector properties in human melanoma metastasis[43]. Few reports also show a positive correlation between PD-1 and Ki-67 in the periphery of virally infected macaques and humans[44,45]. 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 do not require antiretroviral therapy)[46]. 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[11,40,47], our data indicate that, at the target site of chronic inflammatory diseases, an exhausted phenotype does not mean complete loss of function, but rather functional adaptation and development of expansion capacity and possible memory features. In this context, it would be interesting to explore whether the DNA methylation program acquired along with the development of the exhaustion profile in tumor-infiltrating PD-1^{HI} CD8 T cells[48] is shared by locally expanding PD-1-expressing CD8 T cells in chronic inflammatory diseases. Although it is certainly possible that within the PD-1 population there are some exhausted cells, our data show that the PD-1 subset seems to be enriched for cells with an effector/pro-inflammatory phenotype with also low numbers of CD8

T_{TEMRA} cells. In addition, the high percentage of PD-1+ CD8 T cells expressing GzmB, TNFα, and IFNγ suggests that the PD-1+ CD8 T cell subset in SF seems to be a relatively homogeneous population of locally accumulating effectors.

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, supporting the idea that this subset is driven by antigen-specificity, despite no specific restriction of the TCRBV segment is observed. 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 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 a possible local clonal expansion towards cognate antigen(s) at the site of inflammation. This hypothesis was supported 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 type 1 diabetes showing that self-reactive cells with the highest self-antigen affinity express the most PD-1[49], and that gut-derived natural intraepithelial cells expressing PD-1 include strongly self-reactive clones and are restricted by classical major histocompatibility complex molecules[50].

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 (T_{RM}) cells, i.e. a subset of experienced T cells stably residing in tissues and providing local protection upon re-exposure to pathogens[51,52]. Features of T_{RM} cells such as the transcriptional profile[29,30,32], the partial overlap with tumor-derived CD8 TIL, expression of typical surface (retention) markers (i.e. CD69 and CD103), down-regulation of the receptor for sphingosine 1-phosphate *S1PR1*[33] and up-regulation of specific negative co-stimulatory markers as well as the cytotoxic enzyme GzmB[29,30] 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 T_{RM}-like 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 T_{RM} cells[53] have been previously described in the synovial tissue of patients with RA. Therefore, it is possible that immune cells present in SF actually originate from the synovial tissue infiltrate, and are pushed out in the fluid exudate. This locally adapted cell subset is endowed with a constitutively pro-inflammatory/cytotoxic profile and lie in a functional re-activated effector state. However, it is debatable whether PD-1-expressing CD8 T cells represent the activated form of T_{RM}-like cells or rather a state of differentiation of CD8 T cells driven by the inflammatory environment. Moreover, it would be interesting to observe whether this subset is still present in non-inflammatory conditions, and whether T_{RM}-like PD-1-expressing CD8 T cells are indeed enriched in inflamed vs. non-

inflamed tissues, reinforcing the evidence of their potential detrimental role in chronic inflammatory diseases. This hypothesis is supported by the observation that, in human skin, $CD103^+ T_{RM}$ cells (in which PD-1 expression was found enriched) represent the subset with more pronounced effector functions compared to $CD103^- T_{RM}$ cells[31].

Finally, the question arises why the CD8 T cells are not kept in check by the high expression of PD-1, and whether PD-1 can still regulate CD8 T cell functionality and/or proliferative capacity. The ligand of PD-1 (i.e. PD-L1) is expressed by antigen presenting cells (APC) in SF[16] and the PD-1 signalling on CD8 T cells appears to be, at least partially, functional *ex vivo*; however, the soluble form of PD-1 (sPD-1) is present at high levels at this site[54], thus counteracting PD-1-mediated suppression by blocking interaction with APC which explains why this subset of effector cells cannot be suppressed at the site of inflammation. We believe PD-1 is a bystander marker of a subset of locally expanding clonal cells that, despite upregulating inhibitory markers (such as PD-1, LAG-3, CTLA-4, TIGIT), cannot be properly halted due to insufficient activity of the inhibitory signalling. PD-1 signalling leads to downregulation of PI3K activation and subsequent less activation of Akt[55]. Interestingly, Akt has been shown to be hyperphosphorylated in CD8 T cells derived from SF[8], indicating that PD-1 signalling is at least not sufficient to downregulate the PI3K-Akt axis in these cells. It may be even possible that PD-1 signalling is preventing the terminal differentiation and death of these cells as recently shown in a mouse model of chronic viral infection, where the genetic absence of PD-1 led to the accumulation of more cytotoxic but terminally differentiated CD8 T cells[56].

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 not exhausted but rather 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[16,57,58]. Halting migration to the site of inflammation or local depletion of resident effector cells showing tissue-specific harmful potential might have additional substantial therapeutic implications in chronic inflammatory diseases.

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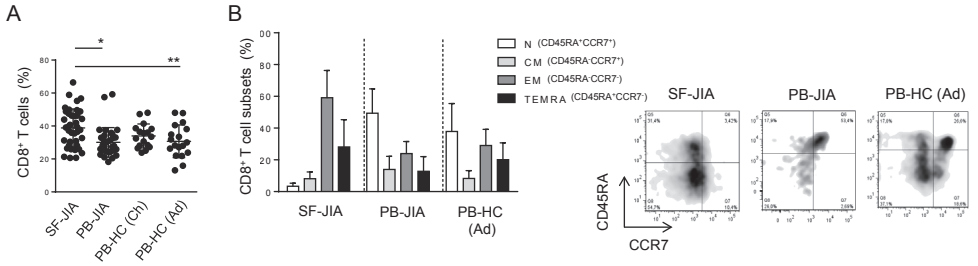
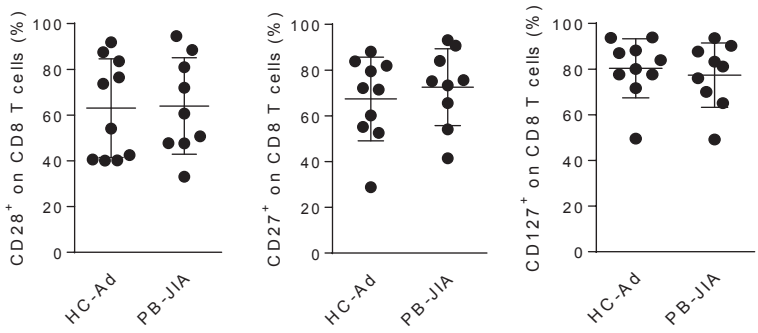
SUPPLEMENTAL DATA



Supplemental Figure 1. 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). n= 7 SF-JIA samples, n=6 PB-JIA samples and n=7 HC-PB samples were used. Data are means ± SD. Representative density plots of CD8 T cell subsets in indicated samples are shown (right panel)

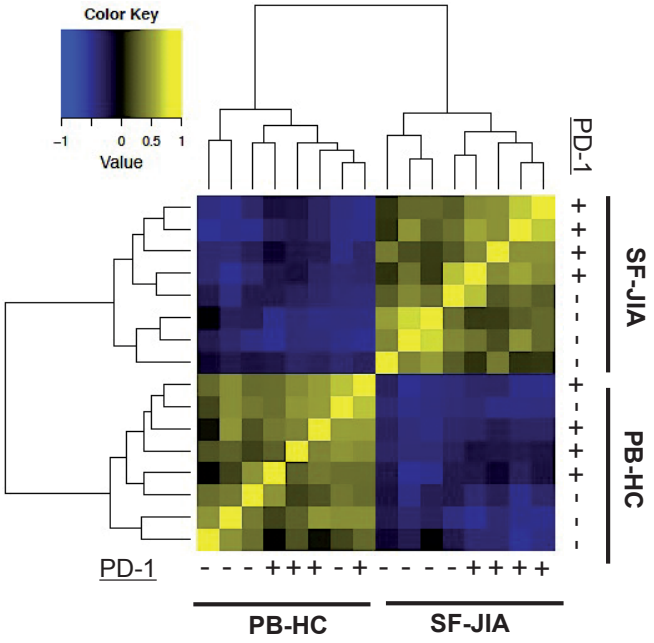
SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-JIA: peripheral blood of Juvenile Idiopathic Arthritis patients; PB-HC (Ch): peripheral blood of healthy control children; PB-HC (Ad): peripheral blood of healthy control adults. N: naive; CM: central memory; EM: effector memory; TEMRA: terminally differentiated effector memory.



Supplemental Figure 2. Comparable CD8 T cell profile 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.

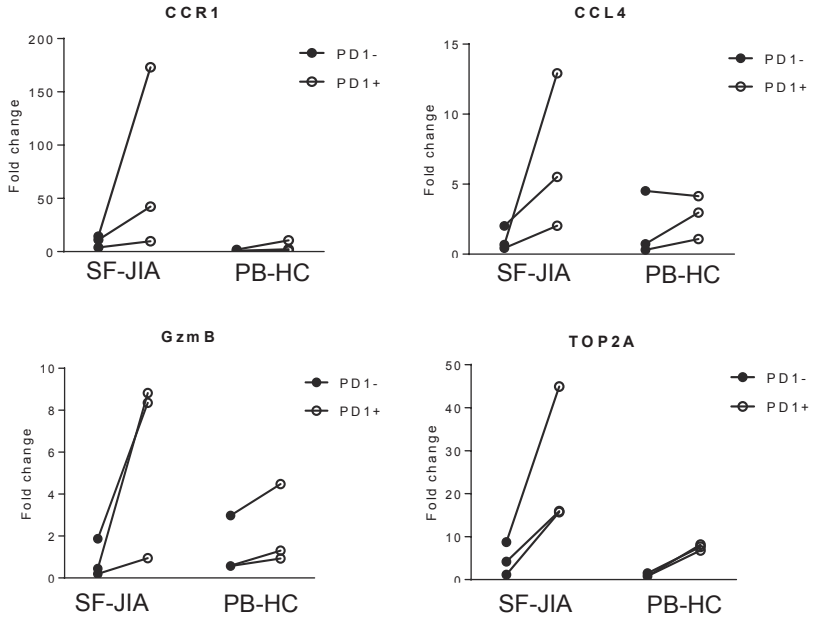
HC-Ad peripheral blood of healthy control adults; PB-JIA: peripheral blood of Juvenile Idiopathic Arthritis patients.



Supplemental Figure 3. 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.

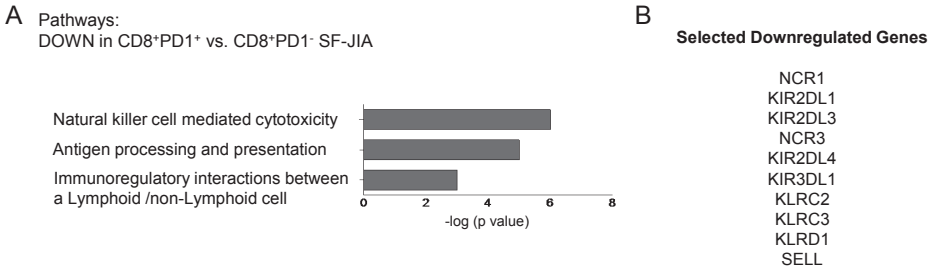
SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-HC: peripheral blood of healthy control adults.



Supplemental Figure 4. Expression levels of genes involved in cell activation.

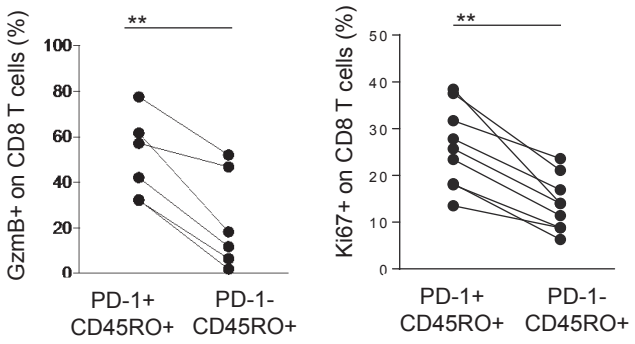
PD-1+ and PD-1- CD8 T cells were sorted from SF of JIA patients and PB of healthy control adults and whole transcriptome sequencing was performed. Expression levels of selected genes up-regulated in PD-1+ CD8 T cells and associated with pathways involved in cell activation are shown.

SF-JIA: synovial fluid of Juvenile Idiopathic Arthritis patients; PB-HC: peripheral blood of healthy control adults.



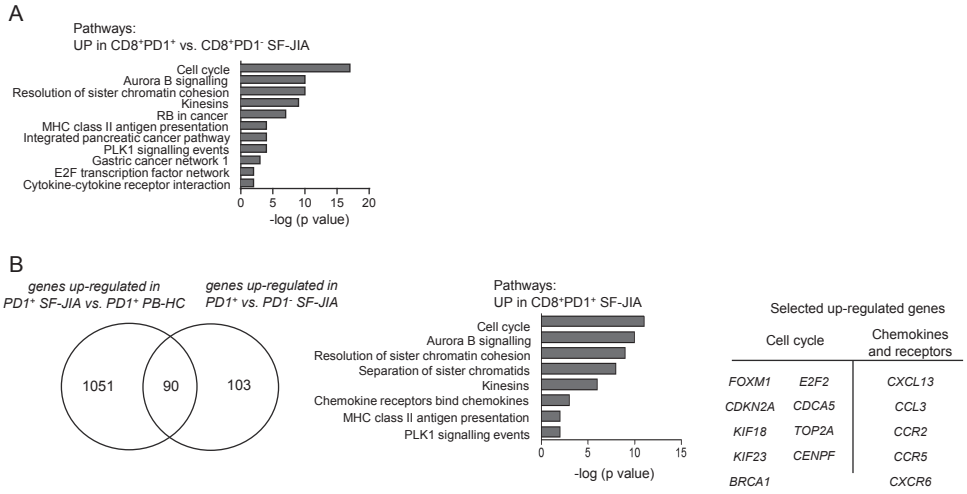
Supplemental Figure 5.

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.



Supplemental Figure 6. PD-1+ CD8 T cells maintain an activated profile regardless of the memory phenotype.

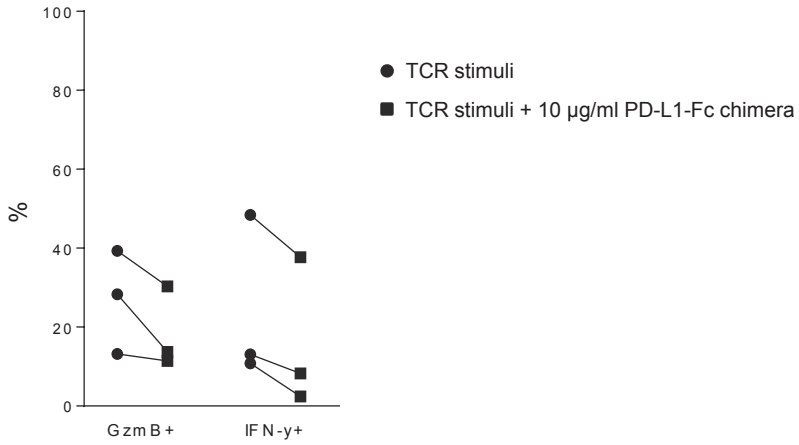
Cytotoxicity (GzmB) and proliferation (Ki67) of memory PD-1$^+$ CD8 T cells was compared with the one of the memory PD-1$^-$ counterpart. **p<0.01, Paired Student's t-test



Supplemental Figure 7. Enrichment of pathways involved in cell activation are evident in PD-1$^+$ CD8 T cells from SF.

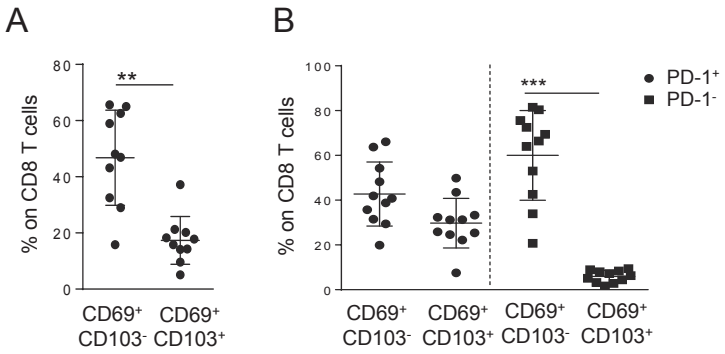
A Pathways enriched when genes up-regulated in PD-1$^+$ compared to PD-1$^-$ CD8 T cells from SF were selected. **B** n=90 genes were shared between the following comparisons: genes up-regulated in PD-1$^+$ from SF-JIA vs. PD-1$^+$ from PB-HC and genes up-regulated in PD-1$^+$ vs. PD-1$^-$ from SF-JIA (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.



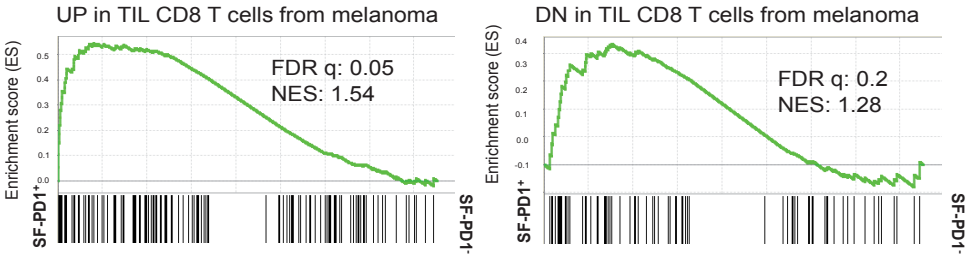
Supplemental Figure 8. Responsiveness to PD-1 triggering.

SF-derived FACS sorted PD-1$^+$ CD8 T cells were exposed to low TCR stimulation (Dynabeads:cell ratio 1:50) in the absence or presence of anti-PD-L1 agonist (PD-L1-FC chimera, 10µg/ml). After 40-hours stimulation, GzmB and IFNγ production were measured upon 4 hrs incubation with Golgistop.



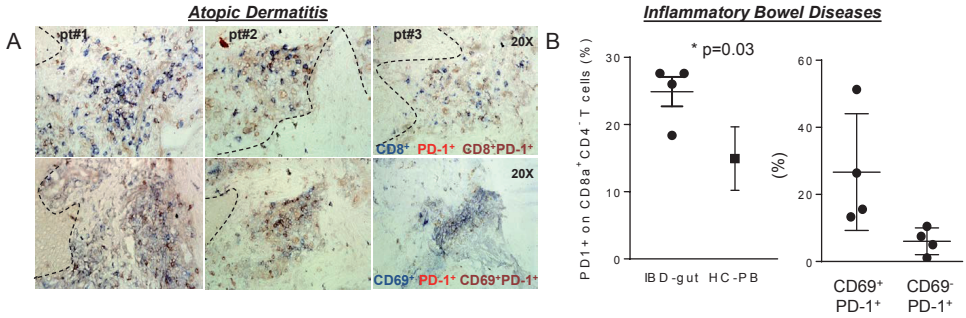
Supplemental Figure 9. Tissue residency markers on CD8 T cells from SF.

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.



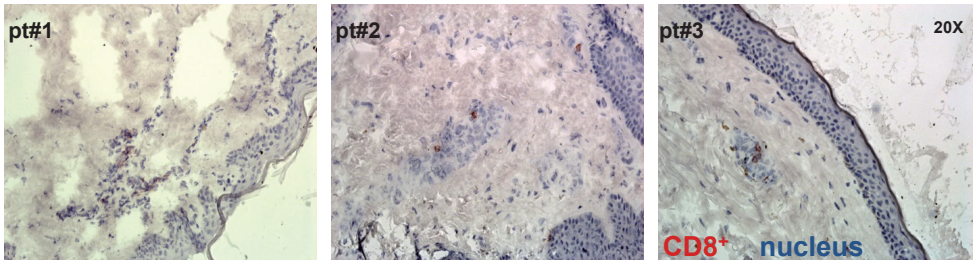
Supplemental Figure 10. Partial enrichment of the gene signature of human tissue-residing CD8 T cells in PD-1$^+$ CD8 T cells from SF.

Enrichment of the previously published gene signature (upregulated genes, left panel; downregulated genes, right panel) of tumor infiltrating CD8 T cells isolated from human metastatic melanoma (Baitsch L, J Clin Invest. 2011) was tested on PD-1$^+$ vs. PD-1$^-$ CD8 T cells from SF.



Supplemental Figure 11. PD1-expressing CD8 T cells display a TRM profile at the target sites of human inflammatory arthritis.

A 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 - T_{RM} - 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. **B** Immune cells were isolated from inflamed gut mucosa of patients with Inflammatory Bowel Diseases (IBD). PD-1 expression on CD8a$^+$CD4$^-$ T cells in the gut mucosa of IBD patients is compared to its expression on CD8 T cells from PB of HC (left panel), unpaired Student's t-test. PD-1 expression on T_{RM} (CD69$^+$) and recirculating (CD69$^-$) CD8a$^+$CD4$^-$ T cells is shown (right panel). *ns*, Paired Student's t-test.



Supplemental Figure 12. Few CD8 T cells are evident in non lesional skin of AD patients.

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

Conserved human effector regulatory T cell signature is reflected in super-enhancer landscape

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Submitted

ABSTRACT

Regulatory T cells (Treg) are critical regulators of immune homeostasis. Increasing evidence demonstrates that environment-driven Treg differentiation into effector Treg is crucial for optimal functioning. However, programming of human Treg under inflammatory conditions remains poorly understood. Here, we combine transcriptional and epigenetic profiling to identify the human effector Treg core signature. Functional autoimmune inflammation-derived Treg display a unique transcriptional profile characterized by upregulation of both a core Treg (FOXP3, CTLA-4, TIGIT) and effector program (ICOS, GITR, BLIMP-1, BATF). We identified specific human effector Treg markers including VDR and IL12Rβ2. H3K27ac occupancy revealed large changes in the (super-)enhancer landscape, including enrichment of the binding motif for VDR and BATF. The observed Treg profile showed striking overlap with tumor-infiltrating Treg. Our data demonstrate that human inflammation-derived Treg acquire a specific effector Treg profile guided by epigenetic changes. The core effector Treg profile is strongly conserved, and fine-tuned by environment specific adaptations.

INTRODUCTION

Forkhead box P3-expressing (FOXP3+) regulatory T cells (Treg) are key players to control aberrant immune responses. Mutations in the *FOXP3* gene lead to severe autoimmunity and inflammation in both mice and men[1–4]. Because of their potential for clinical applications, Treg have been intensively studied over the last decades. Despite this effort there is still a fundamental gap in knowledge, especially regarding the gene expression profiles and epigenetic regulation of human Treg in inflammatory settings.

There is accumulating evidence from mouse models that specific environments such as inflammation or non-lymphoid tissues can induce further differentiation/adaptation into specialized activated Treg subsets, also referred to as effector (e)Treg[5,6] (and reviewed in[7,8]). Characteristic for eTreg appears to be the maintenance of FOXP3 expression, increased expression of several molecules related to their function such as ICOS, CTLA4 and TIGIT, and adaptation to the local environment. For example, recent studies in mice show that in visceral adipose tissue (VAT) Treg play a dominant role in metabolic homeostasis[9,10], whereas Treg in skin can promote wound repair and are involved in local stem cell maintenance[11,12]. It is yet not evident how this specialization in tissues is acquired and regulated at a transcriptional and epigenetic level.

In mice, eTreg have also been demonstrated to be crucial in specific inflammatory settings. Interestingly, inflammatory signals can induce the stable upregulation of typical helper T cell transcription factors such as T-bet, allowing the Treg to migrate to the site of inflammation[13]. Moreover, co-expression of T-bet and Foxp3 is essential to prevent severe Th1 autoimmunity[14]. Studies using transgenic mice have eminently contributed to the knowledge in this field, but how this can be translated to humans remains to be elucidated. Because of the interest in the use of Treg for therapeutic purposes in a variety of human diseases it is highly relevant to gain insight in human eTreg programming in inflammatory settings.

There are indications that eTreg are also be present in humans in both homeostatic and inflamed tissues[15,16] (and reviewed in[17]) and recently a specific signature of tumor-infiltrating Treg was described[18,19]. Pertinent issues that remain to be addressed are how diverse and stable human eTreg programming is in the inflammatory environment, and whether this is regulated at an epigenetic level.

Here, we investigated the gene expression profile and active enhancer landscape of human Treg in an autoimmune-associated inflammatory environment, i.e. synovial fluid (SF) from inflamed joints of Juvenile Idiopathic Arthritis (JIA) and Rheumatoid Arthritis (RA) patients. Transcriptome analysis of SF Treg revealed a classical Treg signature as well as strongly upregulated markers including ICOS, BATF, T-bet, IL-12rβ2 and Blimp-1, indicating eTreg differentiation and adaptation to the inflammatory environment. Importantly, this dual programming was imprinted at an epigenetic level, as demonstrated by H3K27ac chromatin immunoprecipitation (ChIP)-sequencing. These eTreg demonstrated normal suppressive function and enhanced IL-2 signaling. Finally, a substantial overlap between

the SF Treg signature and recently published human tumor-infiltrating Treg signature was found. These findings indicate that human Treg effector programming is epigenetically imprinted and may be commonly induced in inflammatory conditions ranging from autoimmune to tumor settings.

METHODS

Collection of SF and PB Samples

Patients with Juvenile Idiopathic Arthritis (JIA) were enrolled by the Paediatric Rheumatology Department at University Medical Center of Utrecht (The Netherlands). Patients with Rheumatoid Arthritis (RA) were enrolled by the rheumatology outpatient clinic at Guy's and St. Thomas' Hospital NHS Trust (London, UK). A total number of 33 JIA patients and 7 Rheumatoid Arthritis (RA) patients were included in this study. Of the JIA patients n=7 were diagnosed with extended oligo JIA and n=26 with oligo JIA, according to the revised criteria for JIA[20], with an average age of 10.9 years (range 3.2-17.6 years) and a disease duration at the time of inclusion of 5.3 years (range 0.1-15 years). The average age of RA patients was 61 years (range 30-75 years). PB and synovial fluid (SF) was obtained when patients visit the outpatient clinic via vein puncture or intravenous drip, and by therapeutic joint aspiration of the affected joints, respectively. Informed consent was obtained from all patients either directly or from parents/guardians when the patients were younger than age 12 years. The study was conducted in accordance with the Institutional Review Board of the University Medical Center Utrecht (approval no. 11-499/C; JIA) and the Bromley Research Ethics Committee (approval no. 06/Q0705/20; RA). PB from healthy adult volunteers (HC, n = 14, average age 41.7 years with range 27-62 years) was obtained from the Mini Donor Service at University Medical Center Utrecht. PB from n=8 healthy children (average age 11.4 years with range 7.3-15.6 years) was obtained from a cohort of control subjects for a case-control clinical study. Samples were collected in compliance with the Declaration of Helsinki. SF of JIA patients was incubated with hyaluronidase (Sigma-Aldrich) for 30 min at 37°C to break down hyaluronic acid. Synovial fluid mononuclear cells (SFMCs) and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences, AB) and were used after freezing in Foetal Calf Serum (FCS) (Invitrogen) containing 10% DMSO (Sigma-Aldrich).

Flow cytometry and cell sorting (suppression assay)

$CD3^+CD4^+CD25^+CD127^{low}$ cells (Treg) were isolated from frozen PBMC, using the FACS Aria III (BD). Antibodies used for sorting are: anti human CD3-BV510 (Biolegend), CD4-FITC (eBioscience), CD25-PE/Cy7 (BD), CD127-AF647 (Biolegend). To check for FOXP3 expression of the sorted populations anti human FOXP3-eF450 (eBioscience) was used. Read out of

proliferation is performed with following antibodies: CD3-PerCP/Cy5.5 (Biolegend), CD4-FITC (eBioscience), CD8-APC (BD). Total PBMC were labeled with 2µM ctViolet (Thermo Fisher) and cultured alone or with different ratios of sorted Treg (1:16, 1:8, 1:4, 1:2). Cells were cultured in RPMI1640 media containing 10% human AB serum with addition of L-Glutamine and Penicillin/Streptomycin. PBMC were stimulated by 0,1 µg/ml coated anti-CD3 (eBioscience) and incubated for four days in a 96 well round bottom plate (Nunc) at 37˚C. After 4 days cells were stained with CD3, CD4, and CD8 for read out of proliferation by flow cytometry performed on FACS Canto II (BD Biosiences).

STAT5 Phosflow

PBMC and SFMC were thawed and resuspended in PBS (0,5 – 1,0 x 10^6 living cells/tube). Surface staining of CD3 (biolegend) and CD4 (eBioscience) was performed for 25 min at 4°C. Cells were then stimulated with 0 , 1.0 , 10 or 100 IU/ml human (h)IL-2 (Proleukin; Novartis) for 30 min at 37°C, fixated and permeabilized by using buffers from the Transcription Factor Phospho Buffer Set (BD Biosciences). Intranuclear staining of FOXP3, T-BET (eBioscience), CD25 and pSTAT5 (BD Biosciences) was performed for 50 min at 4°C. Data acquisition was performed on a FACS Canto II flow cytometer (BD Biosciences) and data was analyzed using FlowJo Software (Tree Star Inc.).

RNA-sequencing

$CD3^+CD4^+CD25^+CD127^{low}$ and $CD3^+CD4^+CD25^-CD127^+$ cells were sorted by flow cytometry from HC PBMC and JIA patient SFMC and PBMC. Total RNA was extracted using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) as specified by the manufacturer's instructions and stored at -80°C. Sequencing libraries were prepared using the Rapid Directional RNA-Seq Kit (NEXTflex). Libraries were sequenced using the Nextseq500 platform (Illumina), producing single end reads of 75bp (Utrecht Sequencing Facility). Sequencing reads were mapped against the reference human genome (hg19, NCBI37) using BWA (v0.7.5a, mem –t 7 –c 100 –M -R). Differential gene expression was performed using DEseq2 and custom Perl and R (www.r-project.org) scripts. For K-means clustering and PCA analysis, genes with fold change between samples on 10th and 90th quantile at least 1 log2 RPKM and expression at least 2 log2 RPKM in the sample with the maximal expression were used. Gene set enrichment analysis (GSEA)[21] was used to assess whether specific signatures were significantly enriched in one of the subsets. 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 analyzing raw data using GEO2R (NCBI tool) or downloaded from published papers, or self-made base on the H3K27ac data. In particular, the following published data sets were used: human core Treg signature:[22]; effector Treg signature in mice:[23]; tumor-infiltrating Treg signature:[19]; effector Treg genes in mice: gene set GEO: GSE61077; $TIGIT^+$ Treg signature in mice:[24].

Heatmaps and subsequent hierarchical clustering analyses using One minus Pearson correlation were performed using Morpheus software (https://software.broadinstitute.org/morpheus/).

ChIP-sequencing

ChIP-seq was performed as described previously by *Peeters et al.*[25]. In short, PBMC from HC and SFMC from JIA patients were thawed and $CD3^+CD4^+CD25^+CD127$low cells were sorted by flow cytometry. For each sample, cells were crosslinked with 2% formaldehyde and crosslinking was stopped by adding 0.2 M glycine. Nuclei were isolated in 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 1% Triton X-100 and lysed in 20 mM Tris (pH 7.5), 150mMNaCl, 2mMEDTA, 1% NP-40, 0.3% SDS. Lysates were sheared using Covaris microTUBE (duty cycle 20%, intensity 3, 200 cycles per burst, 60-s cycle time, eight cycles) and diluted in 20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% X-100. Sheared DNA was incubated overnight with anti-histone H3 acetyl K27 antibody (ab4729; Abcam) pre-coupled to protein A/G magnetic beads. Cells were washed and crosslinking was reversed by adding 1% SDS, 100mMNaHCO3, 200mMNaCl, and 300 mg/ml proteinase K. DNA was purified using ChIP DNA Clean & Concentrator kit (Zymo Research), end-repair, a-tailing, and ligation of sequence adaptors was done using Truseq nano DNA sample preparation kit (Illumina). Samples were PCR amplified, checked for the proper size range and for the absence of adaptor dimers on a 2% agarose gel and barcoded libraries were sequenced 75 bp single-end on Illumina NextSeq500 sequencer (Utrecht DNA sequencing facility). Sample demultiplexing and read quality assessment was performed using BaseSpace (Illumina) software. Reads with quality score of Q>30 were used for downstream analysis. Reads were mapped to the reference genome (hg19) with Bowtie 2.1.0[26] using default settings. SAM files were converted to BAM files using samtools version 0.1.19. Peaks were subsequently called using MACS-2.1.0[27]. Enriched regions were identified compared to the input control using MACS2 callpeak--nomodel --exttsize 300 --gsize=hs -p 1e-9. The mapped reads were extended by 300bp and converted to TDF files with igvtools-2.3.36 and were visualized with IGV-2.3.34[28]. Differential binding analysis was performed using the R package DiffBind version 1.8.5[29]. In DiffBind read normalization was performed using the TMM technique using reads mapped to peaks which were background subtracted using the input control. Enhancer gene associations were determined as the nearest TSS to the center of the enhancer and super-enhancer locus. BEDtools v2.17.0 was used for general manipulation of peak bed-files[30].Super-enhancers were identified by employing the ROSE algorithm[31] using a stitching distance of the MACS2 called peaks of 12.5kb, peaks were excluded that were fully contained in the region spanning 1000bp upstream and downstream of an annotated TSS (-t 1000). The H3K27ac signal was corrected for background using the input control and subsequently ranked by increasing signal. Super-enhancer gene associations were determined as the nearest TSS to the center of the enhancer and super-enhancer locus. BEDtools v2.17.0 was used for general manipulation

of peak bed-files[30] . For transcription factor DNA binding motif enrichment analysis, we used regions of open chromatin identified by DNAse hypersensitive regions sequencing and overlapping with H3K27ac regions[32] (GSM665839). Motif enrichment analysis was performed using the HOMER software v4.7 (findMotifsGenome.pl; hg19; -size 200). ChIP-seq data for VDR and BATF was retrieved from GEO: GSE89431 and GEO:GSE32465, respectively.

Microarray RA Treg

CD14+ monocytes (purity >98%) were depleted through positive selection using CD14 MicroBeads (Miltenyi Biotec). $CD4^+$ T cells were isolated from the CD14– cell fraction by negative selection (Miltenyi Biotec) and stained with PerCP/Cy5.5-conjugated CD4, allophycocyanin (APC)/Cy7–conjugated CD45RA, Pacific Blue–conjugated CD45RO, fluorescein isothiocyanate (FITC)–conjugated CD127 (BioLegend), and phycoerythrin (PE)–conjugated CD25 (Miltenyi Biotec). $CD4^+CD45RA^-CD45RO^+CD25^+CD127^{lo}$ cells (memory Treg cells), were sorted by fluorescence-activated cell sorting (FACS) analysis using a BD FACSAria II. Sorted Treg cell samples were lysed in 1000 µl of TRIzol (Invitrogen). Chloroform (200 µl) was added, and the samples were then whirl mixed and incubated for 2–3 minutes at room temperature. Following centrifugation (10,000g for 15 minutes at 4°C), the water phase was further purified using the ReliaPrepTM RNA Miniprep system (Promega) per manufacturer's instructions. RNA integrity was confirmed on an Agilent Technologies 2100 bioanalyzer. One hundred nanograms of total RNA was used to prepare the targets (Affymetrix) in accordance with the manufacturer's instructions. Hybridization cocktails were hybridized onto a Human Gene 2.0 ST Array. Chips were scanned and gene expression data were normalized using the RMA algorithm. Gene expression analysis was performed using Qlucore Omics Explorer software, version 3.0.

Statistics

For ChIP-seq and RNA-seq analysis, p values were adjusted with the Benjamini-Hochberg procedure. For ChIP-seq regions with a significantly different H3K27ac signal were defined using a false discovery rate (FDR)<0.05 in case of super-enhancers and FDR <0.0001 in case of enhancer regions. The significance of the protein and cytokine expression was analyzed using Two-way ANOVA with Sidak correction for multiple testing using GraphPad Prism (GraphPad Software).

RESULTS

Suppressive Treg derived from human inflamed joint fluid demonstrate a distinct transcriptional profile with an enhanced core Treg signature.

To determine whether the gene expression profile of human Treg in an inflammatory environment is different from circulating human Treg, $CD3^+CD4^+CD25^+CD127^{low}$ Treg and $CD3^+CD4^+CD25^-CD127^+$ non-Treg were isolated from: synovial exudate obtained from inflamed joints of juvenile idiopathic arthritis (JIA) patients, peripheral blood (PB) from JIA patients with active disease and inactive disease, and PB from healthy children and healthy adults (Figure S1A for gating strategy). More than 90% of the sorted Treg populations were FOXP3 positive (Figure S1B). The transcriptional landscape was determined with RNA-sequencing. An unsupervised principal component analysis (PCA) was performed to study the variability between Treg derived from different environments. Synovial fluid (SF) derived Treg clearly clustered separately from PB derived Treg (Figure 1A), indicating that SF derived Treg have a specific expression pattern compared to the three groups of PB derived Treg.

In accordance with previous publications we confirmed that SF-derived Treg were functional using *in vitro* suppression assay (Figure 1B)[33,34]. As expected, Treg signature genes were significantly enriched in SF Treg compared to SF $CD4^+$ non-Treg (Figure 1C, left panel). Importantly, Treg signature genes were also enriched in SF Treg compared to PB Treg derived from both healthy children and JIA patients (Figure 1C right panel and Supplemental Figure 2A-B). These included Treg hallmark genes important for Treg stability and function such as *FOXP3*, *CTLA4* and *TIGIT*, at both the transcriptional and protein level (Figure 1D and 1E)[24,35,36]. In humans, $CD3^+CD4^+$non-Treg can upregulate FOXP3 and associated markers that as such can serve as markers for T cell activation[5,15,23,37]. We indeed observed a slight upregulation of FOXP3, CTLA4 and TIGIT at the mRNA and protein level in SF non-Treg but this was not near to the levels observed in SF Treg (Figure 1D+E and Supplemental Figure 1A+B), further confirming that SF Treg and SF non-Treg are distinct cell populations. Together these data demonstrate that the inflammatory environment reinforces the Treg-associated program.

Inflammatory environment derived Treg display a specific effector profile.

A pairwise comparison between SF-derived and PB-derived Treg from healthy children revealed many differentially expressed genes, including core Treg markers such as *FOXP3* and *CTLA4*, but also several markers that reflect more differentiated Treg, like *PRDM1* (encoding Blimp-1), *ICOS* and *BACH2* (Figure 2A). Based on recent literature we specifically analyzed the expression of markers that are related to effector T(reg) cell differentiation in mice. Hierarchical clustering analysis confirmed that Treg clustered separately from non-Treg and revealed a cluster of core Treg genes that showed increased expression in all Treg groups including *CTLA4*, *FOXP3*, *IL2RA* (encoding CD25), *TIGIT* and *IKZF4* (encoding Helios) (Figure 2B, box 1). Furthermore, the heatmap revealed a cluster that showed molecular heterogeneity within the Treg groups. Some markers were expressed almost exclusively

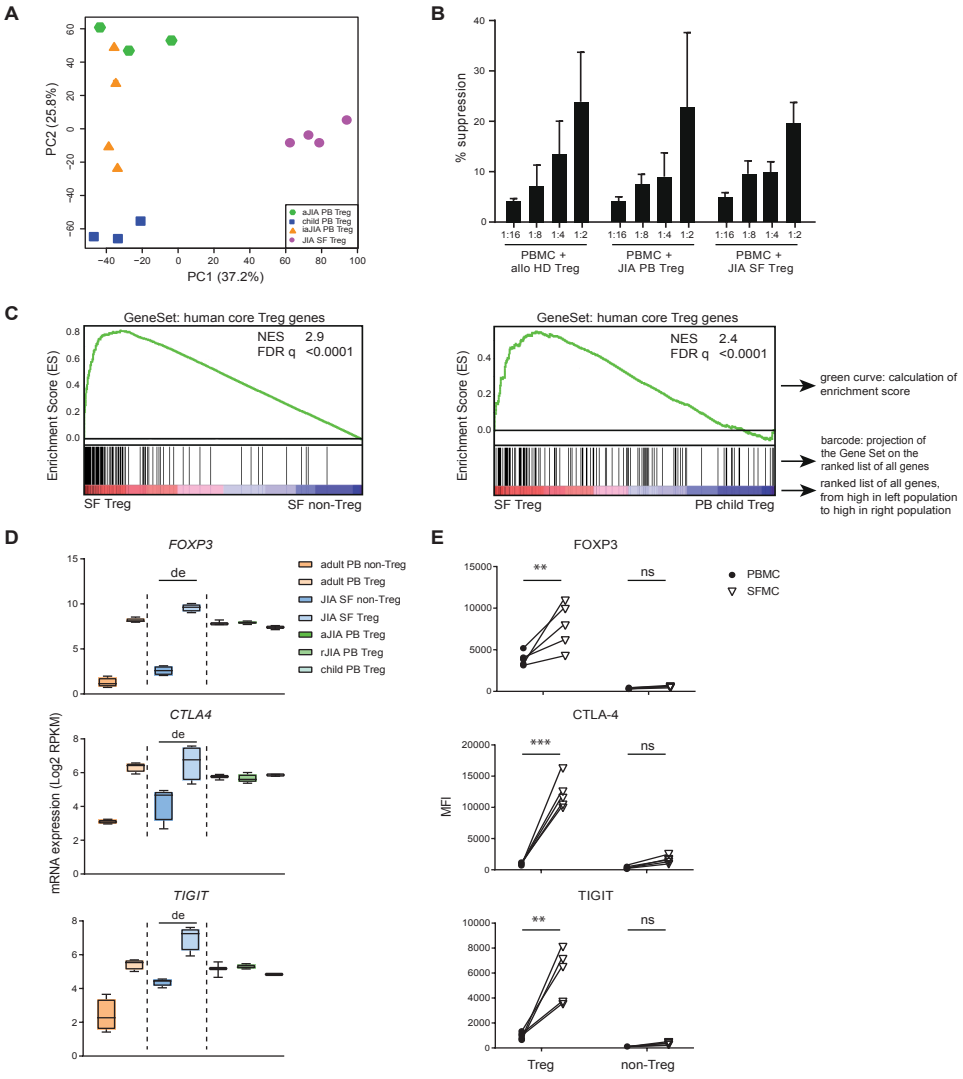

Figure 1. Human Treg from inflammatory site are distinct from PB Treg and have clear Treg characteristics.

A Unsupervised Principal Component Analysis (PCA) of all Treg groups derived from children. **B** Bar charts representing the percentage of suppression of $CD4^+$ T cells by Treg cells from PB of healthy donors, PB of JIA patients and SF of JIA patients. 50.000 ctViolet labeled PBMC from an allogenic healthy donor were cultured with different ratios of Treg for 4 days in anti-CD3 coated plates (n=4, mean+SEM). **C** GeneSet Enrichment Analysis (GSEA) of human core Treg signature genes (as identified in Ferraro et al PNAS 2014) in pairwise comparisons involving SF Treg and SF non-Treg and SF Treg and PB Treg derived from healthy children, represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q). **D** mRNA expression (log2 RPKM) of *FOXP3*, *CTLA4* and *TIGIT* in sorted Treg derived from PB of healthy adults (adult), healthy children (child), JIA patients with active disease (aJIA), JIA patients with inactive disease (iaJIA), SF of JIA patients (JIA SF) and non-Treg derived from PB of healthy adults and SF of JIA patients as determined by RNA-sequencing analysis (de = differentially expressed according to log2 fold change ≥0.6, adjusted (adj)P value ≤ 0.05, minimal baseMean of 10; adj P values *FOXP3* = $1.1E^{-95}$, *CTLA4* = $2.3E^{-05}$, *TIGIT* = $1.9E^{-17}$) (**E**) Median Fluorescence Intensity (MFI) of FOXP3, CLTA-4 and TIGIT in gated $CD3^+CD4^+CD25^+CD127^{low}$ Treg and $CD3^+CD4^+CD25^-CD127^+$ non-Treg derived from paired SFMC and PBMC from 5 JIA patients (** = p<0.01, *** = P<0.001, ns = not significant).

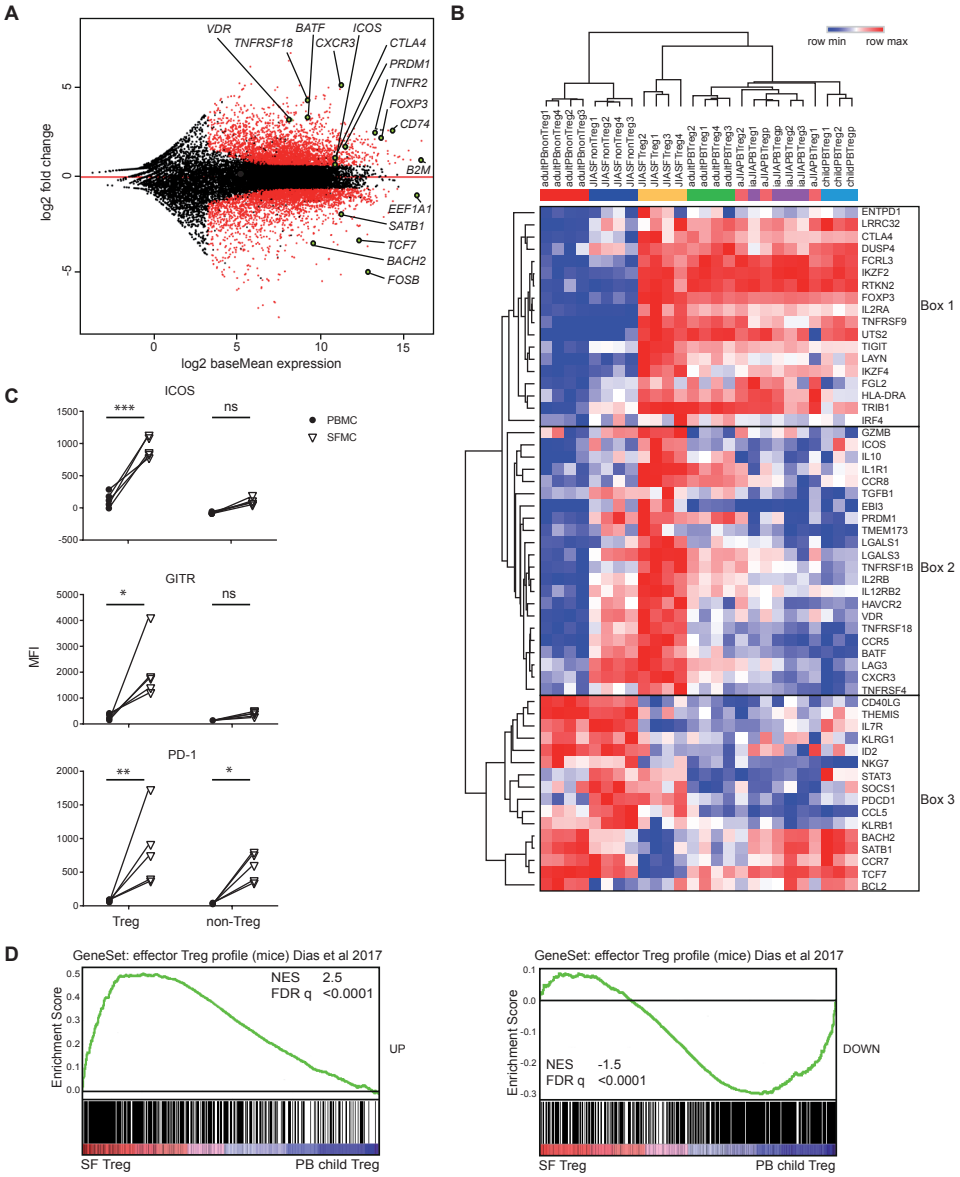

Figure 2. Inflammatory environment-derived Treg demonstrate a specific effector profile.

A MA plot of the differentially expressed genes between SF Treg and PB Treg of healthy children with adjusted p-value <0.05, minimal base mean 10 and minimal Log2 fold change 0.6. **B** Heatmap with hierarchical clustering analysis including all groups measured with RNA-sequencing and on a selection of genes based on recent literature (relative expression of log2 RPKM). **C** MFI of ICOS, GITR and PD-1 in gated $CD3^+CD4^+CD25^+CD127^{low}$ Treg and $CD3^+CD4^+CD25^-CD127^+$ non-Treg derived from paired SFMC and PBMC from 5 JIA patients (* = p<0.05, ** = p<0.01, *** = P<0.001, ns = not significant). **D** GSEA of effector Treg genes in mice (as identified in Dias et al 2017)) in pairwise comparisons involving SF Treg and PB Treg derived from healthy children, represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q).

by SF Treg (including *GMZB*, *ICOS* and *IL10*) whereas others were highly expressed by SF Treg but with shared, albeit lower expression by SF non-Treg (including *PRDM1*, *BATF*, *TNFRSF18* encoding GITR, *TNFRSF1B* encoding TNFR2 and *HAVCR2* encoding TIM-3) (Figure 2B, box 2). A third cluster represents the genes that are most highly expressed in non-Treg (Figure 2B, box 3). Some of the genes in this cluster shared expression with SF Tregl, such as *LAG3*, *CXCR3* and *PDCD1* (encoding PD-1) whereas expression of *CD40LG*, *BACH2*, *SATB1*, *CCR7* and *TCF7* was clearly lower in SF Treg (Figure 2B, box 2 and 3). Interestingly, the more heterogeneously expressed genes listed in box 2 and 3 were previously associated with an eTreg profile, such as *ICOS*, *IL10*, *PRDM1*, *TNRFSF18*, *TNFRSF1B* and *PDCD1*. The increased expression of ICOS, GITR and PD-1 was verified at the protein level by flow cytometry (Figure 2C). Besides the differential expression of these markers, we found a significant enrichment of genes within SF Treg that were recently described in mice to be up- or downregulated in eTreg[23,38] (Figure 2D and Supplemental Figure 2C), confirming the eTreg profile of SF Treg in humans. Collectively, these data demonstrate that autoimmune-inflammation derived human Treg display an eTreg signature.

SF Treg demonstrate adaptation to the interferon-skewed inflammatory environment.

To determine the relationship between the inflammatory environment-derived and PB-derived cells, we performed an unsupervised PCA analysis on the SF Treg and SF non-Treg, and PB Treg from healthy children. PB Treg were clearly separated from SF derived cells, confirming that the environment plays a dominant role in determining the transcriptional landscape (Figure 3A). K-mean clustering analysis showed that both SF Treg and SF non-Treg have increased expression of genes related to inflammation-associated pathways, including pathways linked to cytokine responses such as Interferon and IL-12 (Figure S3A). K-mean, and subsequent gene ontology analysis of SF Treg and all PB Treg groups derived from children demonstrated that pathways associated with Th1 skewing were specifically upregulated in SF Treg (Figure 3B and Supplemental Figure 3B+C). Indeed, the expression of the Th1 key transcription factor *TBX21* (T-bet), the Th1 related chemokine receptor *CXCR3*, and IL-12 receptorβ2 (*IL12RB2*) in SF Treg was increased on both the mRNA as well as protein level (Figure 3C and D). The expression of *TBX21* and *CXCR3* was equally high in both SF non-Treg and SF Treg, whilst *IL12RB2* showed a significantly higher expression in SF Treg (adjusted P value = $6.8E^{-10}$). Accordingly, co-expression of T-bet and FOXP3 protein was found in SF Treg in contrast to non-Treg, thus excluding significant contamination of non-Treg that could contribute to the high T-bet levels observed in SF Treg (Figure 3E). These results indicate that SF Treg exhibit functional specialization to allow selective regulation of specific T helper cell responses at particular tissue sites, as was previously established in mice[9,39–41]. In line with this observation, we found an enrichment in SF Treg of the transcriptional signature of TIGIT expressing Treg which have been recently identified as activated

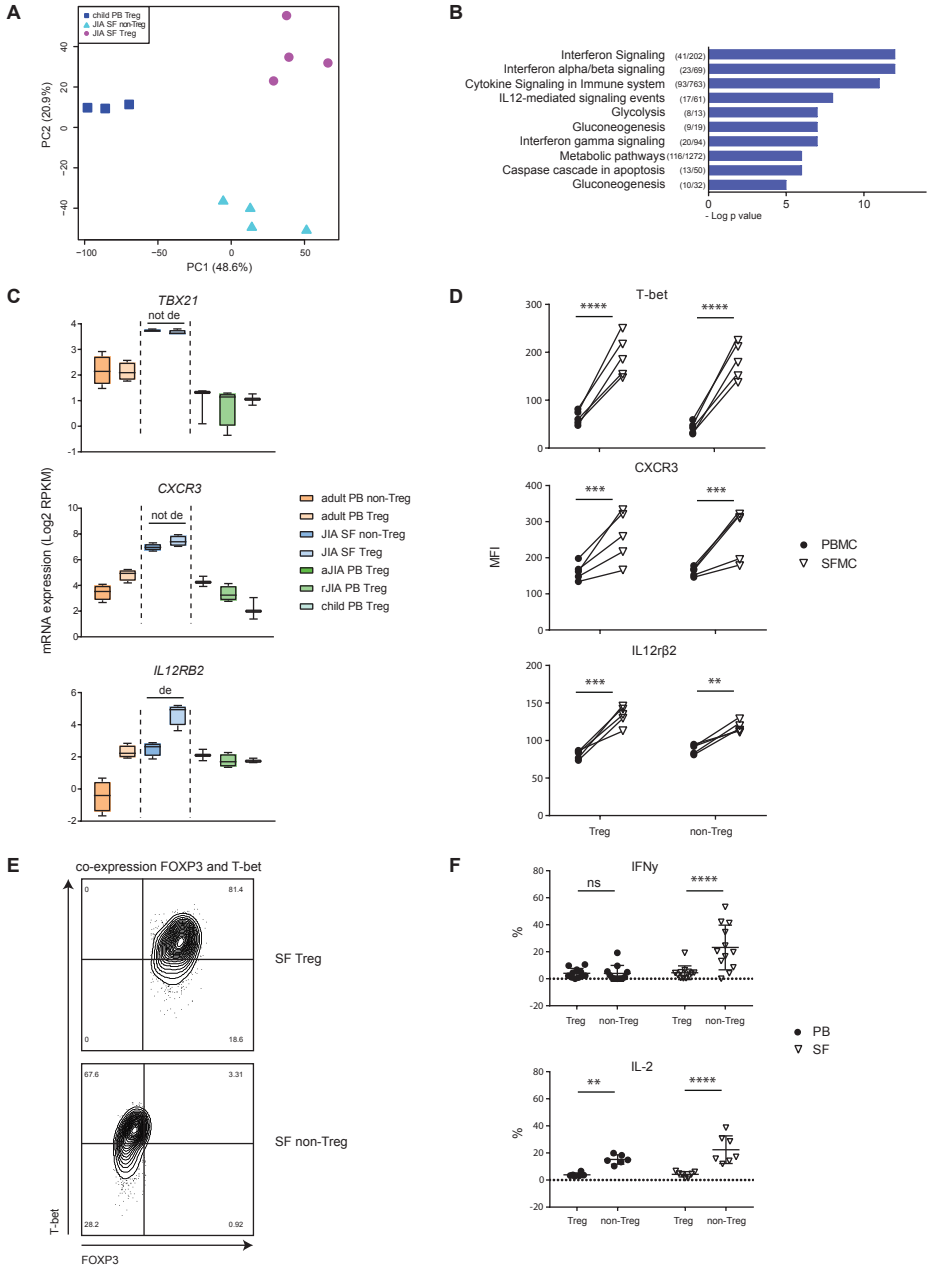

Figure 3. SF Treg adapt to the interferon-skewed inflammatory environment but lack cytokine production.

A Unsupervised PCA of SF derived Treg and non-Treg and PB derived Treg from healthy children. **B** Gene ontology terms related to genes that were specifically upregulated in SF Treg compared to PB Treg derived from children (with active JIA, inactive JIA and healthy children), ranked by enrichment scores. The number of genes measured in the analysis compared to the total amount of genes that are annotated in the gene ontology

term are depicted after the terms. **C** mRNA expression (log2 RPKM) of *TBX21*, *CXCR3* and *IL12RB2* in sorted Treg derived from PB of healthy adults (adult), healthy children (child), JIA patients with active disease (aJIA), JIA patients with inactive disease (iaJIA), SF of JIA patients (JIA SF) and non-Treg derived from PB of healthy adults and SF of JIA patients as determined by RNA-sequencing analysis (de = differentially expressed according to description in Figure 1D). **D** MFI of T-bet, CXCR3 and IL12Rβ2 in gated $CD3^+CD4^+CD25^+CD127^{low}$ Treg and $CD3^+CD4^+CD25^-CD127^+$ non-Treg derived from paired SFMC and PBMC from 5 JIA patients (* = p<0.05, ** = p<0.01, *** = P<0.001). **E** Contourplot of T-bet and FOXP3 expression on $CD3^+CD4^+CD25^+FOXP3^+$ gated Treg and $CD3^+CD4^+CD25^{int/-}FOXP3^-$ gated non-Treg from SFMC (one representative of n=5). **F** Percentage of IFNγ and IL-2 measured in $CD3^+CD4^+CD25^+FOXP3^+$ gated Treg and $CD3^+CD4^+CD25^{int/-}FOXP3^-$ gated non-Treg from SFMC and PMBC (IFNγ: n = 11 PB, n12 SF; IL-2: n=6 PB, n=7SF; ** = p<0.01, **** = p<0.0001, ns = not significant).

Treg that selectively suppress Th1 and Th17 cells in mice[24] (Supplemental Figure 3D).

The high expression of Th1-related proteins raised the question whether the SF Treg may have acquired a Th1 phenotype. However, SF Treg failed to produce both IL-2 and IFNγ (Figure 3F) and SF Treg responded dose-dependently to IL-2 with increasing pSTAT5 levels (Supplemental Figure 3E), a signaling pathway which is pivotal for Treg survival and function[42]. In fact, compared to PB Treg, SF Treg appeared to be even more responsive to IL-2. Altogether, our findings demonstrate that SF Treg are adapted to their inflammatory environment while maintaining Treg key features.

Effector Treg differentiation and inflammation are regulated by the (super-) enhancer landscape.

To explore the mechanistic regulation of the inflammation-adapted effector Treg profile we analyzed the active enhancer landscape of SF Treg. Enhancers are distal regulatory elements in the DNA that allow binding of transcription factors and as such coordinate gene expression. Epigenetic regulation of enhancers is critical for context-specific gene regulation[43]. Active enhancers can be identified by enrichment of a specific histone mark: acetylation of lysine 27 on histone H3 (H3K27ac enrichment)[44]. Chromatin Immunoprecipitation sequencing (ChIP-seq) performed for H3K27ac using SF Treg and healthy adult PB Treg showed a striking difference in enhancer profile and activity (Figure 4A, upper panel). To determine if the transcriptome of SF Treg is reflected at an epigenetic level, H3K27ac ChIP-seq data was correlated to our RNA-seq data from the same cell populations. The genes that demonstrated increased H3K27ac were increased at the mRNA level and *vice versa* (Figure 4A, middle and lowest panel), confirming that gene expression and chromatin-acetylation are interconnected in these cells.

Super-enhancers are large clusters of enhancers that have stronger enhancer activity and specifically regulate genes that define cell identity, both in health and disease[45,46]. Also at the level of super-enhancers, we found a significant enrichment in the transcribed genes with even higher enrichment scores, again indicating that the profile of SF Treg is mediated by epigenetic changes (Figure 4B, middle and lowest panel). The analysis of differential super-enhancer-associated genes revealed 337 different gene loci

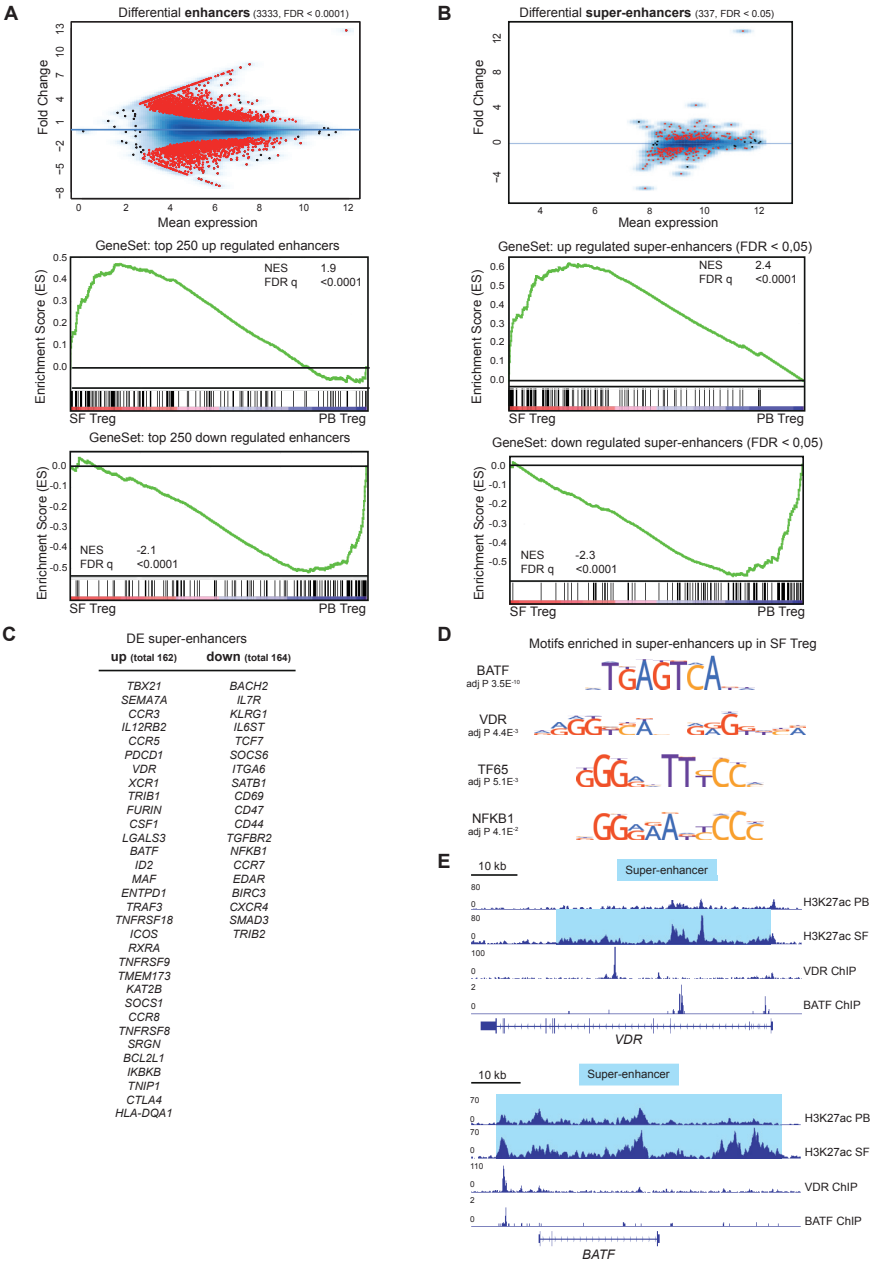

Figure 4. Environment specific effector Treg profile is regulated by the (super-)enhancer landscape.

A MA plots of differentially expressed enhancers (FDR<0.0001) in SF Treg vs PB Treg (upper panel). GSEA of the top 250 upregulated enhancers in pairwise comparisons involving transcriptome data of SF Treg and PB Treg derived from healthy adults, represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q) (middle panel). GSEA of the top 250 downregulated enhancers in pairwise comparisons involving transcriptome data of SF Treg and PB Treg derived from healthy adults, represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q) (lower panel). **B** Same as in (**A**) but for super-enhancers (FDR<0.05; upper panel), and with the upregulated supers (FDR<0.05; middle panel), and with

the downregulated super-enhancers (FDR<0.05; lower panel). **C** Selection of super-enhancers up- and down-regulated in SF Treg compared to PB Treg derived from healthy adults (FDR<0.05). **D** Motifs for transcription factor binding sites that are enriched in the upregulated super-enhancers in SF Treg compared to to PB Treg derived from healthy adults. **E** Gene tracks for *VDR* and *BATF* displaying ChIP-seq signals for H3K27ac, VDR and BATF.

(Figure 4B, upper panel). Specifically, we identified that super-enhancers associated with genes related to canonical Th1 differentiation, such as *TBX21* and *IL12RB2*, are increased in SF Treg (Figure 4C), demonstrating the epigenetic regulation of the environment-associated profile. Core Treg genes encoding the effector molecules *ICOS*, *CTLA4*, *TNFRSF18* and *PDCD1* were associated with increased super-enhancers as well, such as *FURIN* and *ID2* which are more putative functional Treg markers. Importantly, eTreg differentiation was also reflected in the enhancer profile of SF Treg, with differential expression of super-enhancer-associated transcriptional regulators including *BATF*, *BACH2*, *SATB, TRAF3* and *SOCS1*. We also found super-enhancers associated with markers that previously have not been related to (e)Treg differentiation and are specific for human Treg, such as *VDR* (encoding Vitamin D3 receptor), *RXRA* (encoding Retinoic acid receptor RXR-alpha) and *IL12RB2*. Inflammation related homing markers (*CCR2*, *CCR5*, *CCR7*) were also associated with differential H3K27ac levels in SF Treg. Altogether, these super-enhancer-associated genes reflect the adaptation and specialization of Treg within an inflammatory environment.

Motif analysis for transcription factor binding sites in the SF and PB Treg enhancers revealed motifs for Treg-specific transcription factors. These included STAT5 and Myb; a transcription factor recently described as a core component of the effector differentiation program[23]. Recent papers demonstrate BATF, RelA and NFKB1 as crucial regulators for eTregs in mice[47–49]. In support of these data, we also found that the motifs for BATF, TF65 (encoding RelA), NFKB and VDR were significantly enriched within the super-enhancer regions that were specifically upregulated in SF Treg compared to PB Treg (Figure 4D), thereby further strengthening the eTreg profile of SF Treg. Moreover, within the super-enhancers of both the transcriptional regulators VDR and BATF the binding sites for each of them were found, indicating that BATF and VDR regulate their own gene expression and also control other eTreg genes (Figure 4E). Together, these observations demonstrate that the inflammation-adapted effector phenotype of SF Treg is regulated at an epigenetic level, with a super-enhancer profile and enrichment of binding sites for eTreg genes.

The effector Treg profile overlaps with other chronic inflammatory diseases and has striking overlap with the human tumor Treg signature.

To investigate whether the program identified in SF Treg is a universal profile of human Treg exposed to inflammation, we compared our findings with Treg derived from peripheral blood and inflammatory joints of Rheumatoid Arthritis (RA) patients. Indeed, also in Treg derived from the inflammatory exudate of RA patients similar eTreg genes were upregulated (Figure 5A).

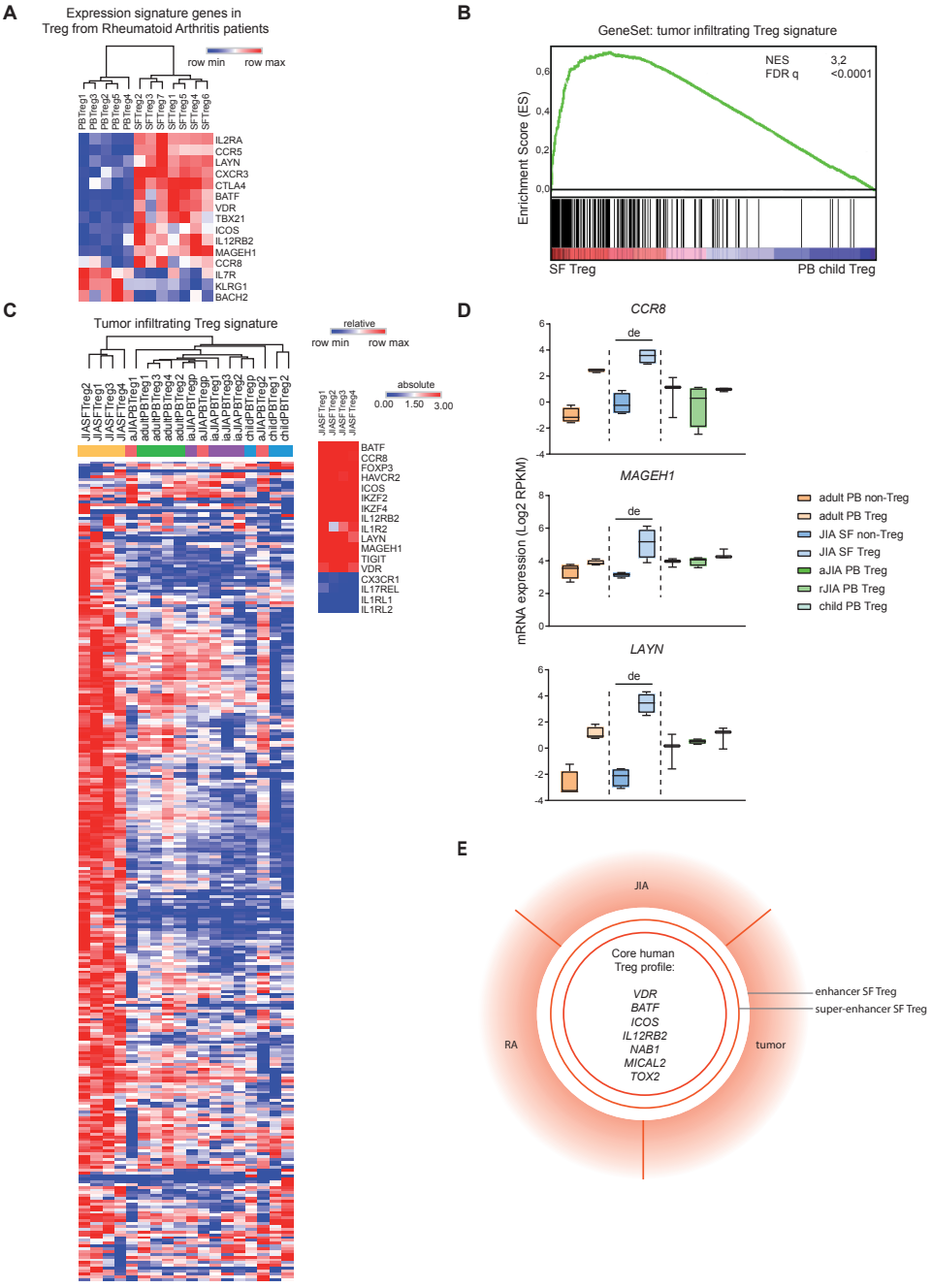

Figure 5. The effector Treg program is universal and overlaps with the human tumor Treg signature.

A Heatmap with unsupervised hierarchical clustering analysis on PB Treg and (partially paired) SF Treg from RA patients measured with a gene array, on a selection of signature genes identified from SF Treg in JIA patients and tumor-infiltrating Treg. **B** GSEA of tumor-infiltrating Treg signature genes (as identified in De Simone et al, Immunity 2016) in pairwise comparisons involving SF Treg and PB Treg derived from healthy children,

represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q). **C** Heatmap with hierarchical clustering analysis including all groups measured with RNA-sequencing on the identified tumor-infiltrating Treg signature genes (as identified in De Simone et al 2016), with relative expression of log2 RPKM. Small heatmap on the right shows the absolute values (log2 RPKM) of a selection of genes in SF Treg. **D** mRNA expression (log2 RPKM) of *CCR8*, *MAGEH1* and *LAYN* in sorted Treg derived from PB of healthy adults (adult), healthy children (child), JIA patients with active disease (aJIA), JIA patients with inactive disease (iaJIA), SF of JIA patients (JIA SF) and non-Treg derived from PB of healthy adults and SF of JIA patients as determined by RNA-sequencing analysis (de = differentially expressed according to description in Figure 1D; adjP values *CCR8* = $2.2E^{-13}$, *MAGEH1* = $1.2E^{-07}$, *LAYN* = $1.2E^{-33}$). **E** Identification of a core human Treg profile based on the overlapping genes upregulated in JIA SF Treg, RA SF Treg (SF Treg vs act JIA/RA PB Treg, q<0.05, log2fold change >0.6x up) and tumor-infiltrating Treg (identified in De Simone et al. Immunity 2016), filtered with the (super-)enhancers upregulated in SF Treg (FDR<0.05 and FDR<0.0001, respectively).

We then compared our data to a recently published human tumor-infiltrating Treg specific signature[19]. Strikingly, the tumor-infiltrating Treg signature was highly enriched in SF Treg (Normalized Enrichment Score 3.2, FDR q <0.0001; Figure 5B). Hierarchical clustering analysis of the described tumor-infiltrating gene signature further revealed separate clustering of SF Treg (Figure 5C), indicating that the high expression of the signature genes is specific for human Treg at a site that is characterized with infiltration of immune cells. We also observed a small set of genes that were not upregulated in SF Treg, e.g. *CX3CR1*, *IL17REL*, *IL1RL1* and *IL1RL2*, implying environment-restricted adaptation (Figure 5C). The three genes that were described as the most enriched and distinctive genes in tumor-infiltrating Treg, *LAYN*, *MAGEH1,* and *CCR8,* were selectively and highly upregulated in SF Treg (Figure 5D). These data demonstrate that the Treg profile we observed is not restricted to the SF exudate from JIA and RA patients. In fact, it likely represents a more global profile of human Treg in inflammatory settings, possibly fine-tuned by environment specific adaptations. From these different datasets we could deduce a core set of genes that appear universally upregulated in Treg in all three settings, and are regulated at an epigenetic level in SF Treg, i.e. they are associated with both an enhancer and super-enhancer that are increased in SF Treg (Figure 5E). This revealed *ICOS* and *BATF* as core markers that were previously related to eTregs in mice. Additionally, the less-known markers in this context, *IL12RB2* and *VDR,* belong to these core genes, which suggests a role for these molecules in human Treg differentiation. To the best of our knowledge, *NAB1*, *MICAL2* and *TOX2* have so far not been linked to Treg in human or mice, although all three independently have been linked to human cancers[50–54]. Collectively, our results demonstrate that infiltrating human Treg in different inflammatory settings share a core effector profile and provide a strong indication that inflammation drives eTreg differentiation in a comparable manner in different environments.

DISCUSSION

The recent discovery of eTreg in non-lymphoid tissues and inflammatory sites in mice have raised questions how human eTreg are transcriptionally and epigenetically programmed in different settings. The present study provides evidence that human Treg in a local inflammatory setting undergo further differentiation into specialized eTregs, with a unique expression profile. This is reflected in the high expression of TBX21 and CXCR3 and core Treg markers such as FOXP3, CTLA4 and TIGIT. Essential Treg features are maintained, such as suppressive function, IL-2 responsiveness and the lack of cytokine production. The transcriptional changes were mirrored at an epigenetic level, both in the enhancer and super-enhancer landscape, demonstrating that the profile is highly regulated. Additionally, we here identify previously unappreciated and specific markers in human eTreg, such as VDR, IL12Rβ2 and BATF. Finally, we demonstrate that the profile is not limited to autoimmune inflammation in JIA and RA, but shares almost complete overlap with tumor-infiltrating Treg. The similarity between a tumor setting and an autoimmune setting might seem counterintuitive, since the former reflects an immune-suppressive environment whereas the latter is associated with immune activation. Both environments however share specific features such as immune cell infiltration and inflammation[55,56]. This overlapping profile might therefore represent a more universal profile of human Treg in inflammatory environments.

Because of the extensive genome wide changes of Treg from an inflammatory environment, it is important to have insights in their functionality. In line with previous reports[33,34], we show that SF Treg are indeed suppressive and additionally highly responsive to IL-2 by phosphorylation of STAT5, excluding impaired IL-2 signaling[57,58]. This advocates against an intrinsic defect of Treg in the inflammatory setting. The maintenance of inflammation may be explained by resistance of local effector cells, which has been demonstrated both in JIA and RA[33,59,60].

In mice, functional specialization of Treg towards Th1 inflammation by upregulation of T-bet is described to be essential to prevent Th1 autoimmunity[13,14]. The remarkable co-expression of T-bet and FOXP3 in SF Treg in our study, with T-bet levels as high as in SF non-Treg, implies that functional specialization can be translated to a human setting. The increased expression of migration markers such as CXCR3 and CCR5 on both transcriptional as enhancer level further strengthens this, as high T-bet expression has been shown to be crucial for migration and thus co-localization of Th1-specific Treg and Th1 effector T cells by upregulation of these chemokine receptors[13,14].

Contamination of Treg with non-Treg can be a concern when sorting *ex vivo* human cells. However, we exclude substantial contamination by non-Treg because *1)* our data show robust differences between the transcriptomes of SF Treg and SF non-Treg, with increased expression of Treg hallmark genes in SF Treg, *2)* sorted Tregs are >90% FOXP3^{+}, *3)* there is clear co-expression of FOXP3 and T-bet on the single cell level, *4)* we show that

SF Treg, while expressing high levels of T-bet, lack cytokine production, which may be caused by the high expression of FOXP3, SOCS1, EGR2 and EGR3 (data not shown)[61,62]. Nevertheless, there may be heterogeneity within the eTreg population, and future single cell RNA-sequencing will shed further light on the eTreg population.

Our study is the first to investigate the transcriptional and epigenetic regulation of eTreg in human inflammatory settings and thus allows us to look at human specific eTreg regulation as well as commonalities between mice and men. The epigenetic landscape of human Treg is little explored, with only one paper studying H3K27acetylation of circulating human Treg and comparing this to mice Treg[63]. *Arvey et al.* conclude that the majority of Treg lineage-specific elements are not conserved between mice and human which further emphasizes the lack of knowledge of the human Treg epigenetic landscape and the need for additional studies, especially in non-homeostatic conditions. We found similarities between mice and men in eTreg differentiation regarding downregulation of *SATB1* and *BACH2*, which are both associated with a super-enhancer in PB Treg but not in SF Treg. Although both SATB1 and BACH2 are crucial for Treg development and the establishment of the Treg-specific super-enhancer architecture, recent reports show that downregulation of both transcriptional regulators is necessary for further differentiation of Treg and to prevent Treg from converting into helper T cells[64–69]. It is known that BACH2 can transcriptionally repress *PRDM1* (encoding Blimp-1) in T cells[68], and in line with this we observed increased acetylation as well as gene expression of *PRDM1* in SF Treg. Moreover, we found BATF as a prominent marker in SF Treg, with increased expression on both transcriptional and epigenetic level, and a binding site in upregulated super-enhancer associated genes. In addition, high expression of BATF was also observed in RA SF Treg and tumor-infiltrating Treg, suggesting that BATF is a key transcriptional regulator in both human and mice eTreg. Similarly, IRF4 is also reported to be essential for eTreg differentiation in mice, and we indeed observed increased mRNA expression and H3K27 acetylation of IRF4. We also found remarkable differences in eTreg signature markers compared to what is known from mice. The most pronounced difference is the upregulation of IL12RB2 at both the transcriptional and epigenetic level, and a binding site in upregulated super-enhancer associated genes, whereas in mice this marker is downregulated in eTreg. Instead, impaired IL-12Rβ2 expression has been reported as a key checkpoint that prevents Treg from fully differentiating towards Th1 cells in mice[39]. However, our data are in line with recent reports that show selective expression of *IL12RB2* in human PB Treg[63,70], suggesting that this marker has different functions in human and mice[71]. Another unexpected finding is the upregulation of VDR in both JIA and RA SF Treg, both on a transcriptional and epigenetic level, as well as in tumor Treg. VDR is not well-studied in the Treg context, although the tolerogenic effects of vitamin D are well-known[72]. Moreover, although not highlighted in the text, high VDR levels were also present in a recent screening of breast tumor-infiltrating Treg[18]. Another striking difference is the absence of *KLRG1* upregulation in human eTreg. In mice, KLRG1 is one of the key markers

to identify eTreg, although it is not crucial for eTreg function[73]. KLRG1 is not upregulated on SF Treg and is associated with a super-enhancer only in PB Treg. Additionally, in our data gene expression of KLRG1 is restricted to non-Treg (both SF and PB). To sum up, we show that human eTreg have similarities in the expression of eTreg differentiation markers identified in mice, but we also found human specific eTreg markers including IL-12Rβ2 and VDR and absence of KLRG1.

Lately, the application of Treg based therapies for autoimmune diseases and in transplantation settings is gaining renewed interest. Promising data from animal models and the first accomplished clinical trials, both with cell therapy involving adoptive transfer as well as with low dose IL-2 administration, pave the way for Treg therapies to reach the clinic[74,75]. Our data show that circulating human Treg are markedly different from their counterparts derived from peripheral sites that are characterized by immune activation. This is reflected in the expression of effector markers and transcriptional regulators, but also in the expression of specific chemokine receptors. In the context of Treg-based therapies it is important to appreciate that specific environments may require adapted Treg, both for migration and function. Moreover, in this study we extensively profiled human eTreg that are abundantly present at sites of inflammation. This information can be used as a basis for follow-up studies that may eventually allow the characterization of small amounts of circulating eTreg, for example to monitor patients undergoing treatment.

In conclusion, our study is the first to uncover the transcriptional and epigenetic program that defines human inflammatory Treg. SF Treg display an environment-adapted as well as an eTreg phenotype that is established at an epigenetic level. Moreover, we describe striking similarities of this program with tumor-infiltrating Treg and SF Treg from RA patients. This revealed a core set of genes that are shared with Treg from affected sites in JIA, RA and cancer and include BATF, VDR and IL12Rβ2.

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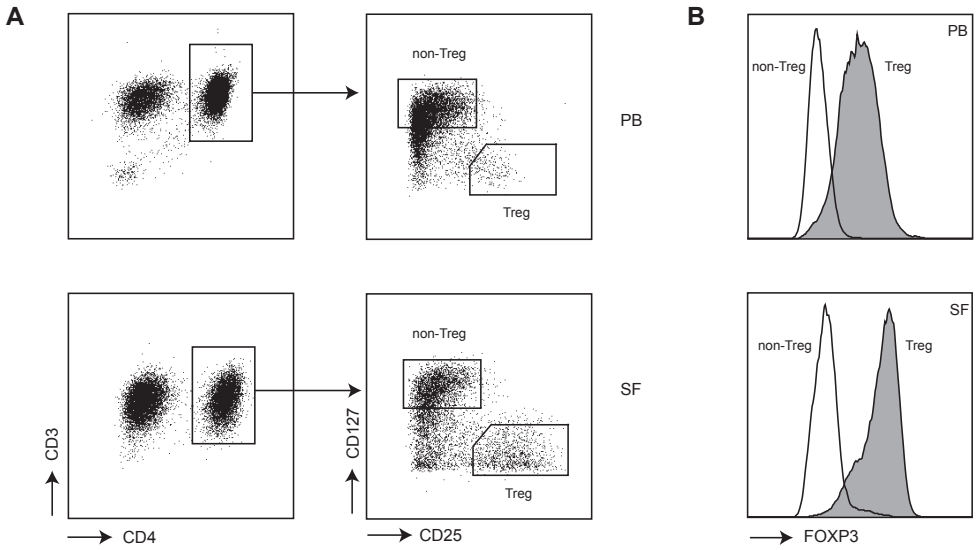
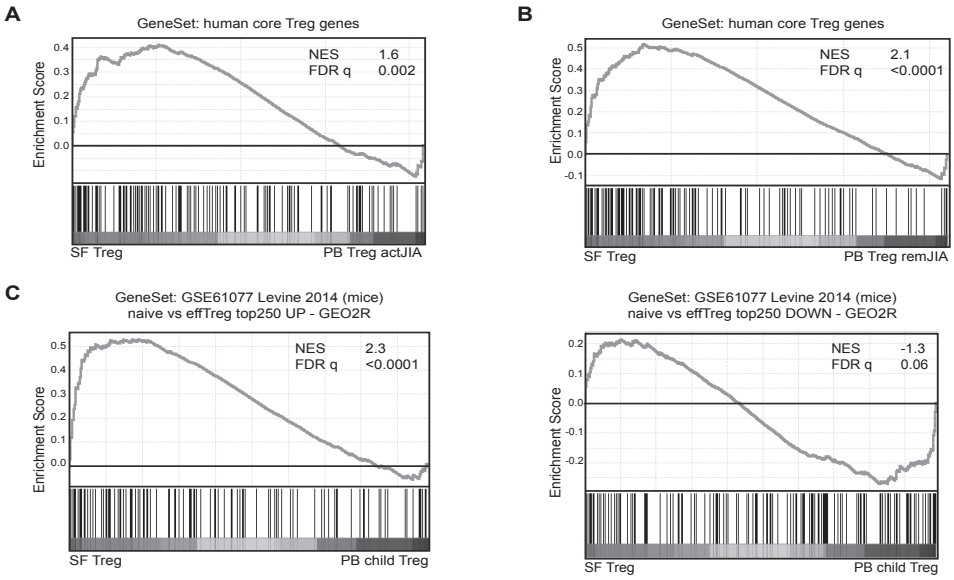
SUPPLEMENTAL DATA



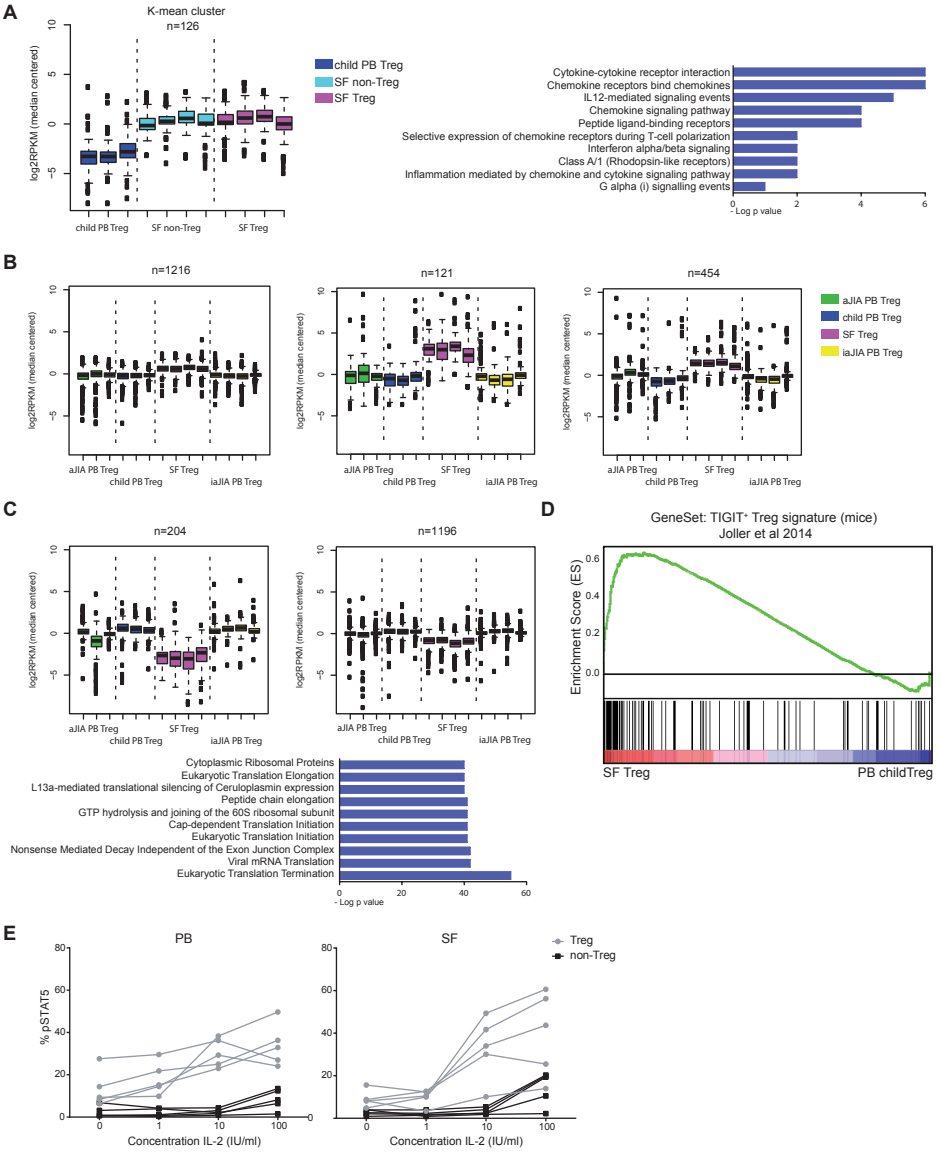
Supplemental Figure 1. Gating strategy and FOXP3 expression of sorted cells

A Gating strategy to sort both SF- and PB-derived Treg and non-Treg. **B** Foxp3 expression of sorted SF- and PB-derived Treg and non-Treg



Supplemental Figure 2. GSEA shows Treg signature and effector Treg signature in SF Treg

A GSEA of human core Treg signature genes (as identified by Ferraro et al PNAS 2014) in pairwise comparison of SF Treg and PB Treg derived from JIA patients with active disease, represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q). **B** Similar as in (**A**) but with pairwise comparison of SF Treg and PB Treg derived from JIA patients with inactive disease. **C** GSEA of eTreg genes (as identified with GEO2R as the top250 genes upregulated (left) or downregulated (right) in naïve vs eTregs, published in Levine et al, Nature Immunology 2014) in pairwise comparison of SF Treg and PB Treg derived from healthy children, represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q).



Supplemental Figure 3. K-mean and GeneSet Enrichment analysis shows adaptation of SF Treg to Th1 environment

A K-mean analysis on PB Treg, SF Treg and SF non-Treg with the cluster representing the genes upregulated in both SF Treg and SF non-Treg (left) and the gene ontology terms for pathways concerning the 126 genes belonging to this cluster (right; ranked on p value). **B** K-mean analysis on all Treg groups derived from children (JIA and healthy) with the clusters representing upregulated genes in SF Treg. **C** K-mean analysis on all Treg groups derived from children (JIA and healthy) with the clusters representing downregulated genes in SF Treg (above) and the gene ontology for pathways (below; ranked on p value). **D** GSEA of TIGIT$^+$Treg signature genes (as identified in mice by Joller at al., Immunity 2014) in pairwise comparisons involving SF Treg and PB Treg derived from healthy children, represented by the normalized enrichment score (NES) and the false discovery rate statistical value (FDR q). **E** Percentage of pSTAT5 positive cells in both PB- and SF-derived Treg and non-Treg in the presence of increasing concentrations IL-2 (n=5).

Chapter 6

Compartmentalization and persistence of dominant (regulatory) T cell clones in autoimmune inflammation

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Submitted

ABSTRACT

Inflammation is characterized by infiltration of multiple immune cells and expansions of antigen-specific T cells. In autoimmune diseases, inflammation is often limited to specific target tissues, but within tissues, multiple sites can be affected. An important outstanding question is whether affected sites are infiltrated with the same (pathogenic) T cell clones and whether these clones persist over time. In Juvenile Idiopathic Arthitis (JIA) it is possible to analyze large number of cells derived from the site of inflammation, i.e. inflamed joints. Here, we performed CyToF analysis and T cell receptor (TCR) sequencing to study immune cell composition and (hyper)expansion of inflamed joint-derived T cells. Samples were taken from different joints affected at the same time, and joints that were affected multiple times during the relapsing remitting course of the disease. CyTOF analyses revealed that the composition and functional characteristics of the immune infiltrates are strikingly similar between joints within one patient. Furthermore we observed a strong overlap between dominant T cell clones, especially Treg, in inflamed joints affected at the same time. Some of the most dominant clones could also be detected in circulation. Finally, these dominant T cell clones were found to persist over time and to expand during relapses, even after full remission of the disease. These data suggest that in autoimmune disease there is auto-antigen driven expansion of both Teff and Treg clones, that are highly persistent and are (re)circulating. Therefore these dominant clones can be interesting therapeutic targets.

INTRODUCTION

A hallmark of autoimmune diseases is inflammation, often localized in specific target tissues. Within those tissues however, multiple sites can be affected such as several locations within the intestine in case of Inflammatory Bowel Diseases (IBD), or multiple joints in Rheumatoid Arthritis (RA) or Juvenile Idiopathic Arthritis (JIA). Investigation of the pathophysiological mechanisms of autoimmune diseases have revealed accumulation of T cells at affected sites, as well as genetic associations with MHC class II alleles (HLA), directing towards T cells as a key component in the pathogenesis[1]. Next to the presence of highly activated $CD4^+$ T cells within the inflammatory environment, increased numbers of $CD4^+CD25^+CD127^{low}FOXP3^+$ (forkhead box P3) regulatory T cells (Treg), capable of suppressing immune responses and fundamental to immune homeostasis, are also present[2,3].

Flow cytometric studies have identified a heterogeneous pool of $CD4^+$ T cell and Treg phenotypes at reactive sites in human autoimmune diseases, that differ from their circulating counterparts[4–8]. In general, it is increasingly appreciated that local immune cells in tissues display a different array of trafficking and functional markers compared to circulating immune cells[9]. Novel technologies such as mass cytometry allow the analysis of cellular heterogeneity with high resolution and can reveal expanding and possible pathogenic cell populations with unique phenotypes.

Studies that asses the T cell receptor (TCR) repertoire have generated evidence for clonal expansions of T cells in a large spectrum of autoimmune diseases[10–15]. These findings suggest that the T cell response at the site of auto-immune inflammation is mounted by specific local antigens, that induce selective expansion and/or migration of antigen-specific clones. However, there are still many unknowns regarding the dynamics and persistence of both pathogenic effector T cells (Teff) and Treg clones during inflammation and disease progression. Mapping of the TCR repertoire with next generation sequencing provides a powerful tool to analyze changes in T cell dynamics.

Like other T cells, Tregs that leave the thymus each express a unique T-cell receptor. The TCR repertoire of peripheral Treg, consisting of both thymus derived and peripheral induced Treg, is as diverse as conventional $CD4^+$ cells while Treg only represent a small fraction of the total $CD4^+$ T cell pool[16–18]. By interacting with a cognate peptide-MHC complex Treg can recognize and act to a specific (auto-)antigen through their TCR, which is central to their thymic development as well as their suppressive function once they left the thymus[19,20]. A restricted TCR repertoire of Treg has been linked to a disturbed immune homeostasis in several studies using transgenic mice, including development of autoimmune diseases and loss of tolerance towards commensal bacteria[21–24], although Treg with a single TCR specificity can also guarantee some protection[25].

In humans hyper expanded Treg TCRβ clones are found at the site of inflammation in JIA[26–28] and in refractory JIA patients this even seems true for circulating Treg[29]. However, the temporal and spatial dynamics of hyper-expanded T cell clones in

chronic inflammation and their relation to disease relapses remain to be established. Furthermore, the presence of hyper-expanded clones suggest dominance of specific antigens present at the target tissue, but knowledge is lacking regarding the antigen specificity of Teff and Treg clones at the site of inflammation. Defining the $CD4^+$ T cell subsets that are expanding in patients is critical to decipher pathogenesis, since they may represent a novel therapeutic target. Moreover, insight into the antigen specificity of local T cells may aid in the discovery of disease-associated autoantigens.

Here, we had the unique opportunity to study autoimmune inflammation 1) at different affected sites at the same time and 2) over time to get a detailed understanding of T cell dynamics during human inflammation. We profiled the T cell composition of inflammatory exudate and peripheral blood of JIA patients from multiple reactive sites using mass cytometry. In addition, we determined the overlap of TCRβ clones between Treg and conventional $CD4^+$ cells (non-Treg) derived from the inflamed site of JIA patients in time and space. Hyper-expanded clones identified at the site of inflammation were highly overlapping between two affected joints that were sampled at the same time. Moreover, the cellular distribution of two inflamed joints within a patient was almost identical, whereas between patients were distinguishable differences. When comparing affected joints during multiple disease relapses there was a large degree of overlap in the dominant TCRβ clones, both Treg and non-Treg. Together, these data indicate re-circulation of T(reg) cells that very likely react to dominant auto-antigens that are continuously present in the affected joint in JIA patients.

METHODS

Collection of SF and PB Samples

Patients with Juvenile Idiopathic Arthritis (JIA) were enrolled by the Pediatric Rheumatology Department at University Medical Center of Utrecht (The Netherlands). A total number of 9 JIA patients were included in this study. Of the JIA patients n=2 were diagnosed with extended oligo JIA, n=2 with rheumatoid factor negative poly-articular JIA, and n=5 with oligo JIA, according to the revised criteria for JIA[30]. The average age at the time of (first) sample inclusion was 13,1 years (range 3,2 – 18,1 years) with a disease duration of 7,3 years (range 0.4 – 14.2 years).

PB and synovial fluid (SF) was obtained when patients visit the outpatient clinic via vein puncture or intravenous drip, and by therapeutic joint aspiration of the affected joints, respectively. Informed consent was obtained from all patients either directly or from parents/guardians when the patients were younger than age 12 years. The study was conducted in accordance with the Institutional Review Board of the University Medical Center Utrecht (approval no. 11-499/C). PB from n=3 healthy children (average age 15,1 years with range 14,7 - 15,4 years) was obtained from a cohort of control subjects for a case-

control clinical study. Samples were collected in compliance with the Declaration of Helsinki.

SF was incubated with hyaluronidase (Sigma-Aldrich) for 30 min at 37°C to break down hyaluronic acid. Synovial fluid mononuclear cells (SFMCs) and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences, AB) and were used after freezing in Foetal Calf Serum (FCS) (Invitrogen) containing 10% DMSO (Sigma-Aldrich).

Flow cytometry and cell sorting

For TCR sequencing purposes, $CD3^+CD4^+CD25^{high}CD127^{low}$ cells (Treg) and $CD3^+CD4^+CD25^{low/int}CD127^{int/high}$ cells (non-Treg) were isolated from frozen PBMC and SFMC, using the FACS Aria III (BD). Antibodies used for sorting are: anti human CD3-BV510 (Biolegend), CD4-FITC (eBioscience), CD25-PE/Cy7 (BD), CD127-AF647 (Biolegend). To check for FOXP3 expression of the sorted populations anti human FOXP3-eF450 (eBioscience) was used.

TCR sequencing

Tregs and non- Tregs were sorted (between 146.191 and 1x10^6 Tregs and between 466.603 and 1x10^6 non-Tregs were obtained) and frozen at -80°C. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) for cell fractions ≥0.2x10^6 cells and the RNeasy Micro Kit (Qiagen) for fractions ≤0.2x10^6 cells following the manufacturer's instructions. cDNA was synthesized using the SMARTer RACE cDNA Amplification kit (Clontech). Amplification of the TCRβ VDJ region was performed using previously described primers and amplification protocols[31]. PCR products were analyzed with a QIAxcel Advanced System (Qiagen). Upon successful amplification, end repair and barcode adapter ligation were performed with the NGSgo®-LibrX and NGSgo®-IndX (GenDx) according the recommendations of the manufacturer. Cleanup of the samples was performed after each step using HighPrep PCR beads and following the recommendations of the manufacturer (GC Biotech, Waddinxveen). Paired-end next-generation sequencing was performed on an Illumina MiSeq system 500 (2 x250 bp) (Illumina).

HLA-typing

For the amplification on NGS the GenDX NGSgo kit was used according to the specifications of the Supplementalementalier (GenDx BV, Utrecht). The processing of the amplicons for sequencing was performed similar as described above.

CyToF

Cells were thawed and stained with a T-cell focused panel of 37 heavy metal-conjugated antibodies (Supplemental Table 1), as previously described[32] and then analyzed by CyToF-Helios. Briefly, PBMCs were stimulated with or without phorbol 12-myristate 13-acetate (150 ng/ml, Sigma-Aldrich) and ionomycin (750 ng/ml, Sigma-aldrich) for 4 h, and blocked with secretory inhibitors, brefeldin A (1:1000, eBioscience) and monesin (1:1000,

Biolegend) for the last 2 h. The cells were then washed and stained with cell viability dye cisplatin (200 µM, Sigma-aldrich). Each individual sample was barcoded with a unique combination of anti-CD45 conjugated with either heavy metal 89, 115, 141 or 167, as previously described[33]. The barcoded cells were washed and stained with the surface antibody cocktail for 30 min on ice. The cells were again washed and re-suspended in fixation/permeabilization buffer (eBioscience) for 45 min on ice. The permeabilized cells were then stained with an intra-antibody cocktail (permeabilisation buffer, eBioscience) for 45 min on ice, before washing and staining with a DNA intercalator Ir-191/193 (1:2000 in 1.6% w/v paraformaldehye, Fluidigm) overnight at 4°C or for 20 min on ice. Finally, the cells were washed and re-suspended with EQ™ Four Element Calibration beads (1:10, Fluidigm) in ultra-pure distilled water at $1x10^6$ cells/ml. The cell mixture was then loaded and acquired on a Helios mass cytometer (Fluidigm) calibrated with CyToF Tunning solution (Fluidigm). The output FCS files were randomized and normalized with the EQ™ Four Element Calibration beads against the entire run, as per the manufacturer's recommendations.

TCR data analysis

Data analysis was performed as described before by[34].

CyToF data analysis

The normalized CyToF output FCS files were de-barcoded manually into individual samples in FlowJo (v.10.2), and down-sampled to equal cell events (5000 cells) for each sample. Batch run effects were assessed using an internal biological control (PBMC aliquots from the same healthy donor for every run). The normalized cells (5000 events) were then clustered with MarVis using Barnes Hut Stochastic Neighbour Embedding (SNE) non-linear dimensional reduction algorithm and k-means clustering algorithm, as previously described[32]. The default clustering parameters were set at perplexity of 30, and $p < 1e-21$. The cells were then mapped on a 2-dimensional t-distributed SNE scale based on the similarity score of their respective combination of markers, and categorized into nodes (k-means). To ensure that the significant nodes obtained from clustering were relevant, we performed back-gating of the clustered CSV file and supervised gating of the original FCS files with FlowJo as validation. Visualizations (density maps, node frequency fingerprint, node phenotype, radar plots) are performed through R scripts and/or Flow Jo (v.10.2). Correlation matrix and node heatmaps are performed through MarVis and PRISM (v 7.0).

Statistics

The nonparametric Mann Whitney (two tail) statistical test was performed in the manual gating of cellular subsets in FlowJo; * $p < 0.05$. The correlation matrix for the node frequency was calculated with nonparametric spearman correlation.

Figures
For the schematic representation sample collection figures are adapted from the following website: https://smart.servier.com/. Venn diagrams are made with use of jVenn[35].

RESULTS

Immune architecture of cellular infiltrates is similar in anatomically distinct inflamed sites

To study the immune cell composition of inflamed joints, we profiled peripheral blood and synovial fluid mononuclear cells (PBMCs/SFMCs) from JIA patients with both knees affected at the time of sampling with mass cytometry. To comprehensively characterize the T cell compartment within the inflamed joints, we set up a T cell centric panel (Supplemental Table 1) comprising 37 phenotypical and functional markers, as well as CD45 barcoding markers to be able to study matched samples simultaneously. This panel included lineage markers (e.g., CD3, CD4, CD8, CD11b, CD14, CD16), T cell migration markers (e.g., CXCR3, CCR6, CCR5, CXCR5), T cell differentiation and activation markers (e.g., CD45RA, CD28, CD69, LAG3, PD1), T cell helper defining cytokines (e.g., IFNγ, IL-4, IL-17A, IL-21), Treg markers (e.g., CD25, FOXP3, CD127, CTLA-4, IL-10) and JIA disease associated cytokines (e.g., TNFα, IL-6).

To decipher the relationship between the marker expression and cellular immune composition, we made use of a data-driven unsupervised T-SNE dimensional reduction and k-means clustering approach to classify cellular populations. Analysis of the immune cells from the SFMC/PBMC compartments revealed a diversity of 22 cellular nodes (Figure 1A, $p < 1e\text{-}21$, and Supplemental Figure 1A and B), broadly segregated by the expression of CD3/CD4, CD3/CD8, CD14/CD11b, HLADR/CXCR5 lineages, T memory marker $CD45RA^{+/-}$, Treg population $CD25^{+}$ $FOXP3^{+}$, and assortment of cytokine markers. Preliminary hierarchal clustering of the median marker expression already revealed a clear demarcation of SFMCs and PBMCs (Figure 1B), and a strong association of immune phenotypes between intra-individual paired knee SFMCs. Furthermore, density maps of immune cellular populations within the T-SNE maps indicates strong dichotomy in the locations of SFMC and PBMC subsets (Figure 1C), with SFMCs occupying the bottom right and the PBMCs occupying the top left regions. A strikingly similar density cellular distribution profile was observed (Figure 1C) in the left and right knee joints of each JIA individual with nearly identical node fingerprints (Figure 1D). The correlation matrix of the entire spectrum of node frequency demonstrated a strong positive correlation between the SFMCs and their left and right joints, and an inverse negative correlation as compared with the PBMC populations (Supplemental Figure 1C). In conclusion, the immune phenotypic architecture of distinct inflamed sites (left and right knee) are remarkably similar, indicating commonality in underlying disease etiology.

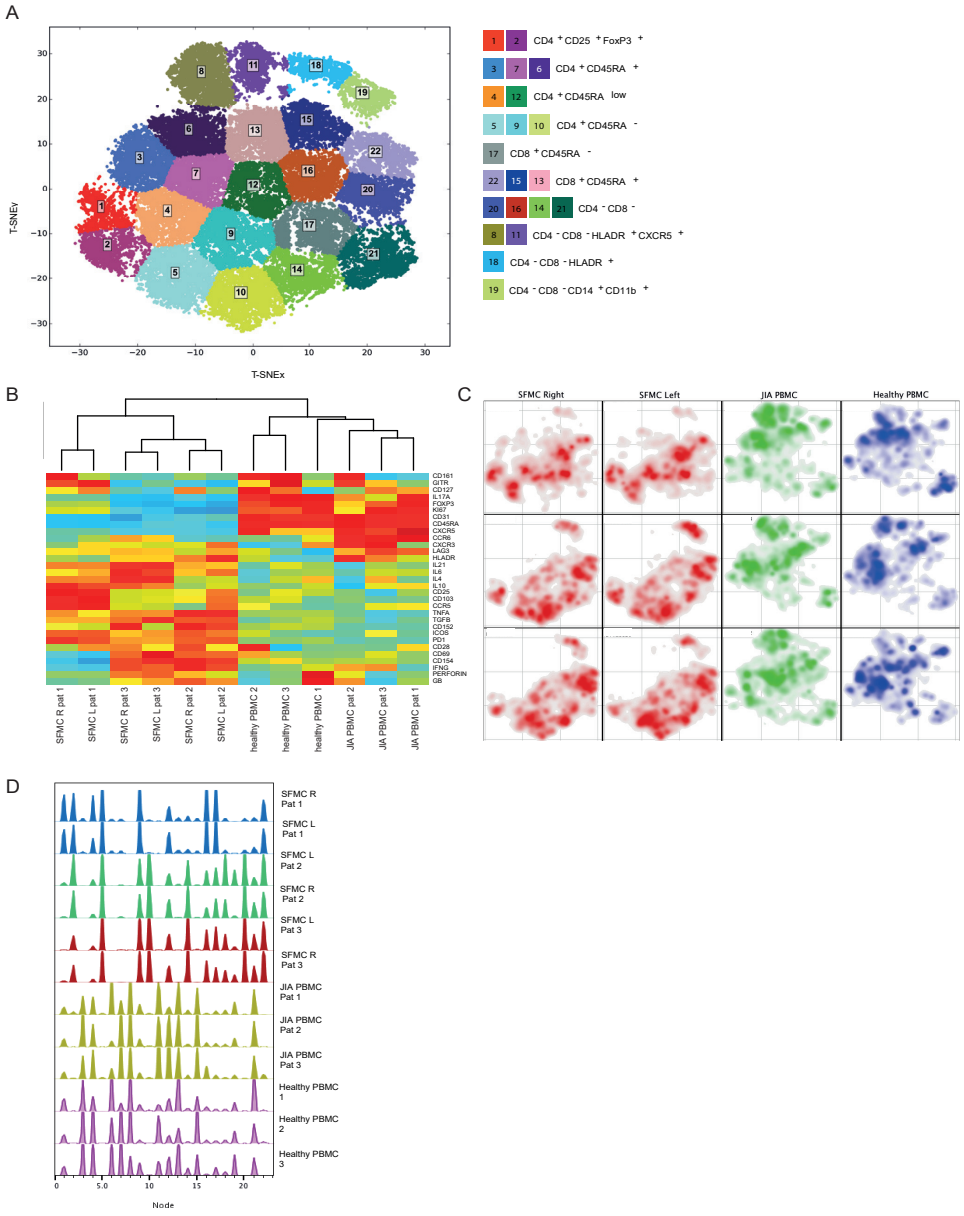

Figure 1. Overall immune architecture in left and right affected joint is very similar but distinct from peripheral blood

A Density maps based on T-SNE dimensional reduction and k-means clustering analysis on SFMC and PBMC samples, resulting in 22 cellular nodes. **B** Preliminary hierarchal clustering on the median expression of all markers. **C** Density maps of immune cellular populations within the T-SNE maps. **D** Node frequency fingerprints showing the distribution across the nodes of SFMCs an PBMCs.

Similarity in immune architecture extends to both T effector and Treg compartments in distinct inflamed sites

Next, we further studied the T cell subsets present in the inflamed joints. The CD4 and CD8 T cell subsets within the synovial environment both reflected the pro-inflammatory environment with strong expression of pro-inflammatory cytokines (TNFα, IFNγ and IL-6), indications of chronic TCR activation (PD1 and LAG3) and a memory phenotype ($CD45RA^-$) as compared with PBMC counterparts (Supplemental Figure 2A and 2B, $p < 0.05$). Remarkably, the cytokine diversity of CD4 memory cells revealed nearly identical profiles for the left and right knee joints for each individual (Figure 2A), with minor inter-individual differences. This trend in cytokine profile was also reflected in the $CD8^+CD45RA^-$ compartment (data not shown). The Treg ($CD25^+$ $FOXP3^+$) population was significantly enriched in the synovial environment (Figure 2B, $p < 0.05$ and Supplemental Figure 2C-D) with enhanced expression of T cell memory ($CD45RA^-$) and Treg activation markers (HLADR and ICOS) as compared with PBMC counterparts. We noted significantly higher proportion of proliferation (Ki67) within the memory Treg compartment as compared with the memory T effector compartment within the synovial environment (Figure 2B, $p < 0.05$), which was further confirmed by flow cytometric analysis (Supplemental Figure 2E). This indicates that Treg belong to the most proliferative T cell subset in the inflamed environment. Furthermore, memory Treg showed very similar profiles of CTLA4/HLADR/ICOS/PD1 expression in the left and right knee joints for each individual with some inter individual differences (Figure 2C).

Altogether, these data demonstrate that within a patient there is a an identical T cell phenotypic and functional profile present at separated inflamed locations, with increased amounts of activated and proliferating Treg populations.

Hyperexpanded T cell clones are shared between left and right joint

We hypothesized that the similarities in T cell phenotype and functional characteristics can be explained by infiltration and expansion of the same T cell clones in both affected joints. To study whether the same cells infiltrate multiple joints, and to be able to distinguish Treg from other $CD4^+$ helper T cells, we sorted similar numbers of $CD3^+CD4^+CD25^+CD127^{low}$ Treg and $CD3^+CD4^+CD25^-CD127^+$ non-Treg (Supplemental Figure 3A). The samples were derived from the same donors and time points as the ones used for CyToF analysis, regarding the first two patients. We examined the T cell receptor beta chain (TCRβ) repertoires of the isolated cells subsets using next generation sequencing technology of the CDR3 region. As expected, within the inflamed environment clonally expanded cells were detected, which was more pronounced for Treg compared to non-Treg (Figure 3A). Comparable with the cellular composition, the distribution of the T cell clones was very similar between left and right joint, both for Treg and non-Treg. Hyperexpanded clonal populations of T cells were further studied by selecting the most abundant 100 unique TCRβ clonotypes, defined by amino acid sequence (Figure 3B). Notably, we found a high degree of sharing between the two affected joints. In addition to the increased

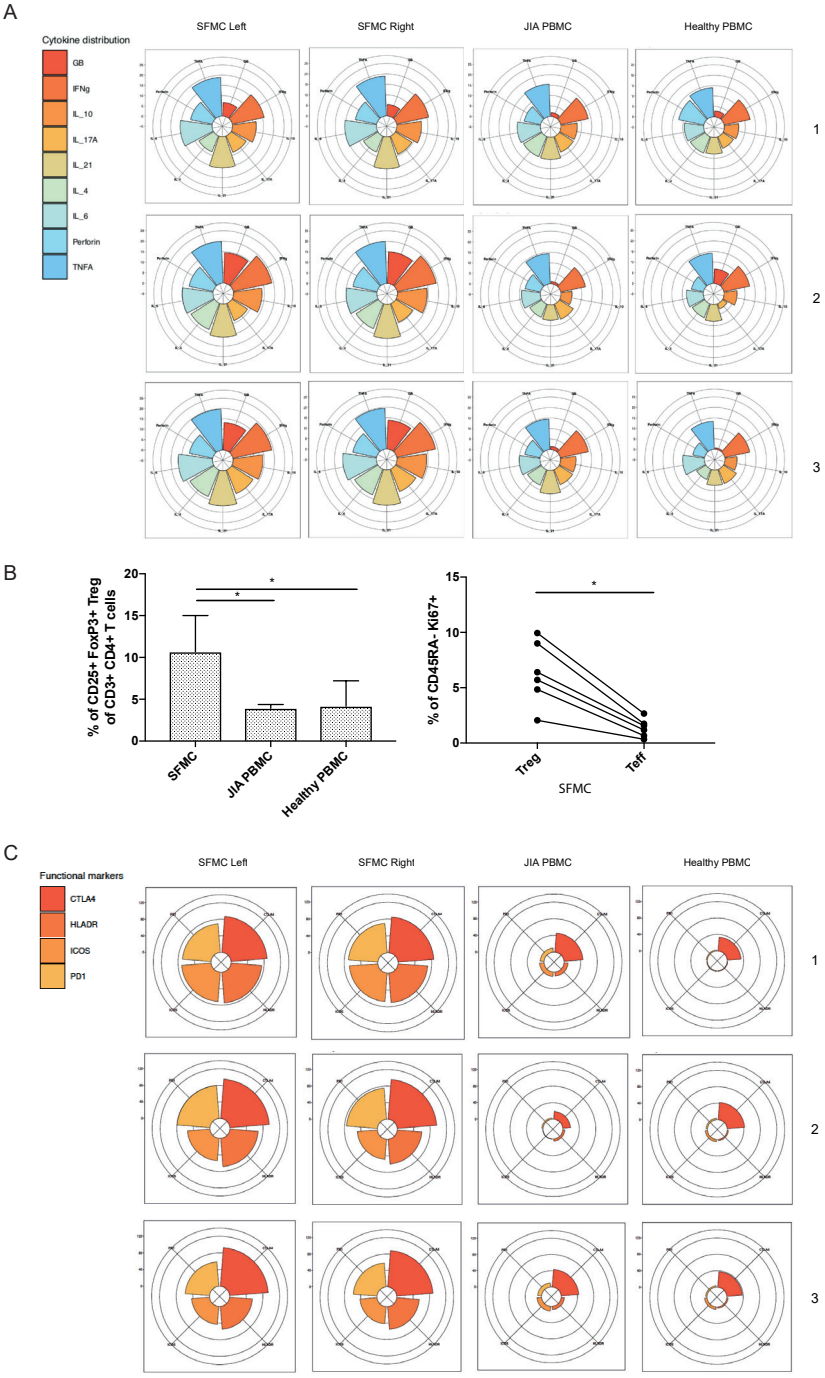

Figure 2. Identical T cell phenotypical and functional profile present at separated inflamed locations

A Cytokine production of $CD4^+$ $CD45RA^-$ memory cells depicted in radarplots. **B** Percentage $CD25^+FOXP3^+$ Treg of $CD3^+CD4^+$ cells in SFMC and PBMC of JIA patients and healthy children, and percentage of $Ki67^+$ cells within $CD45RA^-$ cells in Treg and non-Treg in SFMC (non-parametric Mann-Whitney, * = $p < 0.05$). **C** Expression of functional markers by $CD25^+$ $FOXP3^+$ $CD45RA^-$ cells.

clonality within the Treg compartment, sharing of clones between two locations was also more evident for Treg compared to non-Treg with an overlap of 71 or 79 out of 100 most dominant clones for Treg, for patient 1 or 2 respectively, in comparison to 44 or 66 out of 100 for non-Treg (Figure 3B). Importantly, the most dominant clones, i.e. the clones with the highest frequency, were also detectable in peripheral blood (Figure 3C and Supplemental Figure 3B). The overall overlap between Treg and non-Treg was 2,1% and 3,1% for patient 1 and 2, indicating mostly exclusive usage of TCR and limited Treg plasticity.

In summary, both non-Treg and Treg hyperexpanded T cell clones are shared between inflamed joints. The overlap is most pronounced for Treg, with the highly dominant Treg clones in SF being detectable in circulation.

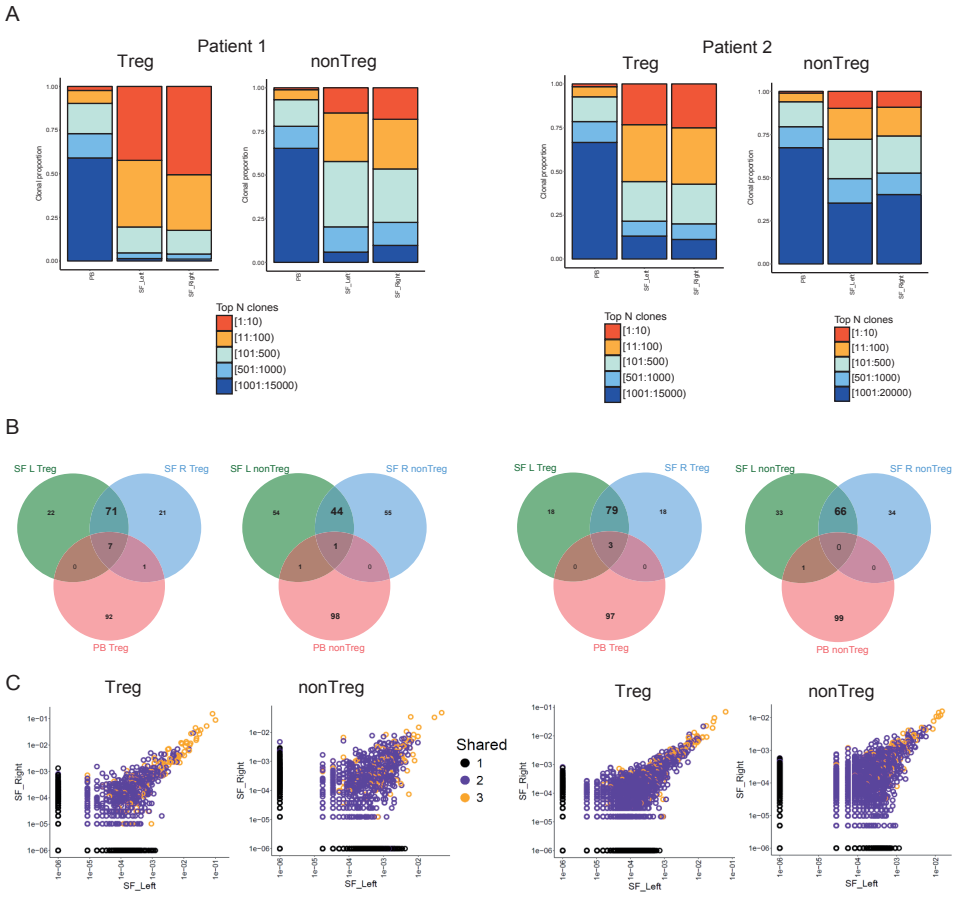

Figure 3. Highly dominant T cell clones are shared in SF from left and right joint and peripheral blood

A Clonal proportions of the TCRβ clones as detected in Treg and non-Treg sorted from PBMC, SFMC left joint, SFMC right joint of two different patients. **B** Venn diagram displaying the 100 most abundant unique TCRβ clones, defined by amino acid sequence, for Treg and non-Treg from patient 1 (left) and patient 2 (right). **C** Frequency plots showing the overlapping Treg and non-Treg clones between left joint derived SF (x-axis) and right joint derived SF (y-axis), with color coding highlighting the clones that are shared with none of the other samples (black circle), shared in two samples (purple circles) and all three samples (PB, SF left, SF right; yellow circle). Left plots display samples from patient 1, right plots display samples from patient 2

Dominant clones persist over time during relapsing remitting disease

We next wondered whether the same dominant clones would be detectable in the inflamed joints over the relapsing-remitting course of JIA. Therefore, we profiled the TCRβ repertoire of samples derived from a patient that had both knees affected, went into remission after the intra-articular steroid injection following SF aspiration, and returned 6 months later to the outpatient clinic with a relapse in the left knee (Figure 4A). Indeed, when we analyzed the 100 most dominant clones of all three SF samples 52 Treg and 48 non-Treg clones were present in all three samples (Figure 4B), and 4 Treg and 7 non-Treg clones were detectable in PB as well (Supplemental Figure 4B). Remarkably, the degree of sharing of T cell clones from the right joint at the first visit with the clones from left joint at the second visit is very comparable to the degree of sharing between the left joints over time. Furthermore, the most abundant clones were found to be the most overlapping between time points, and could also be traced back in circulation (Figure 4C and Supplemental Figure 4B). This implies that dominant clones persist and are (re) circulating during the relapsing-remitting course of disease. To expand this observation, four sequential SF samples from another JIA patient that altogether spanned more than a year were analyzed (Figure 5A). Similar to the previous patient, this patient was in remission in between all SF aspirations. Again TCRβ clones were highly overlapping between all time-points. Of note, 49 out of the 100 most dominant Treg clones were present in all four SF samples (Figure 5B) and the most abundant clones were also detected in peripheral blood (Figure 5C). Thirty-nine out of the 100 most dominant non-Treg clones were detected at all time-points (Figure 5B and Supplemental Figure 4C).

We profiled sequential samples of another 3 patients that also relapsed within 6 to 9 months after the first joint aspiration. These data were in line with the other two patients (Figure 5D), confirming that during relapses largely the same dominant T cell clones are taking part in the local immune response in the inflamed joints of JIA patients.

Minimal overlap on amino acid sequence and HLA level

Based upon the finding that Treg, and to a lesser degree non-Treg, are clonally expanding in the joints of all JIA patients profiled in our study, we wondered whether JIA patients share TCRβ sequences. To study this we first focused on the three patients that have both knees sampled at the same time. The HLA type is a strong determinant in the TCR repertoire, so we wondered whether we would observe overlap on this level. Profiling of the samples using high resolution typing of HLA class I and II loci revealed 24 HLA types, of which 8 are identical and shared between patients (Supplemental Table 2). Five out of 8 HLA loci are encoding MHC class I molecules. Regarding MHC class II, patient 1 and 2 share HLA-DR and HLA-DQ alleles, and patient 2 and 3 share HLA-DP alleles. Since MHC II is of interest regarding our focus on $CD4^+$ T cells, we wondered whether this would result in possible overlap in amino acid sequences as encoded by the identified clones. Analysis of the unique amino acids sequences based on the identified clones in these patients revealed that there are

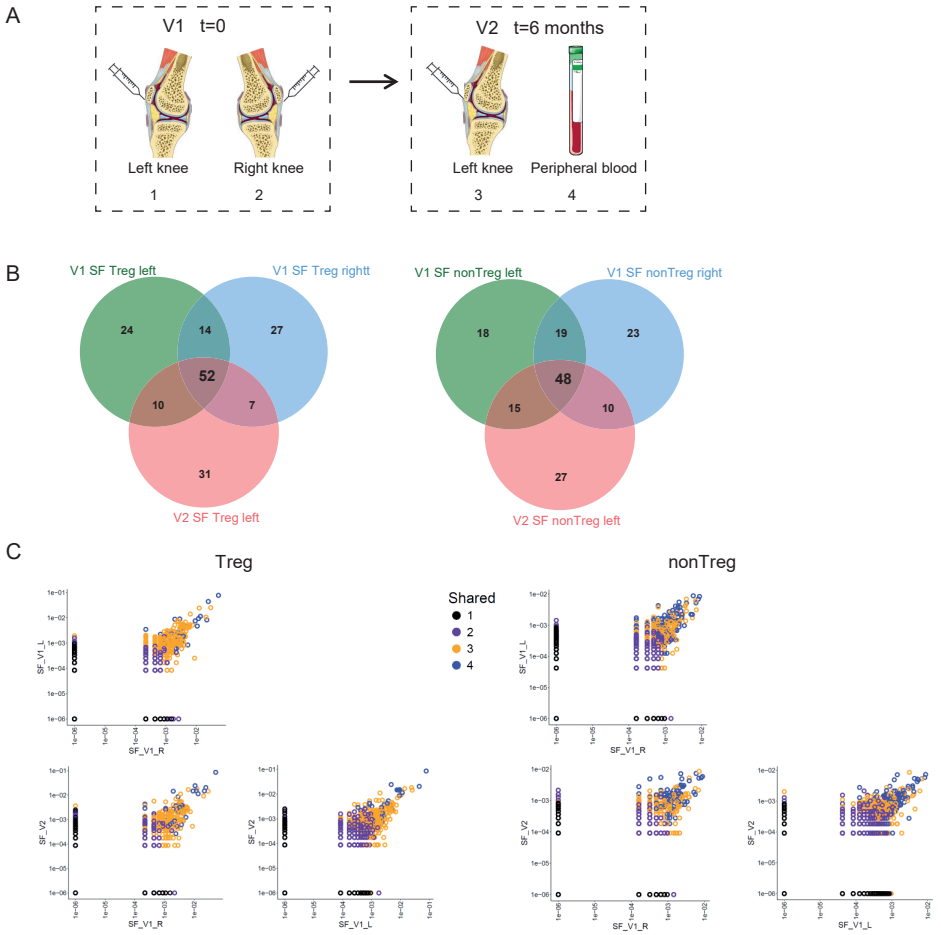

Figure 4. Persistence of T cell clones detected in relapse

A Schematic representation of the sequential samples that are studied. **B** Venn diagram displaying the 100 most abundant unique TCRβ clones, defined by amino acid sequence, for Treg (left) and non-Treg (right) from left and right joint SF from visit 1 and left joint SF from visit 2. **C** Frequency plots showing the overlapping Treg (left) and non-Treg (right) clones between left or right joint derived SF (x-axis) and peripheral blo°od (y-axis), with color coding highlighting the clones that are shared with none of the other samples (black circle), shared in two samples (purple circles), shared with three samples (yellow circle) and shared with all samples (so left and right SF from visit 1 and left SF and PB from visit 2; blue circle)

overlapping unique sequences between patients, although very minimal (129 shared clones represent 0,11% of the total amount of clones from the 3 patients; Figure 6A). This indicates that although within patients the dominance of disease-associated clones is evident, these are not shared between patients. However, further research is needed to firmly draw conclusions.

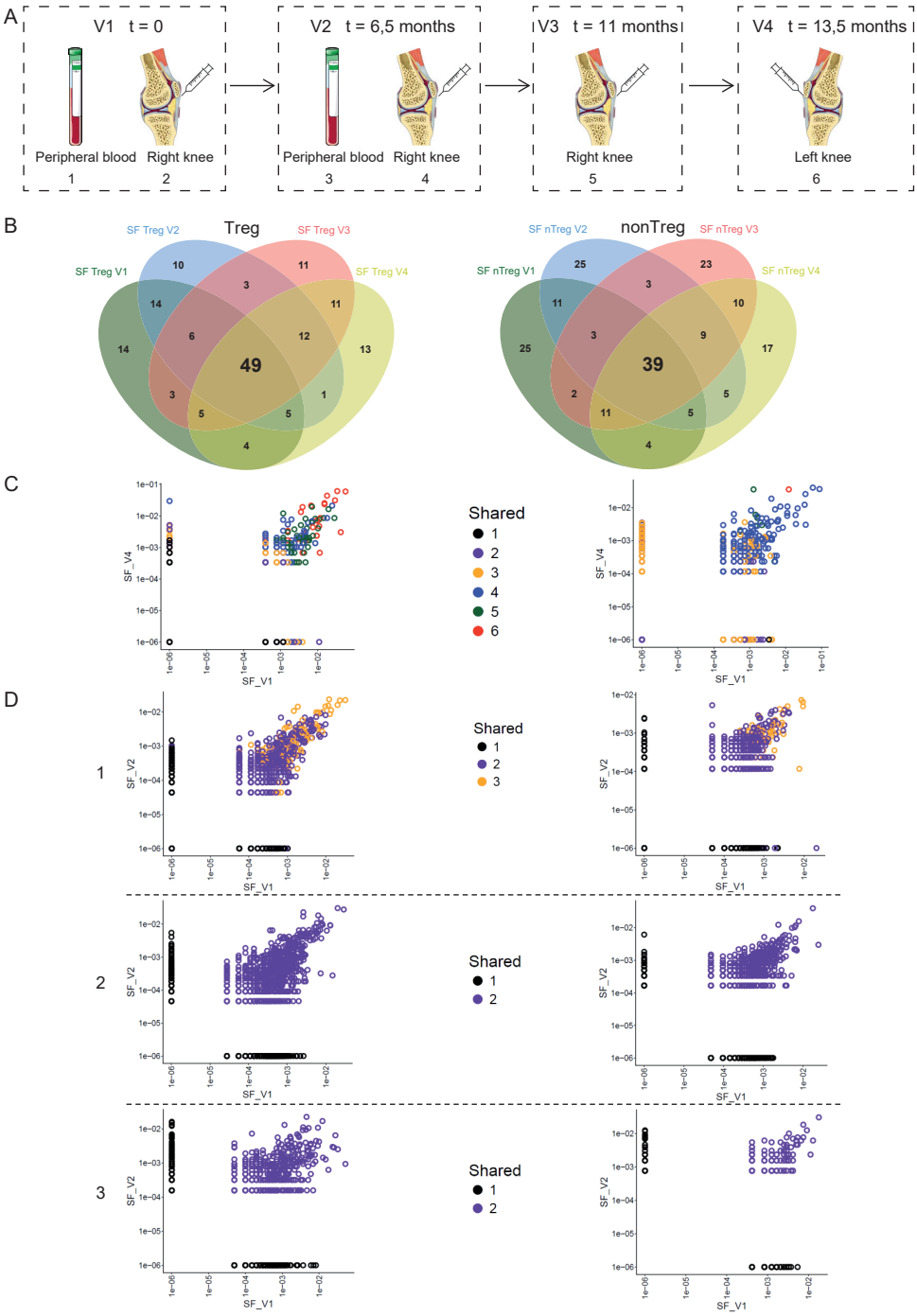

Figure 5. Dominant T cell clones persist in multiple relapses over more than 1 year

A Schematic representation of the sequential samples that are studied. **B** Venn diagram displaying the 100 most abundant unique TCRβ clones, defined by amino acid sequence, for Treg (left) and non-Treg (right) from all 4

sequential SF samples. **C, D** Frequency plots showing the overlapping Treg (left) and non-Treg (right) clones between SF of visit 1 (x-axis) and SF of visit 4 (y-axis) (**C**) or between SF of visit 1 (x-axis) and SF of visit 2 (y-axis) (**D**), with color coding highlighting the clones that are shared with none of the other samples (black circle), shared in two (purple circles), three (yellow circle), four (blue circle), 5 (green circle) and all 6 samples (yellow circle)

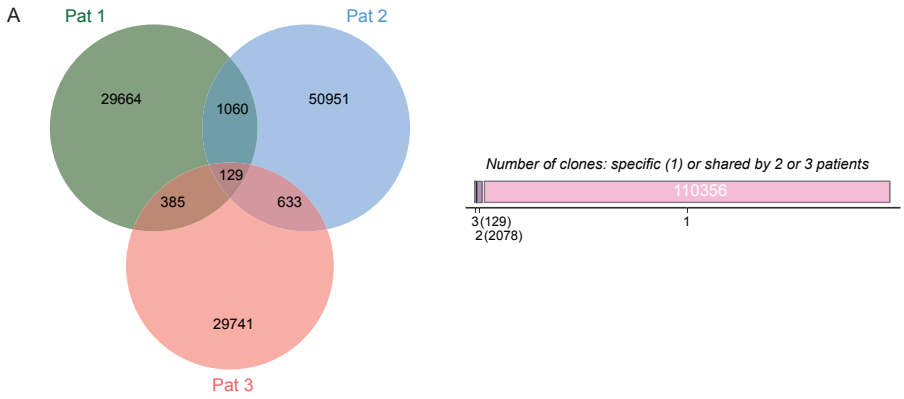

Figure 6. Sequences are scarceley shared between patients

A Venn diagram displaying the unique amino acid sequences based on the identified TCRβ clones in the three patients with SF from both knees sampled at the site time.

DISCUSSION

In this study, we provide the first mass cytometry and TCRβ sequencing analysis of purified Treg and non-Treg in a human autoimmune disease setting that uncovers their spatial and temporal behavior. We show that the architecture of T cell response from distinct inflamed joints is remarkably similar although inflammation often does not start at the same time in both affected joints. The response is characterized by a TCR-induced pro-inflammatory cytokine production by effector cells, as well as expanding Treg with an activated and functional profile. The local immune response is dominated by a limited number of TCRβ clones, of which especially Treg are locally expanding. During relapses largely the same dominant T cell clones are taking part. Altogether, this indicate that there is a strong driving force locally in the joint that heavily skews the TCRβ repertoire, which can be explained by the presence of dominant auto-antigens.

Although the antigen(s) remain elusive, our data provide strong support for the presence of ubiquitously expressed auto-antigens given the observed overlap in dominant clones over time and in space. This is supported by our finding that Treg and non-Treg have a largely distinct TCR repertoire, indicating thymic origin of Treg and thus auto-reactive Treg. The tissue restrictive character of the disease suggest that the antigen would be a joint-specific antigen. However, this restriction might not be applicable, since a paper

using transgenic mice showed that the TCRs that mediate autoimmune arthritis recognize the ubiquitously expressed auto-antigen 60S ribosomal protein L23a (RPL23A)[36]. Also in SF of a subset of RA patients CD4+ T cells producing IFNγ upon stimulation with RPL23A were detected, whereas the arthritogenic T-cell autoantigen is expressed in multiple healthy tissues[36]. Further support for the hypothesis that auto-reactive Treg are present and expanding in SF is provided by a recent study performed in mice with type 1 diabetes. Treg with a demonstrated high degree of self-reactivity were found to be expanding locally in affected pancreatic islets and displayed a specific profile with elevated levels of GITR, CTLA-4, ICOS and ki67, very similar to our observations[37].

Our data demonstrated that dominant T cell clones in SF can be traced back in the circulation. Together with observations that similar T cell clones are detected in multiple affected joints and the obvious overlap in immune cell composition, this strongly suggests that T cells migrate from the joint to peripheral blood and vice versa. This is in line with other recent observations that in arthritis synovial CD4+ T cells and Treg are detected in peripheral blood[27,38,39]. Moreover, their presence has been shown to correlate with disease activity and responsiveness to therapy, with increased amounts of circulating SF clones and/or phenotypes in more severe disease[27,38,39]. Next to that, in refractory JIA and juvenile dermatomyositis (JDM) patients that underwent an autologous hematopoietic stem cell transplantation (aHSCT) as a last resort therapy, the peripheral T cell compartment was shown to be heavily skewed towards dominant T cells clones prior to transplantation, especially in the Treg compartment[29]. After transplantation, the TCR repertoire diversified in responders whereas in the only non-responder a clonal repertoire remained. A renewal of the TCR repertoire of CD4+ T cells and an increased diversity of the Treg in peripheral blood has also been observed in other autoimmune patients undergoing aHSCT. The latter appears to be important for inducing successful remission post-transplantation[40]. This knowledge, combined with our findings that the same T cell clones dominate the immune response at different sites of inflammation and the persistence of the same clones in the relapsing-remitting course of disease, strengthen the possibility to use circulating disease-associated T cell clones for disease monitoring or prognostic purposes. Although recent papers have shed some light on the phenotype of the circulating inflammation-associated CD4+ T cells and Tregs, with HLA-DR+ T cells displaying an activated phenotype and being enriched in SF clonotypes, further phenotyping is needed to fully characterize the profile of the dominant clones[27,38]. Single-cell sequencing with both transcriptome and TCR identification, perhaps combined with index sorting including HLA-DR expression, will give novel and required information to bring this closer to the clinic. Next to that, selective targeting of dominant T cell clones has been recently suggested based upon the finding that in RA synovitis within a single patient is dominated by a limited number of expanding TCRβ clones in multiple joints[15]. In the aforementioned study TCRseq was performed on biopsies (so including all T cells), whereas in our study CD4+ non-Treg and Treg were analyzed separately.

Analyzing T cell subsets separately is crucial, also regarding therapeutic purposes, since specific deletion of Treg at the site of auto-immune inflammation is not desirable.

The correlation of a clonal Treg TCR repertoire and the severity of disease as described above, raises questions to what extent this clonality is contributing to disease. The importance of a diverse repertoire is shown is several mouse models[21–24]. Föhse et al for example have shown that Treg with a higher diversity are able to expand more efficiently compared to Treg with a lower diversity in mice with TCR restricted conventional T cells[22]. It has been suggested that this is due to the TCR diverse Treg cells having access to a more ligands and as a result being able to out-compete the TCR-restricted Treg cells[16]. However, this applies for circulating Treg, and whether this would also be important for Treg in tissues is not known. The finding that tissue Treg residing in healthy tissues also show a considerable oligoclonality regarding their TCR repertoire may indicate that this is a normal feature[41,42]. Of note, it has been shown recently in mice that a diverse Treg repertoire is especially needed to control Th1 responses whereas Th2 and Th17 responses were still suppressed by Treg with a single TCR[25]. This could be an explanation why the Th1 rich SF environment is poorly controlled by the large amount of Treg.

The amount of overlap between Treg and non-Treg is also of interest, as this can provide insight in possible local induction of Treg or plasticity of existing Treg. Several studies have shown that the TCR repertoires of conventional $CD4^+$ T cells and Treg have minimal overlap, indicating distinct rather than convertible cell fates[43–48].
Also at the site of inflammation, where induction of Treg is suspected to be more prominent compared to steady state, we and others find that the repertoires of Treg and conventional T cells is largely exclusive and thus represent distinct lineages[26].

In this study we sequenced the β-chain of the TCR and not the α-chain. The identified dominant TCRβ clones can pair with several α-chains, possibly leading to a different Ag specificity. Future sequencing of both TCR chains will provide insight into the total TCR repertoire. Next to that, we are aware of a possible amplification bias because of a difference in efficiency of PCR primers. However, in our analysis approach we attempted to control as much as possible for such errors to prevent bias in our data. An interesting next step would be to combine single cell RNA-sequencing with identification of the TCR to directly link the expression profile of a given cell to its TCR clonotype.

In conclusion, we show that in SF the immune cell architecture is marked by inflammatory responses of activated effector T cells as well as activated and highly expanding Treg. The remarkable overlap in immune cell composition as well as the dominant clones over time and in space provide indications for a powerful driving force that shapes the local T cell response during joint inflammation. The presence of these inflammation-associated clones in the circulation provide promising perspectives for use in disease monitoring. Further research is needed to pinpoint the driving force and to create opportunities to target disease-specific T cells.

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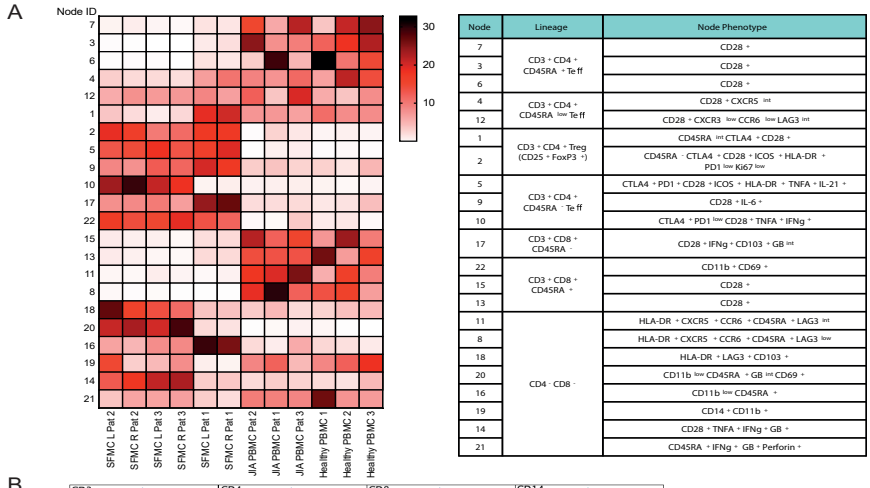
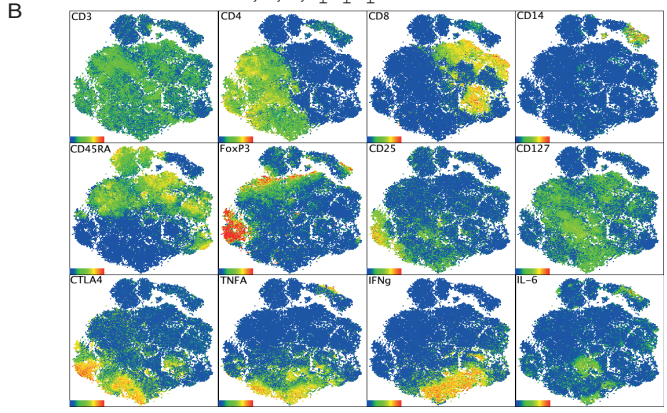
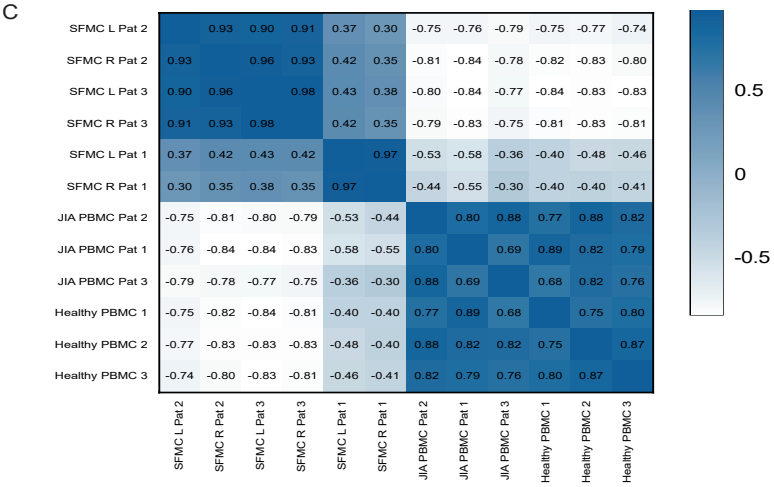
SUPPLEMENTAL DATA



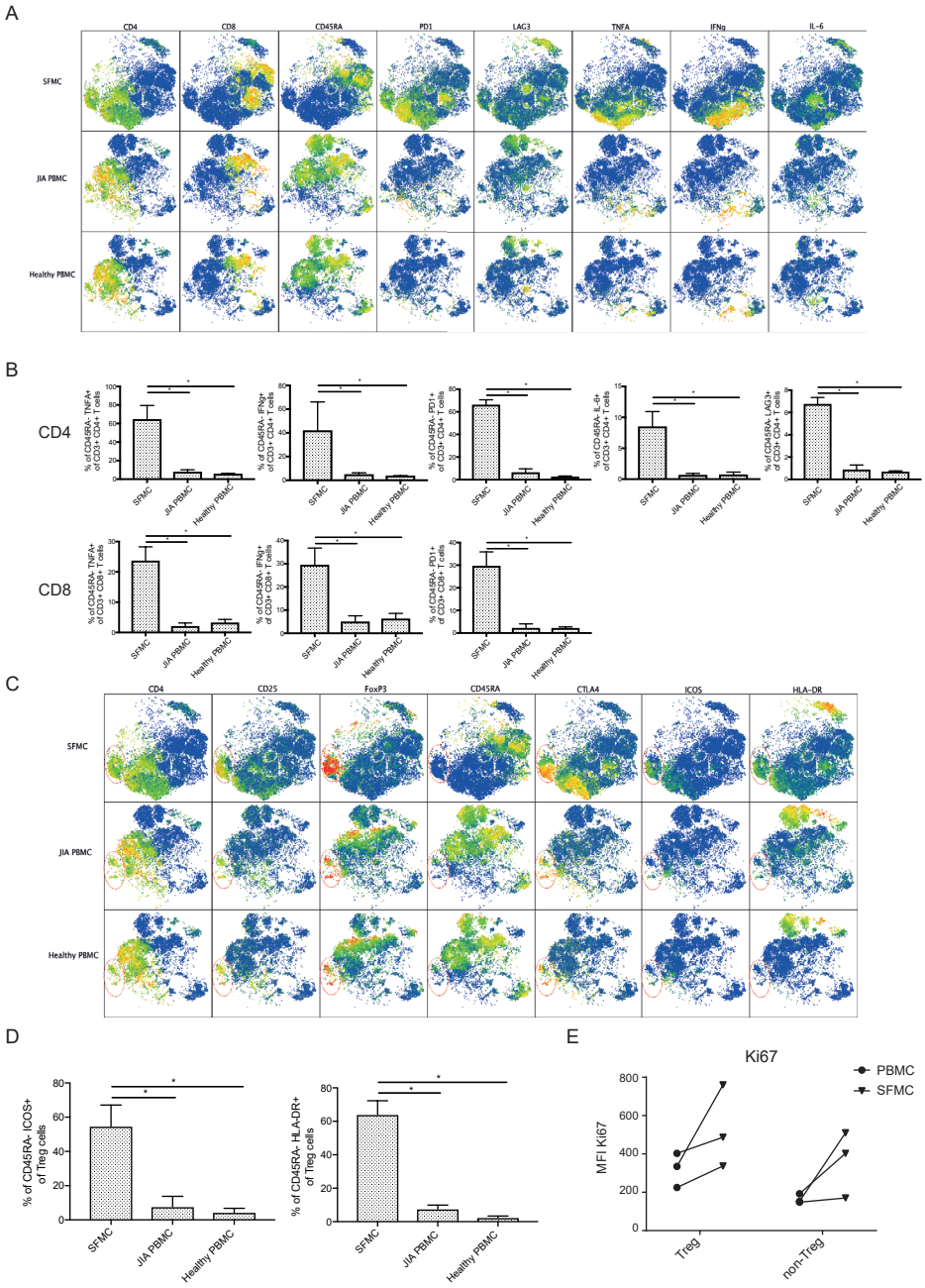
Node	Lineage	Node Phenotype
7	CD3 + CD4 + CD45RA + Te ff	CD28 +
3		CD28 +
6		CD28 +
4	CD3 + CD4 + CD45RA low Te ff	CD28 + CXCR5 int
12		CD28 + CXCR3 low CCR6 low LAG3 int
1	CD3 + CD4 + Treg (CD25 + FoxP3 +)	CD45RA int CTLA4 + CD28 +
2		CD45RA - CTLA4 + CD28 + ICOS + HLA-DR + PD1 low Ki67 low
5	CD3 + CD4 + CD45RA - Te ff	CTLA4 + PD1 + CD28 + ICOS + HLA-DR + TNFA + IL-21 +
9		CD28 + IL-6 +
10		CTLA4 + PD1 low CD28 + TNFA + IFNg +
17	CD3 + CD8 + CD45RA -	CD28 + IFNg + CD103 + GB int
22	CD3 + CD8 + CD45RA +	CD11b + CD69 +
15		CD28 +
13		CD28 +
11	CD4 - CD8 -	HLA-DR + CXCR5 + CCR6 + CD45RA + LAG3 int
8		HLA-DR + CXCR5 + CCR6 + CD45RA + LAG3 low
18		HLA-DR + LAG3 + CD103 +
20		CD11b low CD45RA + GB int CD69 +
16		CD11b low CD45RA +
19		CD14 + CD11b +
14		CD28 + TNFA + IFNg + GB +
21		CD45RA + IFNg + GB + Perforin +





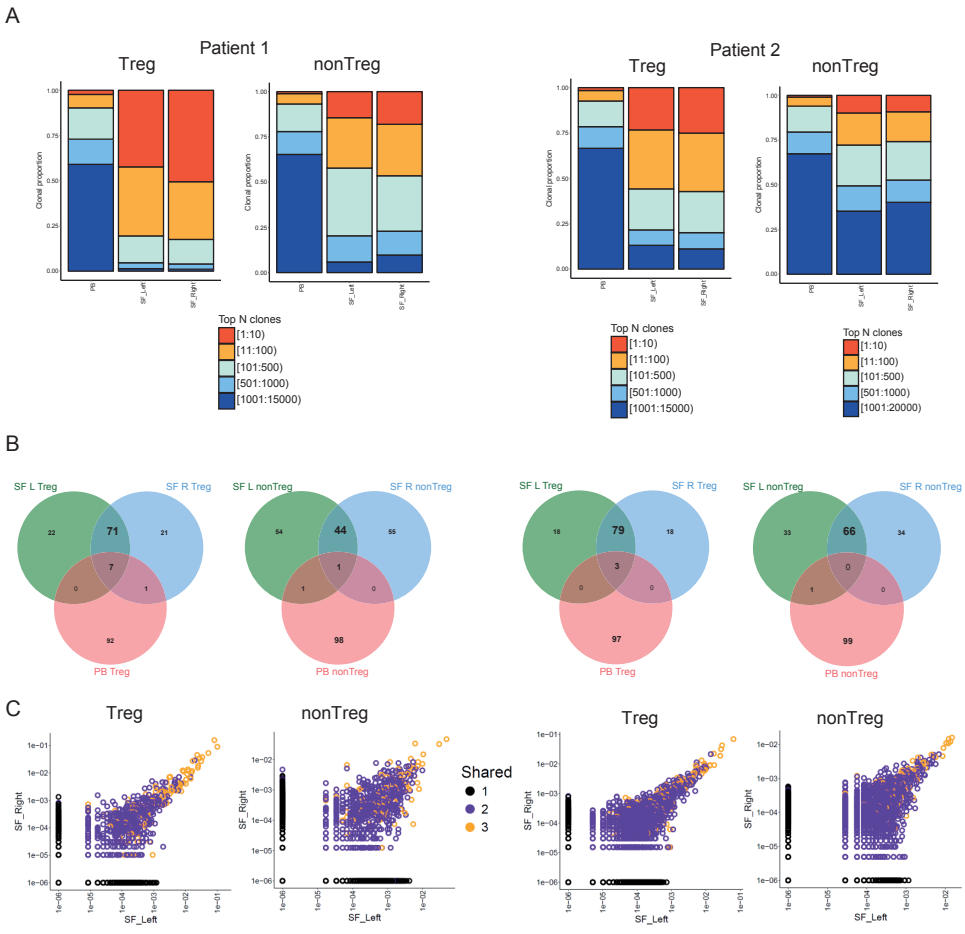
Supplemental Figure. 1 Preliminary analysis reveals correlation between SFMC from distinct joints

A Marker expression of T-SNE dimensional reduction and k-means clustering analysis on SFMC and PBMC samples
B Correlation matrix using spearman correlation of the entire spectrum of node frequency



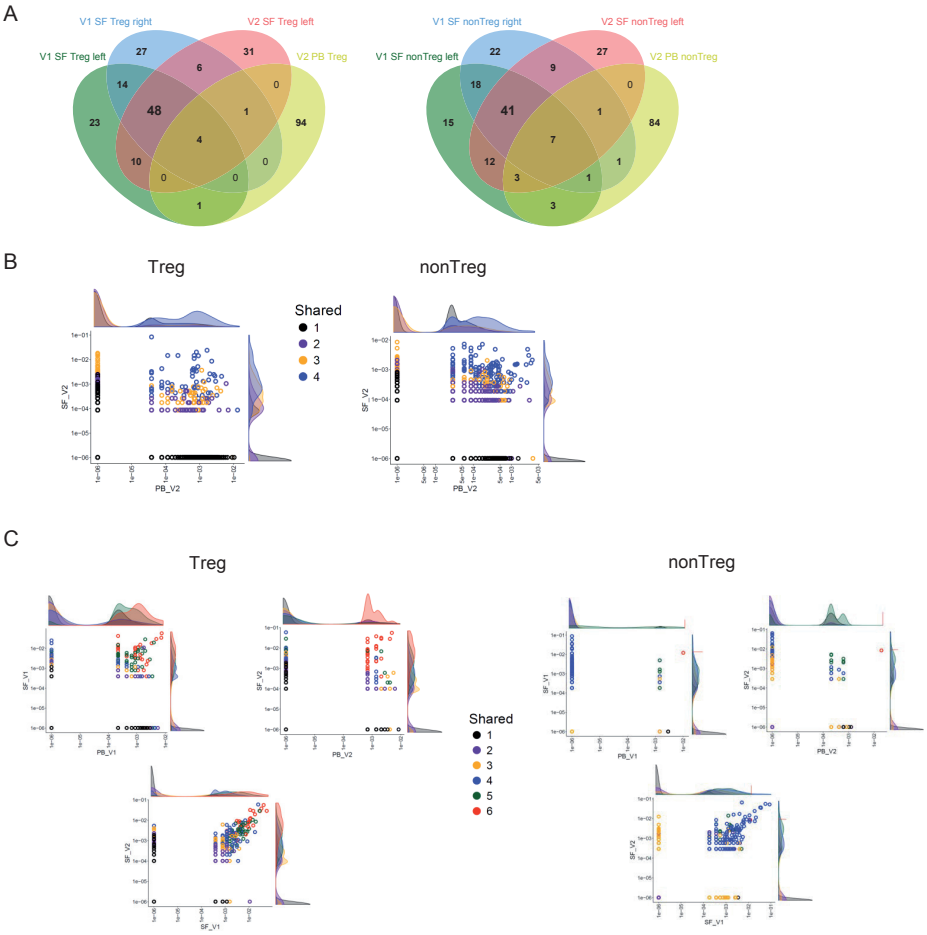
Supplemental Figure 2. SFMC display an activated expression profile

A T-SNE plots showing the expression profile of phenotypical and functional markers in SFMC, PBMC from JIA patients and PBMC from healthy children. **B** Bar charts showing the percentage of specific cell populations within CD4+CD45RA- and CD8+CD45RA- cells (non-parametric Mann-Whitney, * = p <0.05). **C** T-SNE plots showing the expression profile of phenotypical and functional Treg markers in SFMC, PBMC from JIA patients and PBMC from healthy children. **D** Quantification of CD45RA-ICOS+ and CD45RA-HLA-DR+ expression on CD25+ FOXP3+ Treg (non-parametric Mann-Whitney, * = p <0.05). **E** MFI of Ki67 protein expression in Treg and non-Treg as determined by flow cytometry.



Supplemental Figure 3. Overlapping clones are detected in peripheral blood

A Sort strategy of $CD3^{+}CD4^{+}CD25^{+}CD127^{low}$ Treg and $CD3^{+}CD4^{+}CD25^{-}CD127^{+}$ non-Treg using flow cytometry. **B** Frequency plots showing the overlapping Treg and non-Treg clones between left or right joint derived SF (x-axis) and peripheral blood (y-axis), with color coding highlighting the clones that are shared with none of the other samples (black circle), shared in two samples (purple circles) and all three samples (so PB, SF left and SF right; yellow circle)



Supplemental Figure 4. Detection of persisting TCRβ clones over disease course

A Venn diagram displaying the 100 most abundant unique TCRβ clones, defined by amino acid sequence, for Treg (left) and non-Treg (right) from left and right joint SF from visit 1 and PB and left joint SF from visit 2. **B** Frequency plots showing the overlapping Treg (left) and non-Treg (right) clones between peripheral blood from visit 2 (x-axis) and left joint derived SF from visit 2 (y-axis), with color coding highlighting the clones that are shared with none of the other samples (black circle), shared in two samples (purple circles), shared with three samples (yellow circle) and shared with all samples (so left and right SF from visit 1 and left SF and PB from visit 2; blue circle). **C** Frequency plots showing the overlapping Treg (left) and non-Treg (right) clones between two samples (depicted on x-axis and y-axis), with color coding highlighting the clones that are shared with none of the other samples (black circle), shared in two (purple circles), three (yellow circle), four (blue circle), 5 (green circle) and all 6 samples (yellow circle)

Targets	Metal Channel	Clone	Antibody Vendor/Catalogue number
Lineage markers			
CD3	139	UCHT1	Biolegend (300402)
CD4	148	SK3	Biolegend (344625)
CD8	144	SK1	Biolegend (344727)
CD11b	161	ICRF44	Biolegend (301302)
CD16	209	3G8	Fluidigm (3209002B)
CD14	112/114	M5E2	Biolegend (301843)
T helper subsets			
IL-4	156	8D4-8	Biolegend (500707)
IFN-g	168	B27	Biolegend (506513)
IL-17A	169	BL168	Biolegend (512302)
IL-21	151	3A4-N2	Biolegend (513009)
CD161	157	HP-3G10	Biolegend (339902)
T cell functional markers			
CD45RA	171	HI100	Biolegend (304102)
CD69	176	FN50	Biolegend (310902)
CD28	146	CD28.2	Biolegend (302923)
CD152 (CTLA4)	155	BNI3	Biolegend (555851)
CD154 (CD40L)	149	24-31	Biolegend (310835)
HLA-DR	143	L243	Biolegend (307612)
LAG3	159	17B4	Abcam (ab40466)
PD1	147	EH12.2H7	Biolegend (329941)
Ki67	166	20Raj1	Thermofisher/ebioscience (14-5699-82)
ICOS	154	C398.4A	Biolegend (313512)
CD31	172	WM59	Biolegend (303102)
CD103	142	B-Ly7	Thermofisher/ebioscience (14-1038-82)
Chemokine receptors			
CXCR3	163	G025H7	Biolegend (353718)
CXCR5	160	RF8B2	BD biosciences (552032)
CCR5	145	NP-6G4	Abcam (ab115738)
CCR6	170	G034E3	Biolegend (353402)
Treg markers			
CD25	150	M-A251	BD biosciences (555429)
CD127	153	A019D5	Biolegend (351302)
FoxP3	165	PCH10L	Thermofisher/ebioscience (14-4776-82)
GITR	164	621	Biolegend (311602)
TGF-B (LAP)	175	TW4-2F8	Biolegend (349602)
IL-10	158	JES3-9D7	Biolegend (501402)
Cytokines/Enzymes			
TNF-alpha	152	Mab11	Biolegend (502902)
IL-6	162	MQ2-13A5	Thermofisher/ebioscience (16-7069-85)
Granzyme B	173	CLB-GB11	Abcam (ab103159)
Perforin	174	B-D48	Abcam (ab47225)
Barcodes			
CD45-A	89	HI30	Fluidigm (3089003B)
CD45-B,C or D	115, 141, 167	HI30	Biolegend (304002)
Live/Dead /Singlets			
DNA (Singlets)	191/193	Nil	Fluidigm Cell-ID Intercalator-Ir (201192
Cisplatin (Live/Dead)	195	Nil	Sigma-aldrich (479306-1G)

Supplemental table 1. Overview of the T cell panel with 37 markers

Patients	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1	HLA-DPB1
1	*03:01:01:01	*07:02:01	*07:02:01:03	*11:01:01	*03:01:01	*04:01:01
	*68:01:01:02	*07:05:01	*15:05:02	*11:03:01		
2	*02:01:01	*15:01:01:01	*03:03:01:01	*11:01:01	*03:01:01	*03:01:01
	*03:01:01:01	*35:01:01	*04:01:01:01	*15:01:01	*06:02:01:01	*04:02:01
3	*03:01:01:01	*15:01:01:01	*03:03:01:01	*01:01:01	*05:01:01	*02:01:02
		*35:01:01	*04:01:01:01	*13:01:01	*06:03:01	*04:02:01:0
	MHCI			MHCII		

Supplemental table 2. HLA typing results

Chapter 7

T-Cell Compartmentalization and Functional Adaptation in Autoimmune Inflammation: Lessons from Pediatric Rheumatic Diseases

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Submitted

ABSTRACT

Chronic inflammatory diseases are characterized by a disturbed immune balance leading to recurring episodes of inflammation in specific target tissues such as the joints in juvenile idiopathic arthritis. The tissue becomes infiltrated by multiple types of immune cell, including high numbers of CD4 and CD8 T-cells, which are mostly effector memory cells. Locally, these T-cells display an environment-adapted phenotype, induced by inflammation- and tissue-specific instructions. Some of the infiltrated T-cells may become tissue resident and play a role in relapses of inflammation. Adaptation to the environment may lead to functional (re)programming of cells and altered cellular interactions and responses. For example, specifically at the site of inflammation both CD4 and CD8 T-cells can become resistant to regulatory T cell-mediated regulation. In addition, CD8 and CD4 T-cells show a unique profile with pro- and anti-inflammatory features coexisting in the same compartment. Also regulatory T-cells are neither homogeneous nor static in nature and show features of functional differentiation, and plasticity in inflammatory environments. Here we will discuss the recent insights in T cell functional specialization, regulation, and clonal expansion in local (tissue) inflammation.

INTRODUCTION

A typical hallmark of immune-mediated inflammatory diseases (IMIDs) is the intermittent presence of inflammation that manifest in specific target tissues. In the affected tissues activated antigen-specific T-cells with a memory phenotype are present. The combination of memory T cell infiltrated at the affected sites and genetic associations have pointed to T-cells as key players in the pathophysiology of chronic inflammatory diseases. Due to the ongoing inflammation in target tissues there is an increasing risk for collateral tissue damage. Especially in children this can have long-term effects and consequences. Understanding the biology of immune cells that are actively involved in the local inflammatory process is crucial for the development of therapeutic approaches. However, because of technical challenges, the vast majority of data in human studies comes from peripheral blood cells. With the emergence of novel techniques including (single-cell) RNA-sequencing and mass cytometry it is now possible to unravel T cell signatures in a local (tissue) setting. Here, we will discuss the most recent findings on human T cell functional programming and adaptation at the site of chronic inflammation with a focus on pediatric rheumatologic disease.

T-CELLS AT THE SITE OF INFLAMMATION

Inflammation is a process aimed at eliminating the triggering agent, but in chronic inflammation the (auto)antigens persist, inducing a sustained inflammation. When inflammation does resolve, a population of antigen-specific T-cells may remain in the tissue as memory cells and become tissue resident[1], the so called tissue-resident memory T-cells (Trm). This makes them unique compared to central memory, effector memory and naïve T-cells that recirculate within lymphoid organs and the blood and/or lymphatics. A hallmark of Trm cells is their ability to respond quickly and robustly after re-encountering the antigen as well as the expression of inhibitory molecules to keep them in check. In steady-state Trm play an important role in tissue homeostasis and protection. After immune re-activation of Trm, circulating immune cells are attracted to the site of inflammation as well, resulting in local accumulation of antigen-specific memory T-cells. So both Trm and infiltrating T-cells that might eventually become Trm themselves, may actively participate during the inflammatory response. Because of their vigilance and specific localization in tissues, Trm are suspected to play a dominant role in the typical relapsing remitting course of chronic inflammatory diseases. Not much is known about the dynamics and interaction between both memory subsets. An accumulation of both $CD4^+$ and $CD8^+$ T-cells are found at sites of chronic inflammation. However, research on the involvement of T-cells in disease pathogenesis has mainly focused on $CD4^+$ T-cells, whereas $CD8^+$ T-cells have been the focus of the Trm research field. In this review we summarize the findings

on CD8+, CD4+ and FOXP3+ regulatory T-cell (Treg) subsets in the contexts of local (tissue) adaptation and functional differentiation. In line with the divarication of T helper cells, with each subset having a specified function in the immune system, it is now becoming apparent that specialization is also true for T-cells present in different tissues and types of inflammation. The local acquisition of additional or adjusted functions and phenotypes, will be referred to as adaptation.

CD4+ T helper responses in local pathogenesis

Already for a long time CD4+ T have been recognized as central players in the immune-pathogenesis of autoimmune diseases, which is supported by strong associations of rheumatic diseases with MHC class II alleles[2]. The CD4+ T cell population is comprised of several T helper subsets that develop after the T cell receptor (TCR) on naïve CD4+ T-cells interacts with activated antigen presenting cells (APCs). Next to this TCR stimulation, co-stimulation, subsequent signaling and cytokines in the micro-environment are important in determining the fate of a specific subset, by activating signaling molecules that establish a lineage-specific enhancer landscape and lead to the expression of master transcription factors. Those, together with a complex network of accessory transcription factors, can coordinate cellular differentiation programs committed towards a lineage, while simultaneously repressing the developmental programs of opposing Th lineages[3]. For many years the only identified lineages were Th1 and Th2 subsets, with IFNγ producing Th1 cells being specialized in cell-mediated immune responses against intracellular bacteria and Th2 cells producing IL-4 and IL-13 targeting helminths. The discovery of other subsets such as Th17 cells and T follicular helper (Tfh) cells has shifted the paradigm of two opposing lineages[4,5]. Th17 cells, producing various cytokines including IL-17, IL-22 and GM-CSF, induce defense against fungi and extracellular bacteria and are crucial for the maintenance of mucosal homeostasis[6]. Despite their relatively recent discovery, Th17 cells are implicated in the pathogenesis of many human (autoimmune-) diseases. This also accounts for Tfh cells, that provide help to cognate B cells to produce high affinity antibodies and memory B cells and as such control humoral immunity[7]. Many immune-mediated diseases are associated with aberrant Th responses. In the lamina propria of Crohn's disease, the synovial fluid (SF) of Juvenile Idiopathic Arthritis (JIA) and Rheumatoid Arthritis (RA) patients and renal tissue of Systemic Lupus Erythematosus (SLE) patients for example, Th1 cells are implicated in disease pathogenesis[8–11]. In JIA, a mixed Th17/Th1 phenotype is also found in inflamed joints, capable of producing both IL-17 and IFNγ. The presence of this subset correlates with disease activity[12,13] and its IL-17-producing capacity is associated with CD161 expression[14]. Interestingly, this subset seems to be present specifically in inflammatory environments, and can be a transiently induced from Th17 cells upon exposure to IL-12 and/or TNFα[12,13,15]. Whereas the Th1 lineage is shown to be fairly stable, the Th17 lineage is known for its instability and is severely impacted by the environment[16]. In line with this, the mixed Th1/Th17 phenotype likely

derives from Th17 cells instead of Th1 cells. Next to pathogenic Th17 cells, non-pathogenic Th17 cells have been described in autoimmune diseases as well (reviewed in[17]). A recent paper found delayed IL-10 production in about 25% of activated human Th17 clones in culture[18]. This indicates that IL-10 production is an intrinsic property of a subset of Th17 cells after antigenic stimulation, perhaps to regulate and balance the immune response. Transcriptional analysis of the IL-10$^+$ and IL-10$^-$ Th17 clones demonstrated immune-regulatory and tissue-resident properties of IL-10 producing Th17 cells, and a pro-inflammatory profile of IL-10$^-$ Th17 cells with high CCR7 expression, which may indicate circulatory properties[18]. Local pathogenic Th17 cells have been described in multiple autoimmune disease (reviewed in[16]). In muscle of JDM patients and affected kidneys of SLE patients, IL-17 producing cells are increased,[19–21]. One mechanism that explains the elevated production of IL-17 in JIA and SLE is the increased expression of the transcription factor cAMP-responsive element modulator (CREM)α. This induces repression of IL-2 transcription but also epigenetic changes of the IL-17A locus, resulting in enhanced IL-17A promoter activity[22,23]. Although evidence for involvement of the IL-17 signaling pathway in SLE pathogenesis is expanding, direct interference with IL-17 or it's receptor does not seem to be effective, at least in mouse models[24,25]. Also in JIA, IL-17 blockade is not part of standard treatment. This might be related to the pathogenic role of another CD4$^+$ subset, Tfh cells, that is increased in inflamed tissues of RA and SLE patients and located near B cells in affected kidneys in SLE patients[26,27]. Interestingly, STAT-3 and IL-21, signature molecules shared by Th17 and Tfh cells, are heavily implicated in SLE pathology and are capable of inducing autoreactive B cells[28,29]. Altogether, overactive CD4$^+$ T-cells are present at the site of human inflammation and represent a mixed population of which especially Th17 cells show plasticity.

A continuum of Th cell fates

The presence of mixed CD4$^+$ phenotypes found in human sites of autoimmune inflammation is of great interest, but might not be inflammation-specific. Recent studies using novel technologies have revealed a continuum of cell fates rather than limited and distinct Th subsets in healthy tissues. Mass cytometry measuring T cell trafficking receptor and cytokine expression in eight different human tissues revealed tissue-specific and unique T cell phenotypes[30]. This indicates that T-cells cannot be easily classified into separate lineages across human tissues. Furthermore, multi-color cytometry of peripheral blood cells of a healthy human cohort showed substantial subject-to-subject differences in T cell populations that yet remained relatively stable for months within individuals[31]. Age and disease associated genetic polymorphisms were identified as important factors in the identified variation. These publications highlight the importance of age and tissue influences on T-cells (reviewed in[32]). On top of this homeostatic variety, inflammation will probably add another layer of complexity by driving local tissue cells into an adapted phenotype.

CD8$^+$ T helper responses in local pathogenesis

Although long neglected in autoimmune diseases compared to CD4$^+$ T-cells, CD8$^+$ T-cells are equipped with different capacities by which they can contribute to the inflammatory process. For example, CD8$^+$ T-cells have cytolytic activity, produce pro-inflammatory cytokines and can react to self-antigens upon cross-presentation[33]. In several chronic inflammatory diseases CD8$^+$ T-cells are described to be present in the inflamed tissues and increased CD8$^+$ T cell numbers are associated poor prognosis in several rheumatic diseases including JIA, RA and SLE[34–37]. CD8$^+$ T-cells form a diverse group of cells but in contrast to CD4+ T-cells subsets are less well-defined. The phenotype of CD8$^+$ T-cells at the inflamed site of human autoimmune arthritis is heterogeneous, with both pro- and anti-inflammatory features (reviewed in[33]). In SF of RA patients for example, CD8$^+$ T-cells are characterized by increased expression of activation markers (CD80, CD86, CD25), pro-inflammatory cytokines like IL-6 and TNFα, and with a proliferative signature, but also by elevated levels of negative co-stimulatory markers such as TIM-3 and PD-1[36,38,39]. In affected kidneys of SLE patients, the majority of CD8$^+$ T-cells are located in the periglomerular regions where tissue damage occurs, and this infiltrate is correlated with renal injury[40]. Despite multiple studies reporting CD8$^+$ T-cells accumulation in SLE affected tissues, the phenotype of local CD8$^+$ T-cells remains largely unexplored. One study reports a differentiated effector memory phenotype with loss of CD28 on infiltrating CD8$^+$ T-cells, indicating active involvement of these cells in disease pathology[41]. However, although counter-intuitive for effector cells in an autoimmune environment, a recent study shows that kidney-infiltrating T-cells are metabolically and functionally 'exhausted' in three mouse models of lupus nephritis[42]. The term 'exhausted' stems from numerous studies on CD8$^+$ T-cells in chronic viral infections and, to a lesser extent, in cancers. Chronic antigen exposure in these settings goes along with the upregulation of negative co-stimulatory markers such as PD-1 in combination with reduced secretion of effector cytokines and proliferation. This has led to the hypothesis that these cells are terminally differentiated and severely functionally impaired[43,44]. However, recently this concept was challenged by the discovery that PD-1$^+$ CD8$^+$ T-cells are functionally adapted cells able to control the viral load or tumor cells without causing excessive immune pathology, and can be therapeutically reinvigorated by blocking PD-1/PD-L1 interaction[45,46].

Chronic stimulation in auto-immune inflammation: from an exhausted to a Trm phenotype

Not much is known about the functional profile of tissue-specific memory CD8$^+$ T cell in human chronic autoimmune diseases. Like CD8$^+$ T-cells in infectious diseases and tumors, these cells are chronically activated, but the immune balance seems to be shifted towards overzealous effector functions instead of too much regulation. Previous observations from peripheral blood derived CD8$^+$ T-cells show that the transcriptional signature reflecting exhaustion is associated with poor clearance of chronic viral infection, but conversely

predicts better prognosis in multiple auto-immune diseases, including SLE[47]. A recent paper addressing the enriched PD-1$^+$ CD8$^+$ T cell population in SF of JIA patients is the first to study this cell subset locally, derived from the site of inflammation in humans[48]. In this setting, PD-1 expressing CD8$^+$ T-cells maintain their effector function such as pro-inflammatory cytokine production, cytotoxic profile, and use of glycolysis as a metabolic pathway and thereby are suspected to have a detrimental role in autoimmune tissue damage. Strong inflammatory signals, and high levels of soluble PD-1 that block interaction with APCs[49], may overrule or hamper PD-1-signalling in CD8$^+$ T-cells in SF of JIA patients. In line with this, Odorizzi and colleagues have shown that PD-1 expression is not a prerequisite for exhaustion to occur, by using a mouse model of chronic viral infection with genetic absence of PD-1. In this model, PD-1 prevented CD8$^+$ T-cells overstimulation and apoptosis, as the absence of PD-1 led to more cytotoxic but terminally differentiated CD8$^+$ T-cells[46]. It is tempting to speculate that this mechanism may also play a role in local auto-immune inflammation, leading to survival of auto-antigen induced clonally expanding effector cells.

Interestingly, the combined cytotoxic and regulatory profile of CD8$^+$ T-cells, defined by increased expression of effector molecules such as Granzyme B on the one hand, and of negative co-stimulatory molecules on the other hand, is also typical for Trm cells[50]. In line with this, the PD-1$^+$ CD8$^+$ cells in SF were shown to be enriched for a Trm transcriptional profile compared to the PD-1$^-$ CD8$^+$ T-cells from the same environment[48]. Furthermore, Trm are defined by CD69 expression and downregulation of S1PR1, which is also found on PD-1$^+$ CD8$^+$ cells derived from SF of JIA patients. So local CD8$^+$ T-cells from the site of autoimmune inflammation in JIA cannot be classified as exhausted, despite some overlapping features, but share much of their profile with Trm cells. Interestingly, tumor-infiltrating CD8$^+$ T-cells in human cancers also display a Trm profile[51]. Moreover, a recent paper describing the transcriptional, metabolic, and functional signatures of intra-tumoral PD-1$^+$ CD8$^+$ T-cells has revealed that, next to many commonalities such as impaired cytokine production, these cells also differ from exhausted cells as described in chronic infections[52]. The intra-tumoral CD8$^+$ T-cells showed increased proliferation and glycolysis, and lack of enrichment of the exhausted T cell gene signature[52], as was observed in SF of JIA patients. The interpretation now arises that CD8$^+$ 'exhausted' cells are a heterogeneous group of memory cells with diverse differentiation states but all driven by persistence of antigen that induces upregulation of inhibitory receptors, and with functional properties that are influenced by the environment[53,54]. This concept shares many features with the current view on Trm cells as they are also regarded as highly specialized cells with a tissue adapted profile, tightly regulated to prevent excessive tissue damage. The commonalities between CD8$^+$ T-cells at inflammatory sites and the Trm profile suggest interplay between inflammation and tissue residency. Indeed, several studies indicate that inflammation is the trigger for initial homing of Trm cells, by providing the migratory signals needed to direct them to tissues[51]. PD-1 expressing CD8$^+$ T-cells in an inflammatory exudate such as SF of JIA could be tissue derived, but how they developmentally relate to Trm cells

remains unknown. All in all, local $CD8^+$ T-cells situated in the affected tissue of chronic inflammation are heterogeneous with both effector and regulatory responses that are highly influenced by chronic inflammation, possibly with disease specific profiles.

FOXP3 Regulatory T-cells responses in local pathogenesis

Overzealous $CD4^+$ Th responses carry the risk of initiating detrimental pro-inflammatory responses that can result in collateral tissue damage. Thus, the maintenance of immune homeostasis and prevention of immunopathology requires tight regulatory mechanisms. Regulatory T-cells (Treg) are a subset of $CD4^+$ T-cells with unique homeostatic functions. Absence of the Treg master transcription *FOXP3* leads to fatal multi-organ autoimmunity in mice and men[55,56]. The capacity of Treg to dampen immune responses have made them attractive therapeutic targets in diverse settings such as in autoimmune diseases, transplantation, and cancer. The best discriminative surface markers for Treg are high expression of CD25 (IL-2 receptor α) in combination with low expression of CD127 (IL-7 receptor α). Early studies often used solely high CD25 expression to identify or purify Treg, resulting in contamination with activated conventional $CD4^+$ T-cells, and contradicting data.

Effector and polarized Treg

Traditionally, the Treg lineage was considered as a homogeneous group of committed cells with suppressive capacities towards other immune cells. As it increasingly becoming apparent that most, if not all, immune cells have the capacity to adapt to their environment, data from the last decade demonstrated that Treg are perhaps the most heterogeneous in phenotype and function[57]. Their high turnover and sensitivity to environmental signals leads to a large degree of adaptation that allows Treg to control diverse immune responses and even to exert tissue-specific functions. In that respect novel Treg phenotypes have been identified that differ markedly from their naïve recirculating counterparts (central Treg). Liston and Gray have proposed the model of environment-instructed effector Treg and polarized Treg differentiation[58]. Effector Treg, or eTreg, are characterized in humans by $FOXP3^{high}CD25^{high}CD45RA^{low}$ expression representing a small fraction of circulating Treg[59]. They have signs of recent antigen encounter, have an heightened activation status and migratory potential, and express markers similar to activated conventional $CD4^+$ T-cells while maintaining Treg functions[58]. For example, increased expression of FOXP3, as well as typical functional markers such as ICOS and CTLA-4 is observed in eTreg[59,60]. Polarized (tissue-resident) Treg are present in non-lymphoid tissues, express specific homing receptors and exert tissue-specific functions and immune regulation. Treg do so by utilizing the transcription factor program of the population they are suppressing, or tissue specific transcription factors, respectively[57,58,61]. For example, Treg that co-express T-BET next to FOXP3 can efficiently suppress Th1 responses[62], whereas the expression of adipose tissue-specific peroxisome

proliferator-activated receptor gamma (PPARγ) is needed for Treg to control insulin sensitivity[63]. In the latter case, Treg are tissue-resident in a physiological condition and there is an increasing list of tissue specific phenotypes of tissue-resident Treg exerting non-immunological but tissue-homeostatic functions (reviewed in[57]). To what extend the profile of conventional Trm is applicable to tissue-resident Treg remains to be explored.

Using the proper gating strategy for Treg markers, the frequency of Treg in rheumatic diseases is increased at the site of inflammation in JIA and JDM[64–66]. Whether Treg function is impaired in these diseases is still under debate, partly related to differences in phenotyping markers and conditions of *in vitro* assays used to test Treg functionality. Multiple studies have however shown that Treg derived from SF of JIA patients maintain their suppressive function and upregulate Treg functional markers, such as CD25, CTLA4 and GITR, rather pointing towards an eTreg profile[67–69] (Figure 1).

Treg stability

Instability of Treg has long been suspected to play a role in disease pathology. Instability is defined by loss of FOXP3 expression and suppressive function, with a concomitant acquisition of an effector phenotype. The stability of Treg is a contentious issue, with contradicting data from several studies[70]. Multiple mouse models, including genetic fate-mapping models that allow tracking FOXP3 expressing cells, revealed that Treg are fairly stable *in vivo* with a small proportion of cells that lose FOXP3 expression[71–74].

At the site of autoimmune inflammation in humans, FOXP3-expressing Treg that produce pro-inflammatory cytokines have been described[75,76]. In specific tissues however, it is unknown if aberrant adapted Treg add to disease pathogenesis. In this regard, it is important to distinguish between functional plasticity/adaptability and lineage instability. In JIA, a small fraction of SF Treg expresses CD161 and is capable of producing pro-inflammatory cytokines. At the same time, FOXP3 expression remains high and suppressive capacity is also maintained[77,78]. Another paper studying Treg stability in SF of JIA patients, demonstrated that the T cell receptor (TCR) repertoires of Treg is very distinct from conventional T-cells in SF, indicating a different origin and thus excluding a large degree of instability of Treg[79]. On top of that, the same paper showed that Treg need inflammatory signals present in SF to maintain their FOXP3 expression, supporting the idea that local signals in an inflammatory environment can stabilize or even enhance the Treg phenotype. Systemic administration of IL-2 as a therapy to maintain and possibly expand Treg is currently being tested for SLE patients. A recent paper reports on reduced CD25 expression on peripheral blood Treg of SLE patients, that correlates to the reduced production of IL-2 from circulating memory T-cells[80]. Since the increased expression of CREMα leads to reduced IL-2 production of effector T-cells, and IL-2 receptor (CD25) signalling via STAT5 is pivotal for maintained FOXP3 expression in Treg, impaired Treg function could be a consequence[81]. This provides a rationale for Treg targeted therapy by low dose IL-2 administration[82]. However, it is not known

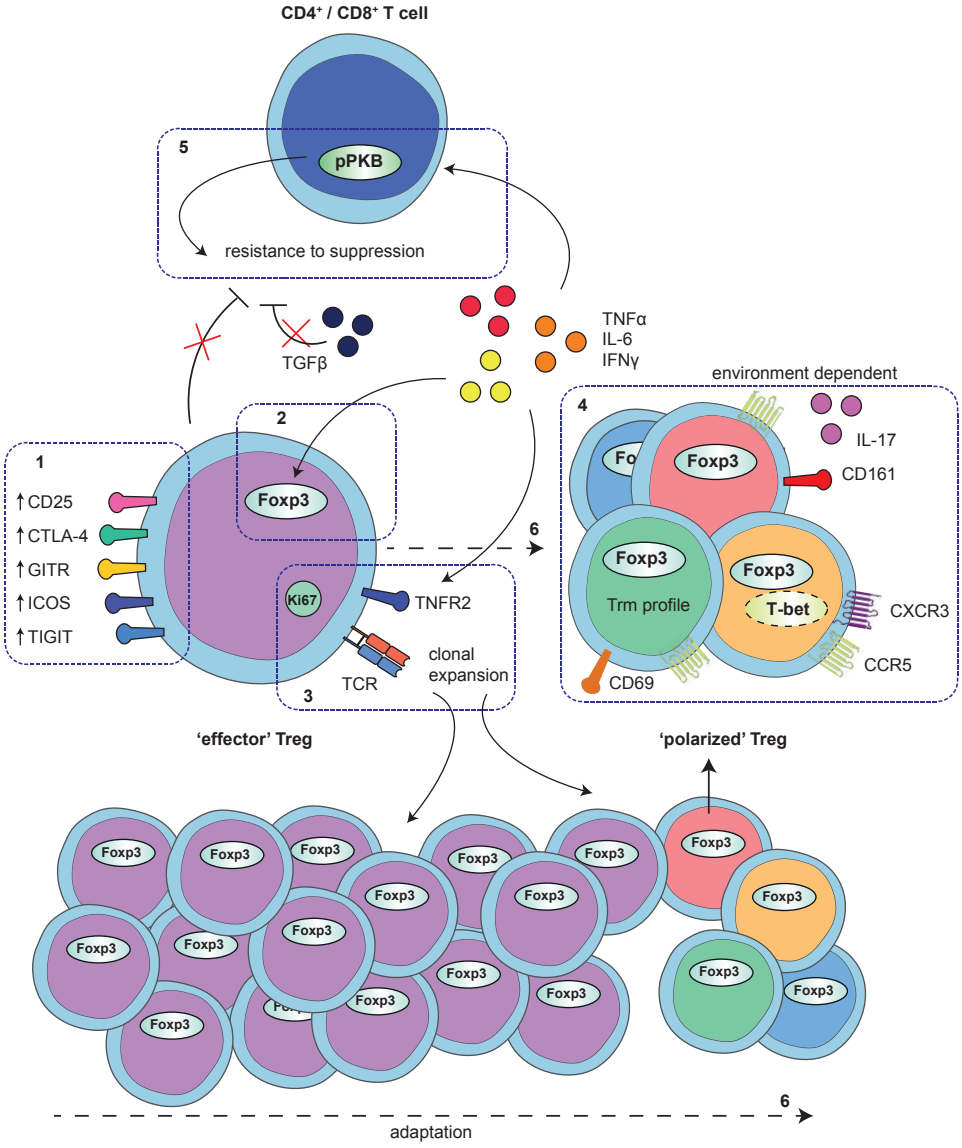

Figure 1. Adaptation of Treg to local auto-immune inflammation.

At the site of human autoimmune-inflammation functional Treg are present that display an effector phenotype. This phenotype is characterized by increased expression of functional Treg markers, including CD25, CTLA4, GITR, ICOS and TIGIT (1) and stable increased expression of FOXP3 and, at least in part, instructed by local inflammatory signals (2). Furthermore, Treg are clonally expanding as is reflected by increased Ki67 expression and a local clonal TCR repertoire, possibly mediated by TNFα signalling via TNFR2 on Treg (3). In addition to the effector profile, Treg may also display a specific environment instructed profile, including e.g. expression of CD161 and IL-17 production, upregulation of Th1- and inflammation- associated markers and chemokine receptors and/or characteristics of a Trm profile (4). These polarization profiles are not exclusive but rather are overlapping, depending on the specific local conditions. Whereas adapted Treg are functional, local cytokines produced by monocytes and fibroblasts also affect CD4+ and CD8+ T-cells, in part by hyper-phosphorylation of

PKB/c-AKT, conferring resistance of local CD4+ and CD8+ T-cells to Treg suppression (5). Overall, the process of Treg adaptation in inflammatory settings is highly influenced by the local environment, most likely starting with an expanding effector population that can be further fine-tuned with environmental adaptations (6)

whether the reduced CD25 expression on Treg also occurs at the site of inflammation.

The stability of Treg is regulated on multiple levels. The acquisition of a specific epigenetic landscape is a strong determinant[83], as well as transcriptional and post-transcriptional regulation of FOXP3. *In vitro* studies have shown that environmental cues can modulate these processes. For example ubiquitination of FOXP3, that targets its proteosomal degradation, is highly regulated by the ubiquitin ligase STUB1 and the deubiquitinating enzyme USP7. *In vitro*, inflammatory stimuli allow STUB1 to bind FOXP3 and promote its degradation, which is further facilitated by the downregulation of USP7[84,85]. However, *ex vivo* gene expression analysis of SF Treg from JIA patients shows stable expression of both proteins (Mijnheer et al., unpublished data). This illustrates that *in vivo* regulation of FOXP3 and Treg function is a highly complex organized process in which multiple proteins are involved. Also, depending on the environmental conditions, different proteins can take part in this network, allowing fine-tuning of the cells to a specific environment and further polarization[72]. Altogether, there are no indications for Treg instability on a large scale *in vivo*, but inflammation does seem to impact Treg by differentiation towards an eTreg phenotype.

Resistance of CD4+ and CD8+ cells to suppression

Unresponsiveness of T-cells to Treg suppression is most likely a normal transient phenomenon during the assembly of an immune response to clear an infectious threat. However, in auto-immune diseases this resistance of local effector T-cells to suppression contributes to a sustained inflammatory response and subsequent disease pathogenesis[86]. In JIA, both CD4+ and CD8+ T-cells from the synovial fluid of affected joints have been found to be intrinsically resistant to suppression[67,68,87]. The same has been described for CD4+ T-cells from SLE patients[88]. The resistance to suppression is at least partly mediated by protein kinase B (PKB)/c-akt hyper-activation, induced by local cytokines. TNFα and IL-6 were found to induce resistance in CD4+ T-cells, whereas TNFα and autocrine release of IFNγ were responsible for the intrinsic resistance in CD8+ T-cells[68,87]. The hyperphosphorylation of PKB/c-akt is interesting in the light of PD-1 signaling, since PD-1 is a strong negative regulator of PKB/c-akt[89]. Apparently PD-1 signaling in SF T-cells is not sufficient enough to downregulate this signaling pathway. Blockade of TNFα can restore the susceptibility of CD4 and CD8 T-cells to suppression, which is in line with the effectiveness of therapies targeting TNFα[87,90]. In IBD, resistance of lamina propria T-cells to Treg-mediated suppression has been described as well. In this setting high levels of SMAD7, causing insensitivity to TGFβ, have been related to the resistance of effector cells[91]. Thus, impaired regulation of

the local immune response involves resistance of effector cells, possibly despite functional Tregs.

Local T cell clonal expansion

When activated T-cells encounter their cognate antigen in the context of co-stimulation and cytokines, specific clones will expand to initiate a robust adaptive immune response. A diverse repertoire of T cell receptors (TCRs) of conventional T-cells allows a response to a multitude of possible pathogens. Also thymic derived Treg need a diverse TCR repertoire to regulate auto-immune responses. The TCR repertoire of Treg, representing only a small fraction of the total T cell pool, is equally diverse as the larger effector pool[92]. The need for a diverse (auto-antigen specific) TCR repertoire of Treg has been illustrated by studies using transgenic mice with a restricted TCR repertoire of Treg. In these models a loss of tolerance towards commensal bacteria and develop autoimmune diseases has been observed[93–96].

At the site of human autoimmune inflammation in both JIA and SLE, clonal T cell expansions of $CD4^+$ T-cell , $CD8^+$ T-cell and $FOXP3^+$ Treg populations are found[41,48,79,97,98]. Remarkably, especially Treg were found to be hyper-expanded, and to express high levels of Ki67. It is possible that dominant auto-antigens present at the affected sites induce this proliferation[68,79]. Local TNFα can also act as a contributing factor to Treg expansion, as TNFα induces Treg proliferation and effector Treg differentiation via TNFR2 signaling[99–102](and Mijnheer et al., unpublished data). Since hyper-expanded Treg clones were demonstrated to display a very distinct repertoire compared to conventional $CD4^+$ T-cells, local induction of Treg from conventional T-cells is unlikely[79]. Tissue resident Treg also show a considerable oligoclonality regarding their TCR repertoire, supporting the notion that tissue Treg and Treg from the site of autoimmune inflammation share typical features[103,104] (Figure 1). Interestingly, in refractory JIA and JDM patients undergoing hematopoietic stem cell transplantation the TCR repertoire of circulating Treg prior to transplantation was also found to be highly clonal. After transplantation, the Treg TCR repertoire became more diverse over time, except for one patient that experienced a relapse[105]. These date suggest that Treg TCR repertoire abnormalities may contribute to disease pathogenesis, possibly by limiting the chance of antigen encounter, thereby being outcompeted by the less restricted Teff.

Recent data from multiple affected joints in RA show that total TCR repertoires are substantially overlapping[106]. In JIA patients, especially PD-1^+ $CD8^+$ T-cells were shown to have a clonal repertoire, with a high overlap in dominant clones between different affected joints[33,52]. The overlap may be explained by the presence of common antigens that drive the disease at multiple sites, and/or (re)circulation of dominant T-cell clones. In affected kidneys of SLE patients expanded $CD8^+$ T-cells clones were found to be present for years in sequential biopsies[41], suggesting long-term persistence of dominant T-cell clones. It is tempting to speculate that these dominant T-cell clones play a role in disease relapses, but further studies are need to conclude this.

CONCLUSION AND FUTURE PERSPECTIVES

The expanding field of T-cells, including the discovery of multiple Th subsets as well as observations of mixed phenotypes in inflamed tissues, has made the classification of subsets increasingly complex[4]. This complexity, however, likely represents human immunity that is continuously exposed to multiple microorganisms and environmental conditions, in contrast to highly controlled mouse models that have contributed to most of the current knowledge[30,107]. A more nuanced view on T-cells in tissues now arises, with fine-tuning of immune cells to the local environment allowing tailored responses explaining the observed diversity in phenotypes. When inflammation becomes chronic this fine-tuning of T-cells might be unrestrained or insufficient, and as a result cause or contribute to pathogenesis. Potential determinants in this process could be the strength and frequency of TCR stimulation, as well as the presence of absence of CD4 help or co-stimulation, whereas local metabolites also seem to be important. How this is regulated exactly, and what the importance is of different factors remains to be answered, as well as the question what is different in affected human tissue in disease versus healthy human tissue. The possibilities of gaining new insight are enormous, with newly developed high-throughput technologies that require only small numbers of cells and that allow for analysis of rare T-cell populations from small tissue samples. Single cell sequencing combined with TCR sequencing and mass cytometry on whole tissues will give a deeper understanding about the heterogeneity of T-cells present in human autoimmunity. This, together with smart use of patients samples, such as sequential sampling and sampling from multiple affected sites, will provide novel insights and undoubtedly improve the therapeutic options for patients with rheumatic diseases.

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Chapter 8

General discussion

T cell regulation during an inflammatory response is of vital importance to avoid tissue damage and to ultimately achieve immune homeostasis. In chronic inflammation the regulatory mechanisms fail, but the process underlying this failure are incompletely understood. The recent insights into the unprecedented width of T cell adaptation have added a new layer of complexity. In this thesis we have dissected local immune regulation and T cell programming and adaptation in inflamed joints of JIA patients. In chapter 7 our published findings are discussed in the context of recent literature. In this chapter I will discuss unpublished data and remaining issues and put them in a broader perspective.

TREG INSTABILITY

The issue of Treg instability (i.e loss of function and Treg core signature), especially in inflammatory conditions, has long been, and still is, a matter of debate[1,2]. Contradicting data have made it difficult to draw firm conclusions, as is also discussed in **chapter 7**. Maintenance of FOXP3 expression and suppressive function are needed to prevent Treg instability and thus immune dysregulation. In **chapter 3** and **5** we show that Treg in a human autoimmune inflammatory environment maintain FOXP3 expression and suppressive function. Moreover, in **chapter 5** we show that both core Treg functional and effector (e)Treg markers are expressed at high levels, all guided by epigenetic imprinting, indicating differentiation rather than instability of Treg in response to local inflammation. We do observe a certain level of flexibility, with SF Treg displaying striking high levels of Th1-related markers such as T-BET, CXCR3 and IL12Rβ2, but the maintenance of Treg features shows that this is different from the previously reported plasticity of Treg, with loss of FOXP3 expression and the acquisition of effector functions under inflammatory conditions[3–6]. It is important to realize that most data on Treg instability are obtained from transgenic mice under extreme conditions such as lymphopenia, or studied in *in vitro* induced Treg, which have been shown to be unstable and to not acquire a full epigenetic Treg program ([7]; S. Sakaguchi, personal communication). Therefore these settings do not seem to mirror the *in vivo* situation in humans. Human Treg that produce pro-inflammatory cytokines have also been described, but suppressive function is maintained[8,9]. In conclusion, convincing evidence for Treg instability in human disease at sites of inflammation is missing. In my opinion it is rather the capability of Treg to adapt to their environment that explains the heterogeneity in phenotypes and functions of tissue and inflammatory Treg.

PERIPHERAL TREG INDUCTION

Selective markers for thymus-derived Treg versus peripherally induced Treg are still lacking and *in vivo* induced Treg seem to undergo a similar differentiation as thymic Treg, hence it is impossible to discriminate these cells even on a transcriptional or epigenetic level up. However, TCR repertoire comparison can provide an indication for the possible relationship between Treg and $CD4^+$ non-Treg. In **chapter 6**, we show that SF Treg have a clonal repertoire and that TCR repertoires of SF Treg and SF $CD4^+$ non-Treg are distinct with limited overlap. This suggest that there is no or minimal Treg induction derived from naïve $CD4^+$ T cells or conversion from non-Treg at the site of inflammation. Particularly, if local conversion would be an important feature, one would expect a certain level of overlap between $CD4^+$ Treg and non-Treg. Other recent publications support distinct TCR repertoires of Treg and non-Treg in SF of JIA patients, as well as in germinal center reactions in response to inflammation[9–11]. Together with the results obtained in **chapter 5** this is suggestive of antigen-induced activation and differentiation of thymus-derived Treg, shaped by the local environment. However, we cannot completely exclude a small contribution of induced Treg. In studies in mice it has been shown that peripherally induced Treg are specifically important for maintaining homeostasis at mucosal surfaces[12,13]. This was achieved by dissecting the roles of the conserved non-coding DNA sequence (CNS) elements at the *FOXP3* locus. CNS1 appeared to be largely dispensable for thymus-derived Treg, whereas its deletion markedly reduced Treg numbers in gut and lung and caused spontaneous Th2-like inflammation. Therefore, peripherally induced Treg are assumed to be of vital importance for immunological tolerance to non-pathogenic environmental antigens like food or commensal microbiota, in line with their induction from naïve $CD4^+$ T cells by non-self-antigens. Recent reports have even shown that local Treg induction is microbiota-driven by specific metabolites such as butyrate, and locally induced Treg in turn serve as an essential feedback mechanism affecting the composition and function of microbial communities[14–16]. Since deletion of CNS1 did not lead to autoimmune responses, systemically or locally, the role of preventing such responses is ascribed to thymus-derived Treg[12]. Aforementioned induced Treg refer specifically to *in vivo* induced mouse Treg, that have been found to stable like thymus derived Treg but unlike *in vitro* induced Treg[7]. There is a lot of interest for the use of *in vitro* induced Treg for therapeutic purposes, but the instability shown by rapid loss of FOXP3 for example is a major drawback[7,17]. Although there is increasing knowledge about the conditions that can lead to *in vivo* induction of Treg, the underlying mechanisms that establish a stable Treg phenotype, including a Treg transcriptional and epigenetic landscape, in peripherally induced Treg remain poorly understood. Therefore, although *in vitro* induced Treg can be of interest for studying possible induction methods, one should be cautious using *in vitro* induced Treg as a substitute for *in vivo* Treg. Studying human Treg phenotype and function requires *ex vivo* analysis of these cells. Also regarding more fundamental research, involving gene

regulation for example, the use of *ex vivo* Treg, perhaps next to *in vitro* induced Treg, will result in more reliable and relevant results.

FUNCTIONALITY OF TREG

Next to stable FOXP3 expression, suppressive capacity is a hallmark feature of Treg. The best possible way to identify human Treg functionality is by using an *in vitro* suppression assay. Purified Treg, based on high expression of CD25 and low expression of CD127 can be co-cultured with responder cells[18,19]. In **chapter 3** we performed such a so-called suppression assay to study functionality of Treg derived from SF. Here we used total PBMC or SFMC as responder cells, instead of the often used $CD4^+$ $CD25^-$ T cells, to give a more relevant reflection of the *in vivo* situation. Total PBMC or SFMC were stained with a cell tracer dye to determine cell proliferation. Cytokine production was analyzed by measuring the intracellular cytokines in the responder cells or by measuring secreted cytokines in culturing medium. Using this assay, we found that SF Treg are not intrinsically defective. Since resistance to suppression can occur, as was also revealed in **chapter 3**, taking along reference allogeneic total cells such as PBMC of a different donor can control for this phenomenon. However, when using human samples such as patient material, the amount of cell is often limiting and purification of high numbers of Treg can be challenging. This is especially the case when tissue samples are being used. We have tested and optimized the suppression assay for such purposes and even using 10.000 responder cells we see suppression of proliferation (Figure 1A, Mijnheer & Scholman, unpublished data). Using a 1:2 ratio of responder cells to Treg this means that only 5.000 purified Treg are needed. The minimum of responder cells to be able to observe suppression of cytokine production is 25.000 cells (Figure 1B, Mijnheer & Scholman, unpublished data). Next to the level of FOXP3 expression, studying the expression of eTreg markers such as ICOS, VDR and BATF (**chapter 5)** may provide information about the activation of Treg. Moreover, the analysis of environment-specific markers such as T-BET and IL12Rβ can give insight into the degree of local adaptation.

TREG DIFFERENTIATION AND ADAPTATION

It is becoming evident that a complex regulatory machinery allows for the Treg differentiation process that involves integration of various environmental cues. Already during thymic development of Treg multiple steps are involved in the differentiation towards a bona fide Treg that might be illustrative for further Treg differentiation. Here the strength of TCR and co-stimulation and the availability of IL-2 determine the lineage commitment by induction of specific transcription

factors[20,21]. However, at the basis of each cell of the body is the same genetic code, and the identity of a cell is regulated by binding of transcription factors to specific genomic regions such as promoters, enhancers or super-enhancers. The chromatin structure should be 'open' at those specific sites to allow the expressed transcription factors to bind. So in case of developing Treg the chromatin structure should allow for the induced transcription factors to bind. This is accomplished in part by the genome organizers

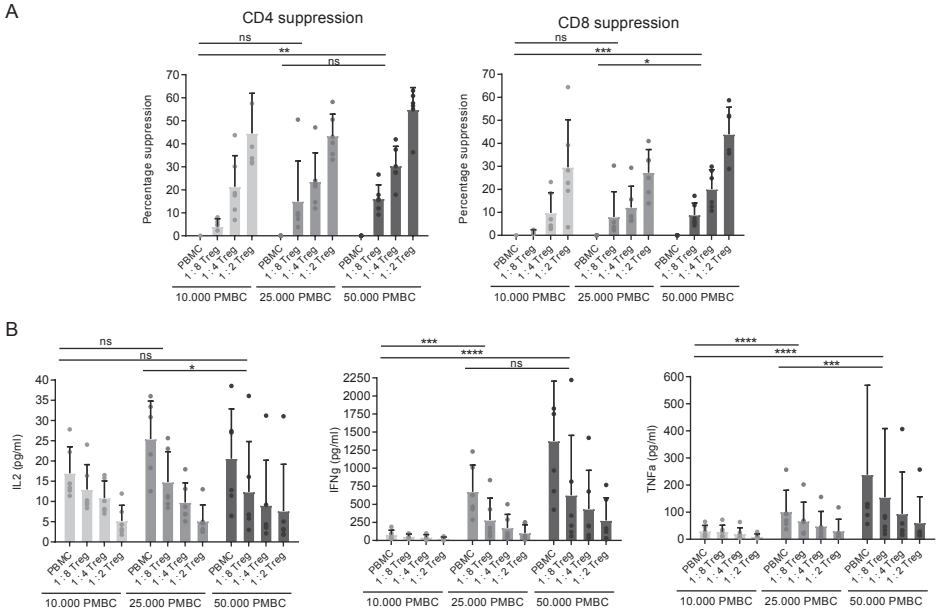

Figure 1. Optimization of a low cell number Treg suppression assay (part of UCAN-U assay standardization).

Dose dependent suppression of allogeneic total PBMC analyzed by CD4 (left) and CD8 (right) proliferation (**A**) or by measurement of secreted cytokines in the culture medium by Luminex technology (**B**), using various cell numbers. A two-way ANOVA with multiple comparisons is performed, * = p ≤ 0,05 – **** = p ≤ 0,0001

SATB1 and BACH2 that activate super-enhancers that are associated with Treg signature genes such as CTLA-4[22–24]. The preparation of this epigenetic landscape occurs in the thymus, even before FOXP3 is expressed, but also seems to occur in Treg that are induced *in vivo* in the periphery[20]. FOXP3 and TCR signaling are needed to maintain and reinforce this Treg specific transcriptome, and as such ensure Treg function[25–27]. This interplay of epigenetic and transcriptional regulation during Treg development provides a stable basis and is likely typical for the further differentiation and specialization of Treg. In **chapter 5** we show that Treg in an inflammatory environment have an altered chromatic

landscape based on H3K27ac pattern compared to circulating Treg, with eTreg and environment-specific changes. So on top of the core Treg genes, an additional program of genes and enhancers are associated with this differentiation. A recent report studying DNA methylation of tissue Treg derived from several healthy tissues in mice revealed a model for tissue Treg that integrate epigenetic changes from multiple differentiation steps; the first being reprogramming during thymic development (as described above), the second being modifications that strengthen a core tissue Treg profile and the third representing environment-specific reprogramming that allow fine-tuning[28]. A similar step-wise differentiation on a transcriptional level has also been recently proposed for visceral adipose tissue-Treg as well as splenic Treg, both in mice[29,30]. Interestingly, this concept shows a lot of resemblance with our identified transcriptional and epigenetic profile of human inflammation derived-Treg that show (1) a classic Treg profile, (2) an effector profile and (3) environment specific changes (Figure 2). Thus a dynamic regulation on both transcriptional and epigenetic level is involved in Treg specialization and allows tissue specific fine-tuning.

Whereas the role of TCR affinity during thymic differentiation is well known, its role in peripheral Treg function is less clear. A recent study addressed this issue in a mouse model of type 1 diabetes using cell from the pancreatic islets[31]. The authors found that a high degree of self-reactivity is crucial for tissue-specific Treg function in an autoimmune environment. This highly suppressive Treg subset is characterized by elevated levels of GITR, CTLA-4, ICOS and T-BET. Moreover, the TCR-signal strength appeared to be important for the induction of T-BET expression in Treg, whereas the role for IFNγ in this induction was minor. The similarities of the profiled Treg from the site of autoimmune inflammation in mice with our data in **chapter 5 and 6** regarding SF Treg are striking. Next to that, we show in **chapter 6** that SF Treg very likely react to a dominant autoantigen and are of thymic origin and thus are self-reactive. The model of this high-TCR affinity induced profile with increased levels of classical Treg markers as well as T-BET could very well apply of SF Treg as well. Also enhanced levels of ki67 and the lack of pro-inflammatory cytokine secretion are in line with what we observed in SF Treg. Together, this sheds new light on the role TCR affinity during extrathymic Treg specialization and the overlapping profile suggest that this also occurs in a human setting. Why regulation fails despite the presence of these specialized Treg is of interest. In the aforementioned study transfer of the highly self-reactive Treg largely protected mice from developing type 1 diabetes, whereas this was not the case when transferring low self-reactive Treg[31]. As the infiltrating Treg population consists of a broad range of self-reactive Tregs, the authors suggests that the local accumulation of this mixed functional and less-functional Treg causes insufficient control despite high Treg numbers. Resistance of effector cells to Treg suppression induced by the local inflammatory milieu, as we have shown in **chapter 3**, can also be a contributing factor.

THE OVERRULING EFFECT OF INFLAMMATION

The presence of tissue resident memory T cells (Trm) in many different tissues and their versatility with a unique profile for each tissue, and the expanding list of tissue Treg that have highly tissue specific functions illustrate the enormous ability of immune cells to adapt. Whereas the environment in each tissue can be very different, inflammation is a more general phenomenon. In **chapter 5** we describe that the transcriptional profile of SF Treg to a large extent overlaps with tumor-infiltrating Treg (Figure 2). Just like in an affected joint in JIA patients, the tumor environment is characterized by inflammation and thus immune cell infiltration[32,33]. This might result in a temporal loss or delay of tissue-specific programming of immune cells because the inflammation process is so dominant. In line with this, we discovered a Trm profile of $CD8^+$ $PD\text{-}1^+$ in SF in **chapter 4** that is also displayed by $CD8^+$ T cells in tumors[34,35]. The overarching factor is inflammation, suggesting that the local programming is a largely inflammation-driven process.

Related to the observed similarities in tumor and arthritis is perhaps the occurrence of autoimmune pathologies such as rheumatic diseases as the main immune-related adverse event that can develop under cancer immunotherapy[36]. This encompasses the therapeutic agents also known as checkpoint inhibitors that are monoclonal antibodies targeting regulatory checkpoint molecules such as PD-1 and CTLA-4. By eliminating these brakes through therapy, regulatory mechanisms are disarmed and effector cells of the immune system are 'released' to attack tumor cells. This appears to be a promising approach to target cancer patients. However, the systemic administration leads to the activation of immune cells throughout the body. The occurrence of rheumatic diseases, but also colitis and skin inflammation underscores the commonalities in physiological response of T(reg) cells to control immune cells in cancer and to maintain tissue-specific homeostasis.

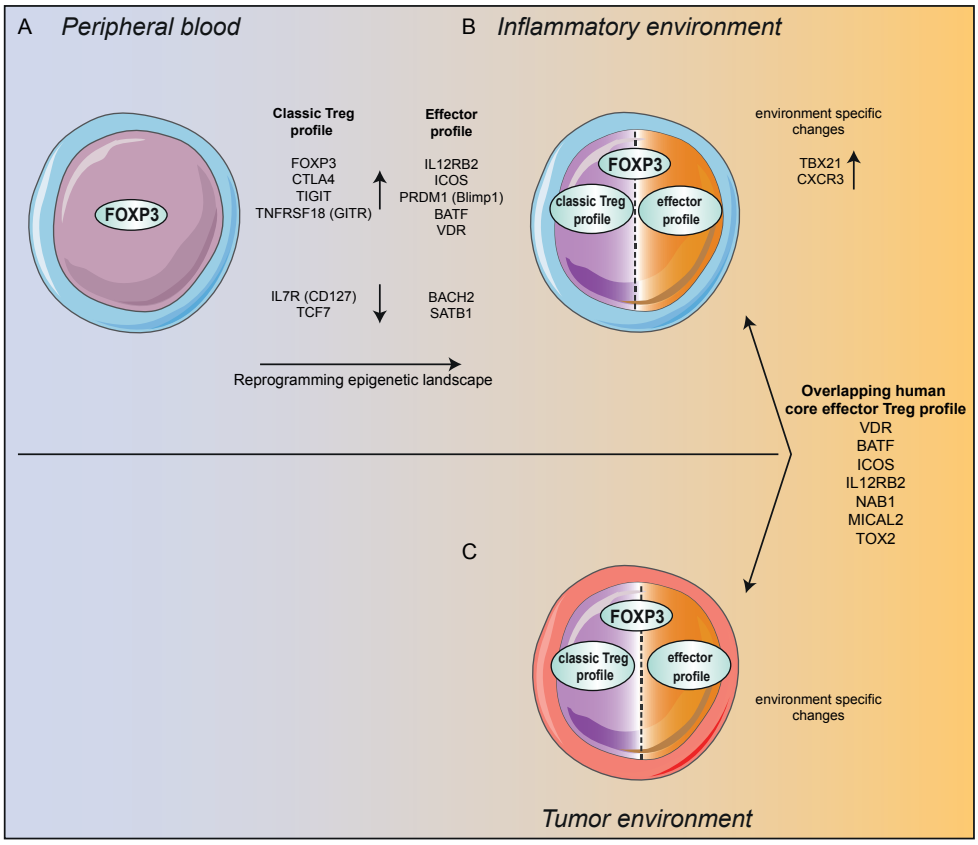

Figure 2. Summary of the main findings from Chapter 5

Circulating Treg in peripheral blood (**A**) can be identified by the expression of FOXP3 and classical Treg markers such as CTLA4 and TIGIT. Ex vivo H3K27ac, transcriptome and flow cytometry protein profiling of SF-derived Treg (**B**) revealed an upregulation of the classical Treg markers, as well as upregulation of effector (e)Treg markers. These included Blimp-1, Batf and ICOS, that have also been identified in mice as critical in the differentiation towards eTreg. We identified VDR and IL12Rβ2 as human-specific eTreg markers. The downregulation of the genomic organizers Bach2 and Satb1 likely allows the differentiation towards an effector profile. High expression of T-BET (encoded by TBX21) and CXCR3 reflect environment-specific changes. Comparison of this eTreg profile to signature genes identified in human tumor-infiltrating Treg 37 (**C**) revealed a striking overlap in profile. Together these data uncover a conserved human eTreg profile that is fine-tuned by the specific environment.

T CELL CLONALITY IN INFLAMMATION

We observed a clonal TCR repertoire of $CD4^+$ T cells, Treg and PD-1^+CD8^+T cells at the site of inflammation in JIA, described in **chapters 4 and 6**. Whereas clonal expansion at a site of inflammation is expected, the degree of clonality of especially Treg is surprising. Next to that, the dominance of the same clones in time and in multiple affected joints is striking. The persistence of clones in combination with the tissue resident like profile of SF T cells raises questions regarding the relation between the two. Cardinal features of Trm cells are their retention in tissues after inflammation has resolved, also in the absence of antigen, and their rapid actions after re-encounter of the antigen[38]. We show persistence of (pathogenic) T cell clones in the context of relapse-remitting autoimmune diseases, of patients that display rapid flares and show disease specific symptoms in well-defined target tissues. These are strong indications for the involvement of tissue resident cells in disease pathogenesis, but further research is needed to draw any conclusion. The presence of the same clones in different joints, as well as in peripheral blood, indicate migration of disease-specific T cell clones. Whether this means migration out of the joint into the (lymph)circulation and possibly into another joint, or rather migration from the local 'triggering' lymph node is not clear. The observation that multiple clones are overlapping, and immune architecture was found to be nearly identical **(chapter 6)**, make it tempting to speculate that T cells migrate out of the joints and recirculate. However, this migratory capacity goes against the tissue resident profile of the SF T cells as described above. A recent paper shows that $CD8^+$ T cells in mice can exit non-lymphoid tissue after infection to become Trm in secondary lymph nodes[39], and $CD4^+$ T cells seem to have a greater capacity to migrate out of tissues after infection (D Masopust, personal communication). This may provide an explanation why identical TCRβ SF T cell clones are detected in several joints, also over time.

Our finding that locally expanding T cell clones are also detectable in circulation **(chapter 6)**, may be of interest for clinical applications. First of all monitoring of these cells may have a prognostic value. Secondly, identification of disease-specific pathogenic T cell clones may open avenues for targeted treatment. To link specific T cell clones to a pathogenic phenotype and to select specific targets, single cell RNA-sequencing in combination with single cell TCR-sequencing of local T cells would be an important next step.

At the end of **chapter 6** we studied overlap in HLA-alleles between patients, as well as overlap in the measured amino acid sequences of the TCRβ. We found minimal overlap on these levels, but this can be largely explained by differences in HLA profiles among patients. Based on two recent papers presenting analytic tools to identify epitope-specific groups of different TCRs that share core sequence similarities[40,41], we performed a pilot analysis with a newly developed pipeline (collaboration Prof. R. de Boer and Dr. A. Pandit). Identification of identical amino acid sequences within the identified TCRs

provides insight into the epitope-specificities and thus antigen recognition. So within a sample we looked whether unique clones share multiple amino acids with each other, so-called motifs. So far, the analysis has only been performed on Treg TCR clones, derived from three patients with SF from both knees that are described in **chapter 6**. Whereas in peripheral blood no networks of similar amino acid motifs were detected, in SF samples several large networks of clones appeared, which encoded similar motifs spanning 5 or even more amino acids (Figure 3, unpublished data). Given the amount of nucleotides necessary to encode these amino acids, this reveals a high degree of overlap. As expected, these motifs were overlapping between left and right joint. Strikingly, the motifs were also highly overlapping between the six SF samples derived from the three patients. For example, the motifs "NSLPH" and "GANVLT" were shared among all six SF samples. Further investigation into the origin of the motifs revealed that the identified motifs are all encoded on the J segment, and are more or less germline encoded with 1 or 2 mutations. There was no preferential J usage and the J segments that are encoding the overlapping motifs are not expressed at a high incidence. Moreover, the motifs are not found in the top 10 most dominant clones. Since the pipeline is still under development and therefore the data is incomplete, it is difficult to already draw conclusions. The data so far are suggestive for ubiquitous expressed auto-antigens that are disease-specific. Future analyses of Treg and conventional T cells in different patient groups will shed light on epitope-specific networks and will bring the hunt for antigens that drive disease an important step closer.

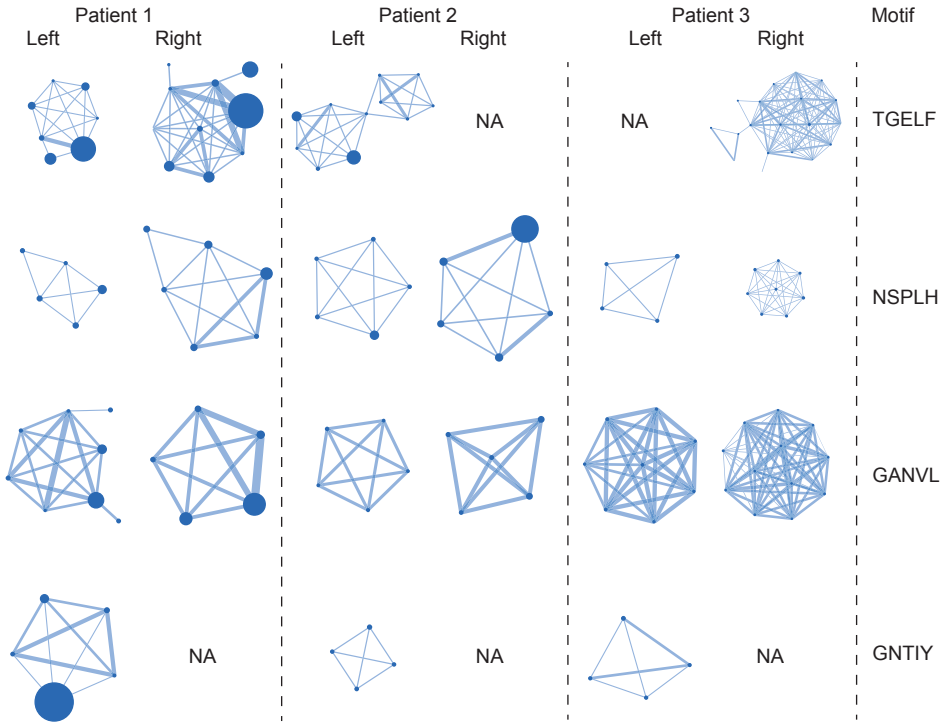

Figure 3. Clusters of TCRs that share sequence motifs (immune fingerprints) within and between patients

Immune fingerprints from different patients are depicted, identified in SF derived from inflamed left and right knees of JIA patients. The right column shows the sequence motif corresponding to the immune fingerprints shown in each row.

FACTORS THAT INFLUENCE ADAPTATION

The multiplicity of factors that determine the profile of T cells in (chronic) inflammation is intriguing, and even more what shifts the balance from health to disease. An overview of the local processes that influence T cell phenotype and function in the joint of JIA patients are shown in figure 1 of chapter 7. In this thesis, we identified specific profiles of activated, clonally expanding T cells at the site of inflammation, namely PD-1^{+}CD8^{+} T cells with an effector as well as Trm profile (chapter 4) and highly clonal Treg displaying an environment adapted eTreg profile on both transcriptional and epigenetic level (chapter 5 and 6). The presence of a complex regulatory machinery that allows this differentiation is shortly described above regarding Treg, but the importance of (environmental) cues initiating this process are also more and more known. For example literature is piling up

about the influence of TCR signalling in multiple stages of T cell development. Mouse data suggest that the degree of affinity of self-Ag during T cell-peptide/MHC interaction in the thymus also seems to already program $CD4^+$, $CD8^+$ and Treg responsiveness in the periphery[31,42–44]. In the periphery this differentiation seems to be further shaped by the strength and/or frequency of TCR-peptide/MHC interactions[30,43,45]. However, no data are currently available in the human setting. Another well-known factor, that also impacts downstream TCR signalling, are signals from co-stimulatory and co-inhibitory receptors via the PI3K–AKT pathway[46]. For example, the balance between the two opposing signals dictate the functional profile of $CD8^+$ T cell in chronic infection and autoimmune diseases[47]. Expression of the receptors itself does not equalize signalling as we have shown in chapter 4, where PD-1 is expressed at increased levels on $CD8^+$ T cells but the presence of soluble PD-1 might prevent signalling[48], and perhaps results in insufficient downregulation of the PI3K-Akt axis. A third factor is cytokines that have an established role during priming of T cells by activated APCs. During an inflammatory response, T cells are exposed to a plethora of cytokines with a specific but dynamic composition and dosage. For example, we profiled the synovial fluid for their cytokine content, revealing a soup of highly expressed proteins that, as expected, differed markedly from the content found in peripheral blood (Figure 4, unpublished data). Pinpointing the contribution of each one of them is a demanding and almost impossible task in an *in vitro* setting. Recent computational approaches to identify the capacity of T cell differentiation in response to the complex extracellular environment provided promising results, revealing classical T cell lineages as well as novel T cell phenotypes[49–51]. Integration of multiple data sets, such as receptor expression on different immune cell subsets as well as extracellular content profiles, can provide novel insight into local (pathological) differentiation processes. Another important influence on T cell phenotype and function is the availability of local metabolites. This topic goes beyond this thesis, but it certainly has an impact by affecting cellular metabolism. Since the profiling of cells from the site of inflammation in chapter 4 and 5 revealed specific metabolic programming, T cell metabolism is briefly discussed below.

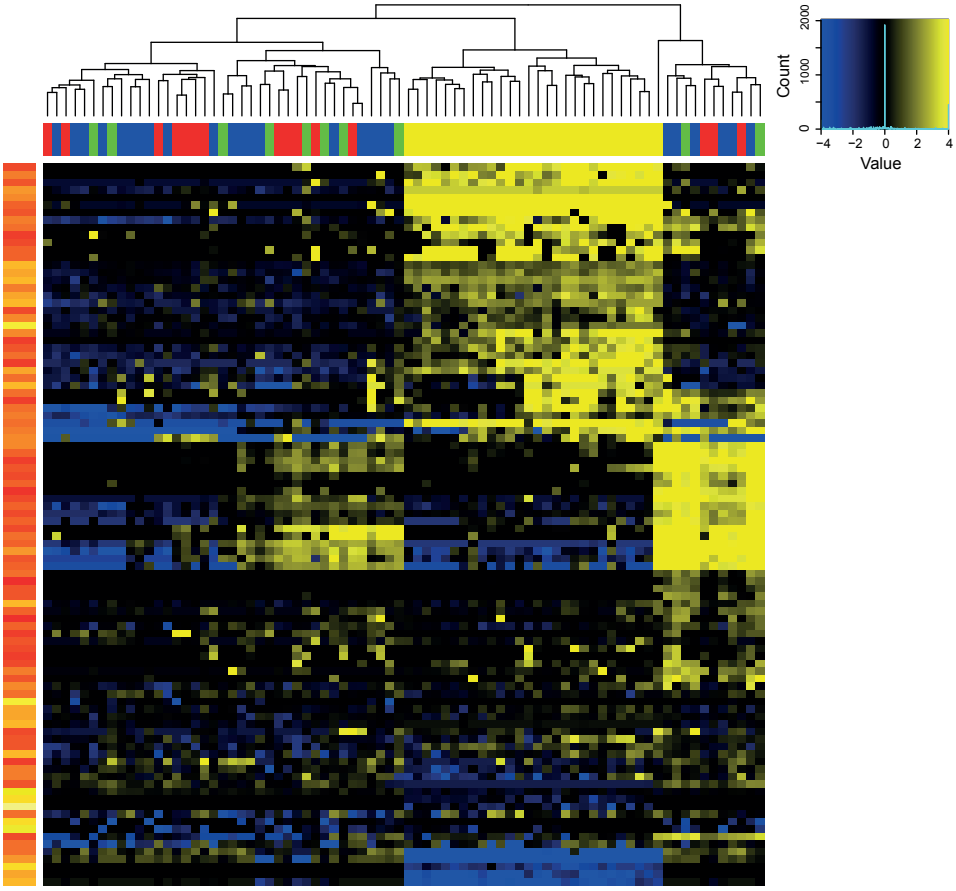

Figure 4. Hierarchical clustering analysis on a broad panel of proteins identified in SF and PB plasma from JIA patients

The profiling of 109 proteins (cytokines, chemokines, growth factors) in 30 SF samples and 50 PB plasma samples of JIA patients. The heatmap shows relative expression with median expression per protein depicted in the left bar. Hierarchical clustering is performed on the samples.

T CELL METABOLISM IN REGULATION AND ADAPTATION

Metabolism is increasingly being recognized as a dominant factor determining T cell fate and function. As the transition from naïve T cells to effector cells upon recognition of the cognate antigen is energy demanding, metabolism needs to be adjusted. Moreover, during tissue adaptation, cellular metabolism needs to be adjusted accordingly to the tissue environment. A key molecule in controlling cellular metabolism is mammalian target of rapamycin (mTOR). In general, naïve T cells use mitochondrial oxidative phosphorylation (OXPHOS) to generate energy (i.e. adenosine triphosphate, ATP). Upon antigen encounter and subsequent TCR and co-stimulatory signals mTOR complex 1

(mTORC1) is activated through the PI3K-Akt pathway[52]. The consequence of PI3K/Akt/mTOR activation is upregulation of glucose transporter Glut1 and increased aerobic glycolysis, shifting the balance towards higher dependence on glycolysis in conventional T cells[53]. Moreover, mTOR has shown to be crucial for the differentiation of $CD4^+$ T cells into effector lineages[54]. The role of mTOR in Treg is obscure, but the prevailing model is that mTOR activation impedes Treg function and induction[54,55]. The inhibition of mTOR in $CD4^+$ T cells results in increased fatty-acid oxidation that favours the induction of Treg[56,57]. However, this interpretation is based on studies assessing Treg differentiation *in vitro,* or *in vivo* using genetic targeting in mice[58]. Moreover, we now know that Treg are not terminally differentiated cells when they leave the thymus, but differentiation and specialization occurs in the periphery and in tissues to an extend that is similar to, or even reaches further than conventional T cell differentiation. Knowledge about human T cell metabolic profiles in tissues or inflammatory environments is scarce[58], probably due to technical limitations such as the amount of cells needed for proper metabolic analysis. We also faced this issue, but as is shown in **chapter 4**, we were able analyse the level of glucose consumption in SF-derived $CD8^+$ $PD\text{-}1^+$ cells *ex vivo* using Seahorse technology, with selected large patient samples. Our findings that these cells engage increased glycolysis fits their effector profile and function, and argues against an exhausted profile of local human $CD8^+$ T cells in autoimmune inflammation. Moreover, transcriptome analysis of Treg, presented in **chapter 5**, revealed enrichment of genes involved in glycolysis as one of the top pathways when comparing the transcriptomes of SF-derived Treg to PB-derived Treg (from both healthy adults and JIA patients – data not shown). Therefore, we used the same samples as described in **chapter 4** to also sort SF- and PB-derived Treg and $CD4^+$ non-Treg to perform pilot experiments analysing the metabolic profile. SF T cells showed increased glycolysis compared to PB T cells, and strikingly, the levels of glycolysis were at least as high in Treg as compared to $CD4^+$ non-Treg (unpublished data). Whereas glycolytic activity in Treg has been primarily associated with Treg dysfunction[59], several recent papers provide evidence that increased glycolysis is needed for eTreg/tissue Treg differentiation and migration to inflammatory sites[60–62]. This also fits with proposed models of a dynamic role of mTOR in Treg (and T cell) differentiation, allowing differentiation into effector stage and memory stage[63–65]. Moreover, different metabolic profiles have been observed in thymus-derived Treg compared to *in vitro*-induced Treg, again highlighting the difference between the two subsets[66]. Of interest is the finding that under hypoxic conditions, like an inflammatory environment, well-known signalling pathways may have different outcomes. Studies on mice with hypoxia-inducible factor 1 (HIF-1α)-deficient T cells have revealed that HIF-1α induces glycolysis in Th17 cells and suppresses Treg differentiation by mediating FOXP3 degradation[67,68], whereas another study showed that during inflammatory conditions characterized by hypoxia HIF-1α induces FOXP3 expression and ensured Treg function next to inducing glycolysis[69]. The latter might apply to the inflammatory environment in the joint as well. Furthermore, danger signals provided by TLR triggering can directly

induce expression of Glut1 in Treg[70]. Altogether, this reveals context-dependent metabolic adjustments of Treg *in vivo* that are influenced by both cell-intrinsic and -extrinsic factors[58]. In addition, this highlight the need for research on human T cells *ex vivo* from various tissues and conditions to provide insight and as such provide a rationale and direction for development of novel therapeutic strategies[71]. In that respect, studying the metabolome of SF and tumour exudate would be an interesting follow up on our findings from **chapter 5**.

FUTURE PERSPECTIVES

The field of (T cell) immunology is rapidly evolving, with the discovery of several new immunological concepts in the last decades. An important step towards improved patient care is to translate findings from mice or *in vitro* studies to local immunological mechanisms in patients. Fundamental immunological research using patient material is essential to achieve this. Throughout the discussion I have mentioned important scientific next steps that are, in my opinion, necessary to expand our knowledge on human disease pathology, especially chronic inflammation. Of clinical interest is for example monitoring (pre)clinical disease by detection of disease-associated T cell clones in peripheral blood. Another future perspective might be specific targeting of pathogenic T cell clones, in circulation or tissues. Moreover, the expansion and persistence of T cell clones during the inflammatory response locally, provides a rationale for early aggressive therapy in JIA, as is suggested for RA as well. Although direct links to patient care are not always obvious at first sight when performing fundamental immunological research, new insights into local disease processes are the basis for progress in treatment and care of patients.

HIGHLIGHTS

- T cells derived from affected joints of JIA patients show a differentiated and environment-adapted profile that differs markedly from circulating T cells on epigenetic, transcriptional, metabolic and protein expression levels
- Functional regulatory T cells from synovial fluid of inflamed joints display an adapted profile with a conserved effector Treg marker expression including FOXP3, TIGIT, ICOS, BLIMP-1, and VDR, as well as environment-specific markers including CXCR3 and T-BET
- The transcriptional signature of human tumor-infiltrating Treg overlaps almost completely with Treg derived from an autoimmune inflammatory environment, i.e. SF
- PD-1-expressing CD8 T cells from SF are not functionally exhausted, but are clonally expanding cells with heightened effector capacities
- Resistance of effector cells to Treg-mediated suppression due to hyper activation of PKB/c-AKT in the joint of JIA patients contributes to local immune dysregulation
- There is an extensive overlap in T cell immune architecture and specific dominant T(reg) cell clones in anatomically distinct inflammatory sites within a patient
- Dominant, locally expanding $CD4^+$ T(reg) cell clones persist over the relapsing-remitting course of autoimmune disease and can be traced in circulation
- Dominant $CD4^+$ T cell clones that are locally expanding in the joint are present in the circulation

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Addendum

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NEDERLANDSE SAMENVATTING

AUTO-IMMUUNZIEKTES

Een ontstekingsreactie is een natuurlijke reactie van het lichaam, meestal gericht op een onbekende indringer zoals een bacterie of een virus. Wanneer er een indringer wordt ontdekt geven lokale weefselcellen alarmsignalen af en kunnen lokale cellen van het immuunsysteem andere immuun cellen aantrekken om zo de indringer te verwijderen. Tijdens een ontstekingsreactie zijn verschillende soorten cellen van het immuunsysteem betrokken, ieder met een eigen rol, om zo de veroorzaker te verwijderen. Een ontstekingsreactie kan ook ontsporen en langdurig, ofwel chronisch, worden doordat de veroorzaker niet verwijderd wordt bijvoorbeeld, of doordat het zich richt tegen lichaamseigen stoffen. Bij een auto-immuunziekte is dat laatste het geval. Door deze chronische ontstekingsreactie is er een groot risico op weefselschade. Veel auto-immuun ziektes zijn weefselspecifiek, dat wil zeggen dat ze alleen in bepaalde weefsels voorkomen. Bij diabetes type I worden specifiek de cellen die insuline maken in de alvleesklier aangevallen, terwijl bij de ziekte van Crohn de ontstekingen in de darm plaatsvinden. Een ander voorbeeld van een auto-immuun ziekte is (jeugd)reuma, waarbij de ontstekingen in de gewrichten plaatsvinden. Hierbij kan dus schade aan de gewrichten ontstaan, die vooral voor jonge kinderen in de groei levenslange klachten kunnen geven. Tijdig medisch ingrijpen is nodig om deze weefselschade te voorkomen.

De huidige therapieën zijn erop gericht om ontstekingsreacties te onderdrukken. Er is alleen nog veel onduidelijk over hoe die ontstekingsreacties in weefsels precies verlopen en welke rollen de verschillende immuun cellen aannemen als ze in de aangedane weefsels zijn. In muismodellen is het mogelijk om immuun cellen uit de aangedane weefsels te halen en te onderzoeken, maar bij mensen is dit een stuk lastiger. Veel onderzoek wordt daarom gedaan op cellen afkomstig uit bloed en cellen die zijn gekweekt in het laboratorium. Hoewel dit een waardevolle bijdrage kan zijn aan nieuwe ontdekkingen, is recent bekend geworden dat immuun cellen sterk beïnvloedt worden door hun omgeving, dus het weefsel waar zich in bevinden. In het geval van (jeugd)reuma komen patiënten met regelmaat naar de polikliniek, zeker als ze klachten hebben. De reumatoloog kan dan, als het gewricht erg gezwollen is, de klachten verlichten door het ontstekingsvocht uit het gewricht te halen en er meteen medicatie in te injecteren. Het ontstekingsvocht dat ook wel synoviaal vocht wordt genoemd kan dan, na toestemming van de patiënt, onderzocht worden in het laboratorium om zo auto-immuunziektes bij mensen beter te begrijpen.

T CELLEN

De cellen die het eerste betrokken zijn bij een ontstekingsreactie zijn cellen van het aangeboren immuunsysteem, die vooral reageren op algemene alarmsignalen. De cellen die vervolgens naar het aangedane weefsel gaan zijn cellen van het verworven immuunsysteem. Deze reactie is iets trager, omdat deze cellen specifiek afgestemd zijn op de indringer en een op maat gemaakte reactie kunnen geven. Daarbij onthouden deze cellen de indringer door het ontstaan van 'geheugen' cellen. Geheugen cellen blijven na een ontstekingsreactie achter in het weefsel en circuleren in het bloed en lymfe, waarbij ze bij een volgende ontmoeting met dezelfde indringer deze snel en efficiënt kunnen aanpakken. T cellen en B cellen zijn onderdeel van het verworven immuunsysteem. Het is aangetoond dat bij (jeugd)reuma patiënten vooral T cellen zich ophopen in het synoviaal vocht, en dat ze belangrijke rol spelen in het ontstekingsproces.

Er bestaan verschillende types T cellen, grofweg onder te verdelen in $CD4^+$ regulatoire T cellen, $CD4^+$ T helper cellen en $CD8^+$ cytotoxische T cellen. Regulatoire T cellen (Treg) reguleren de immuun respons door deze af te remmen om zo weefselschade te voorkomen, terwijl T helper en cytotoxische T cellen de immuun respons gaande houden en actief en gericht 'foute' cellen aanvallen. De afstemming tussen al deze cel types is erg belangrijk, en lijkt in veel ziektes zoals reuma en kanker lokaal ontregeld. Hoewel bekend is dat T cellen belangrijk zijn in verschillende ontstekingsreacties, is er onvoldoende diepgaande kennis over hoe T cellen zich gedragen in ontstekingen in weefsels bij mensen.

ONDERZOEKEN IN DIT PROEFSCHRIFT

Het doel van dit proefschrift is om te ontdekken hoe T cellen in een lokale chronische ontsteking in mensen geprogrammeerd zijn en zich gedragen. We hebben daarbij vooral gekeken naar T cel regulatie en de aanpassing van T cellen aan chronische ontsteking.

In **hoofdstuk 2** hebben we de effecten van de huidige standaard therapieën op Treg samengevat, om zo inzicht te krijgen in de rol van Treg in het ziektebeloop van (jeugd)reuma. Treg, met hun remmende functie op andere immuun cellen, worden als veelbelovende therapeutische toepassing gezien in onder andere auto-immuunziektes. Door te bespreken wat er bekend is over de effecten van de huidige therapieën op Treg willen we inzicht geven in hoeverre het zinvol kan zijn om een op Treg gebaseerde therapie te ontwikkelen voor reuma.

In **hoofdstuk 3** hebben we de functie van lokale Treg uit het synoviaal vocht onderzocht, door de testen of ze in staat zijn lokale en bloed cellen af te remmen. Door cellen te gebruiken uit zowel het bloed als het synoviaal vocht kunnen we de rol van de ontstekingsomgeving op Treg functie onderzoeken. We hebben ontdekt dat Treg uit

het ontstekingsvocht prima in staat zijn immuun cellen uit het bloed te onderdrukken, maar dat de immuun cellen in het synoviaal vocht niet gevoelig zijn voor deze remming. Dit laatste komt door specifieke signaalstoffen die aanwezig zijn in het ontstekingsvocht. Deze ongevoeligheid voor remming van immuun cellen kan dus een aanknopingspunt zijn voor nieuwe therapieën. Daarbij laat het zien dat in het lokale ziekteproces Treg uit zichzelf goed functioneren, in tegenstelling tot wat vaak werd aangenomen, maar dat de ontstekingsomgeving ontregelend werkt bij jeugdreuma.

In **hoofdstuk 4** ligt de nadruk op de cytotoxische T cellen, waar nog maar weinig van bekend is in de setting van auto-immuunziektes. Van chronische virale infecties en tumoren is bekend dat $CD8^+$ cellen die PD-1 als eiwit op de oppervlakte hebben uitgeput zijn en niet goed meer functioneren. Ons onderzoek laat zien dat dit niet het geval is in de aangedane weefsels van verschillende auto-immuun ziektes, maar dat $CD8^+$ $PD\text{-}1^+$ cellen juist erg actief en agressief zijn en zo dus mogelijk een belangrijke bijdrage aan weefselschade leveren. Dit pleit voor een gerichte aanpak van deze cellen in (jeugd) reuma en andere auto-immuunziektes.

In **hoofdstuk 5** hebben we met behulp van recent ontwikkelde technieken, RNA- en ChIP-sequencing, bestudeerd hoe Treg uit het ontstekingsvocht "geprogrammeerd" zijn. Dit hebben we gedaan door te kijken welke delen van het DNA, de genetische code, actief zijn en kunnen leiden tot het maken van een eiwit. Het DNA is namelijk in nagenoeg alle cellen van het lichaam hetzelfde, maar per type cel verschilt het welke delen van het DNA op slot zitten en welke 'aan' staan. Als een stuk van het DNA 'aan' staat kan dit stukje code, ook wel gen genoemd, worden afgelezen waarna het bijbehorende eiwit wordt gemaakt. Deze eiwitten bepalen uiteindelijk hoe een cel zich gedraagt en welke functies de cel heeft, zoals een zenuwcel heel anders is dan een huidcel met hetzelfde DNA. Wij hebben ontdekt dat Treg uit het synoviaal vocht anders geprogrammeerd zijn dan Treg uit het bloed. Treg uit het synoviaal vocht zijn meer gespecialiseerd en aangepast aan de ontstekingsomgeving. Onze data hebben we vergeleken met recent verkregen data over Treg die aanwezig zijn in verschillende types tumoren in mensen. Daarmee hebben we een profiel ontdekt dat uniek lijkt te zijn voor Treg in een menselijke ontstekingsomgeving, met veel overeenkomstige genen en eiwitten gericht op specialisatie van de cellen maar ook met omgevingsafhankelijke verschillen.

In **hoofdstuk 6** hebben we de samenstelling van de immuun cellen aanwezig in het ontstekingsvocht van jeugdreuma patiënten onderzocht met een nieuwe techniek, CyToF. Daarnaast hebben we het T cel receptor repertoire van de lokale T cellen in kaart gebracht door middel van T cel receptor sequencing. De T cel receptor zit op iedere T cel maar is uniek per gemaakte T cel. Deze receptor is verantwoordelijk voor de specifieke herkenning van zijn doelwit cel. Door het repertoire van T cel receptoren in kaart te brengen kunnen we inzicht krijgen in de dynamiek van de T cel respons. Dit onderzoek is uitgevoerd op cellen uit ontstekingsvocht wat gelijktijdig van beide knieën verkregen is, en van ontstekingsvocht wat meerdere keren over de tijd verkregen is van

dezelfde patiënten. Hierdoor hebben we nieuwe, fundamentele inzichten gekregen in de specifieke T cel responses die in het aangedane gewricht plaatsvinden. Vooral van de Treg zijn veel cellen met dezelfde T cel receptor aanwezig in het ontstoken gewricht. Ook zijn dezelfde T cellen aanwezig en blijkt de samenstelling van immuun cellen bijna identiek in de verschillende aangedane gewrichten. Daarbij meten we dezelfde specifieke T cellen in synoviaal vocht tijdens ziekte recidieven, ook wanneer deze over langere tijd van meer dan een jaar plaatsvinden. Deze data wijzen erop dat er een specifiek doelwit in de gewrichten aanwezig is waarop dezelfde T cellen, beide Treg en T helper cellen, reageren tijdens het ontstekingsproces. De T cellen die in grote mate aanwezig zijn in het ontstoken gewricht zijn ook terug te vinden in het bloed. Mogelijk kan het meten van deze ziekte specifieke T cellen in bloed nuttig zijn om het ziekteproces goed te volgen en er een gericht behandelplan op toe te passen. Vervolg studies zijn nodig om dit beter uit te zoeken.

In **hoofdstuk 7** wordt de recente literatuur over lokale T cel aanpassing aan ontstekingen bediscussieerd, in reumatische aandoeningen bij kinderen. Er zijn de laatste jaren zijn veel nieuwe inzichten over T cel specialisatie, onder andere door nieuwe technieken. Daardoor weten we nu dat T cellen zich specialiseren zodra ze zich in een weefsel begeven, met name Treg, en dat dit nodig is voor deze cellen om lokaal goed te kunnen functioneren. Er is nog weinig bekend over hoe deze aanpassing exact in zijn werk gaat, vooral tijdens ziekte, zoals ontstekingen.

Samenvattend heeft het onderzoek in dit proefschrift nieuwe inzichten gegeven over hoe T cellen zich gedragen en geprogrammeerd zijn in auto-immuun ontstekingen bij mensen. Daarmee hebben we nieuwe mechanismes ontdekt over hoe dit deel van het menselijke immuunsysteem werkt tijdens deze ontstekingen, en wat relevante cellen of processen zijn om aan te pakken met een behandeling. Verder onderzoek zal nuttig en nodig zijn om deze ontdekkingen te vertalen naar een (aangepaste) behandeling van patiënten.

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Daarna kwam er de Van Wijk groep; wat fijn om zulke leuke collega's te treffen. Arjan and Alessandra, we ruled as SF Kings and Queens! Different personalities with shared projects, and it worked out very well. Arjan, we hebben veel samengewerkt en zelfs samen 9 maanden een student begeleid; dank dat je altijd bereid was om te helpen, relaxed blijft, en fijn dat we ook over niet werk-gerelateerde dingen goed kunnen praten. Ale, thanks for all the fun we had in the lab during endless SF isolations and sorting sessions, and of course thanks for your input into our projects. Your drive and perseverance has already taken you far, I'm impressed. Judith, jij ook dank voor alle (SF) hulp in het lab en jouw inbreng tijdens meetings, en voor de nodige PhD frustratie-deel momentjes. Knap hoe je er vol voor gaat, succes met de laatste maanden! Theo, fijn om jou mijn eerste jaren als collega gehad te hebben, wat is er bij jou veel veranderd sindsdien; binnenkort nog een grote verandering hoorde ik, gefeliciteerd! Eveline, ik weet onze eerste 'samenwerking' nog goed; samen FACS antilichamen titreren, ik tijdens mijn stage en jij als beginnende PhD in het lab. Wat gezellig dat we daarna telkens weer samen optrokken, ook als kamergenootjes. Dank voor alle gezelligheid, goede gesprekken en samenwerkingen. Yvonne, heel leuk om ook weer met jou samen te werken! Eelco, knap hoe jij je vol enthousiasme op je PhD stort; de sort-nachten lijken bijna verleden tijd, succes met alles! Lisanne, ik zal jouw Antwerpen-presentatie avontuur niet snel vergeten… veel dank nog daarvoor, ik sta nog steeds bij je in het krijt. En heel veel succes met de rest van je PhD, dat gaat helemaal goedkomen. Jose, leuk dat je bij onze groep gekomen bent, een waardevolle aanvulling, heel veel succes met de iDisco en andere projecten. Marlot wat een topper ben jij! Een hele aanwinst dat jij bij onze groep gekomen bent. Je bent een harde werker, verantwoordelijk, enthousiast en heel sociaal. Daarbij is het onmogelijk om niet in de lach te schieten als jij heel beeldend een verhaal verteld. Ik vond het fijn om samen met jou de 'rebuttal' experimenten op te pakken, door jouw enthousiasme zag ik het telkens toch wel weer zitten. Ik ben heel blij dat jij mijn paranimf wil zijn. En ik hoop je landhuis (met gracht) binnenkort echt een keer te bewonderen.

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Dit boekje is tot stand gekomen door meerder samenwerkingen. T cellen sorteren is bijna core business geweest van mijn boekje; dank Pien en Jeroen voor jullie hulp! Ook dank aan de luminex facility; o.a. Rianne, Mariska en Wilco. Michal, your contribution has been essential, thanks for all your help and advice, and for tolerating my 'spam'. Aridaman, your enthusiasm for the TCR data have led to a nice collaboration that really helped getting more knowledge out of the data; thanks for all your help and for answering all my questions again and again.

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CURRICULUM VITAE

Gerdien Mijnheer was born on January 26th 1986 in Zwolle, the Netherlands. In 2004 she completed secondary education at CSG Dingstede in Meppel. The same year she started her study BioMedical Sciences at Utrecht University where she received her Bachelor degree in 2009. She subsequently started her master study, track Infection and Immunity, at Utrecht University. During a research internship in the lab of Prof. Dr. Berent Prakken (Pediatric Immunology department, Wihelmina Kinderziekenhuis Utrecht) she developed her fascination for translational research. There she studied T cell regulation in synovial fluid of Juvenile Idiopathic Arthritis patients. In the lab of Prof. Dr. Jos van Strijp at the Medical Microbiology department, UMC Utrecht, she performed an internship where she studied immune evasion mechanisms of *Staphylococcus aureus*. Gerdien completed her master's degree (cum laude) in 2011. The same year she started her PhD under supervision of Dr. Femke van Wijk and Prof. Dr. Berent Prakken (Laboratory of Translational Immunology, UMC Utrecht) of which the results are presented in this thesis. Gerdien currently works as junior PhD coordinator of the Infection & Immunity program of the Graduate School of Life Sciences at Utrecht University. Gerdien is married to Eddy Voogd and they have two sons: Nathan (2011) and Matthijs (2014).

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