Involvement of microRNAs in T-cell immunity
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Document Version
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Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5

Locally expanding Treg cells display a Th1 like chemokine receptor pattern in rheumatoid joints

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Manuscript submitted
ABSTRACT

Introduction: Regulatory T (T_{reg}) cells are known to accumulate in inflamed joints of rheumatoid arthritis (RA) patients, but little is known about the mechanisms behind this process. Therefore, we performed a comprehensive study of the chemokine receptors, proliferative activity and apoptosis of T_{reg} cells in peripheral blood (PB) and synovial fluid (SF) of RA patients.

Methods: We analyzed peripheral blood samples of RA patients and age-matched healthy controls (HCs), and paired synovial fluid samples of RA patients. Naive T_{reg} cells (FOXP3^{low}CD45RA+), memory T_{reg} cells (FOXP3^{high}CD45RA-), naive conventional T (T_{conv}) cells (FOXP3-CD45RA+) and memory T_{conv} cells FOXP3^{low/CD45RA-}) were enumerated by flow cytometry. T_{reg} cells were assessed for expression of CXCR3, CCR4 and CCR6, the proliferation marker Ki-67 and for binding of the apoptosis marker annexin-V. Sorted SF T_{reg} cells were analyzed for expression of the anti-apoptotic factor Bcl-2 and anti-apoptotic/pro-proliferation microRNA-21 by RT-PCR.

Results: Numbers of circulating memory T_{reg} cells and memory T_{conv} cells were decreased in RA patients when compared to HCs. RA patients had normal numbers of circulating naive T_{reg} cells and increased numbers of naive T_{conv} cells. Total memory CD4+ T cells, and memory T_{reg} cells in particular, were increased in RA SF, whereas naive T_{reg} cells and naive T_{conv} cells were hardly detected. Memory T_{reg} cells expressed similar chemokine receptors as memory T_{conv} cells in PB and SF. Overall, all memory subsets in RA were characterized by a predominance of a Th1-like chemokine receptor pattern over a Th17/Th22 pattern. SF T_{reg} cells more frequently expressed the proliferation marker Ki-67 than memory T_{conv} cells, whereas only few annexin-V+ apoptotic cells were found in RA SF. In accordance with these findings, SF T_{reg} cells displayed high levels of Bcl-2 and microRNA-21.

Conclusions: Memory T_{reg} cells mimicking the chemokine receptor pattern of memory T_{conv} cells accumulate in rheumatoid joints. Here, memory T_{reg} cells thrive well, as indicated by their substantial proliferation and survival. The latter findings were consistent with the high expression of anti-apoptotic Bcl-2 and anti-apoptotic/pro-proliferative microRNA-21 by SF T_{reg} cells. Taken together, these data provide novel insights into the mechanism behind T_{reg} cell accumulation in rheumatoid joints.
INTRODUCTION

Rheumatoid arthritis (RA) is a common auto-immune disease characterized by chronic synovial inflammation that ultimately leads to destruction of affected joints. The synovial inflammation in RA is mediated and maintained by a multitude of immune cells, such as T cells, macrophages and fibroblast-like synoviocytes [1]. Several studies have suggested that the persistence of synovial inflammation may reflect a poor suppressive effect of regulatory T (T\textsubscript{reg}) cells on conventional T (T\textsubscript{conv}) cells [2-5]. Both functional defects of T\textsubscript{reg} cells and resistance of T\textsubscript{conv} cells to T\textsubscript{reg} cell-mediated suppression have been implied in the pathogenesis of RA [3-5].

Studies on circulating numbers of T\textsubscript{reg} cells in RA patients have generated conflicting results. The majority of studies found decreased or normal proportions of circulating T\textsubscript{reg} cells in RA patients, whereas a minority found increased proportions [2, 3, 6-10]. Part of the discrepancy may result from differences in flow cytometric gating strategies applied in these studies. Importantly, Miyara et al. have shown in a convincing way that analysis of FOXP3 in combination with CD45RA allows reliable identification of CD45RA+FOXP3\textsuperscript{low} naive T\textsubscript{reg} cells (i.e. resting T\textsubscript{reg} cells) and CD45RA-FOXP3\textsuperscript{high} memory T\textsubscript{reg} cells (i.e. activated T\textsubscript{reg} cells) [11, 12]. This gating strategy allows for the exclusion of FOXP3\textsuperscript{low} conventional memory CD4+ T cells capable of producing IL-17. Using this method, Kim et al. recently found decreased numbers of memory T\textsubscript{reg} cells in peripheral blood of RA patients, whereas naive T\textsubscript{reg} cell numbers were not modulated [13].

In contrast to findings in peripheral blood, there seems to be general agreement that T\textsubscript{reg} cells accumulate in the synovial fluid of RA patients [2, 6-8]. Previously, Cao et al. have proposed that T\textsubscript{reg} cells are decreased in peripheral blood of RA patients, because these cells migrate towards rheumatoid joints [6]. However, little is known about the chemokine receptors that allow T\textsubscript{reg} cells to migrate into rheumatoid joints. Recent data indicate that T\textsubscript{reg} cells can follow T\textsubscript{conv} cells into the tissues by mimicking the chemokine receptor pattern of T\textsubscript{conv} cells [14]. T\textsubscript{reg} cells with chemokine receptors characteristic for T helper (Th) 1 cells (CXCR3+), Th2 cells (CCR4+CCR6-CXCR3-) and Th17/Th22 cells (CCR4+CCR6+CXCR3-) have been identified [14]. Whether T\textsubscript{reg} cells have a similar chemokine receptor pattern as T\textsubscript{conv} cells in rheumatoid joints remains to be elucidated.

Following migration, the accumulation of T\textsubscript{reg} cells in synovial fluid of RA patients may further depend on their proliferative activity and apoptosis rate. Limited data exist on proliferation of T\textsubscript{reg} cells in rheumatoid joints. In one study, Herrath et al. found that many FOXP3+ T\textsubscript{reg} cells in RA synovial fluid express the proliferation marker Ki-67 [4]. In addition, it has been shown that synovial T cells are resistant to apoptosis and express high levels of the anti-apoptotic factor Bcl-2 [15-17]. To what extent T\textsubscript{reg} cells in rheumatoid joints are refractory to apoptosis and express Bcl-2 remains unknown.
Emerging data indicate that proliferation and cellular survival of T cells are influenced by epigenetic regulation through microRNAs. MicroRNAs are non-coding RNAs that regulate the translation of proteins by silencing of messenger RNA. A microRNA that has been implied as an important regulator of proliferation and apoptosis is microRNA-21. Although microRNA-21 has been mostly studied in cancer cells, we and others have described the pro-survival and proliferation-supportive effects of this microRNA in T cells [18-20]. So far, the expression of microRNA-21 has not been studied in Treg cells of RA patients.

In the current study, we sought to mechanistically explain the accumulation of Treg cells in rheumatoid joints. We hypothesized that Treg cells home towards RA synovial fluid through similar chemokine receptors as conventional CD4+ T cells and further expand locally. We first assessed the presence of naive and memory Treg cells in synovial fluid of RA patients with active disease. Next, we determined whether the chemokine receptor pattern of synovial fluid Treg cells converges with the typical pattern of Th1, Th2 or Th17/Th22 cells. Subsequently, we analyzed synovial fluid Treg cells for their proliferative activity and the percentage of apoptotic cells. Finally, we studied the expression of the anti-apoptotic factor Bcl-2 and anti-apoptotic/pro-proliferative microRNA-21 in synovial fluid Treg cells.

RESULTS

Decreased proportions of memory Treg cells in peripheral blood of RA patients

To identify naive and memory Treg cells in peripheral blood and synovial fluid, we used a combination of the markers CD45RA and FOXP3 (Fig.1A and B). The proportions of circulating naive Treg cells were similar in RA patients and healthy controls (Fig.1C). In contrast, the proportions of naive CD4+ T conventional (Tconv) cells were increased in RA patients, as previously observed by us (Chalan et al., PlosONE, in press). The proportions of circulating memory Treg cells, FOXP3low and FOXP3- memory Tconv cells were all decreased in RA patients. Results were quite similar for the absolute numbers of the five CD45RA and FOXP3 defined CD4+ T cell subsets, although only the decrease of memory Treg cells reached statistical significance (Suppl.Fig.1). In the synovial fluid, naive Treg cells were absent and naive Tconv cells nearly absent (Fig.1C). In contrast, memory Treg cells, FOXP3low and FOXP3- memory Tconv cells were abundant in the synovial fluid of RA patients. Although the proportions of circulating Treg cells in RA patients were modestly decreased in comparison to FOXP3low and FOXP3- memory Tconv cells (Fig.1C), proportions of memory Treg cells were relatively more increased in the synovial fluid than proportions of the two memory Tconv cell subsets (Fig.1D).
Locally expanding Treg cells have a Th1 like chemokine receptor pattern in rheumatoid joints.

Figure 1. Flow cytometric analysis of naive and memory Treg cells. (A) Gating strategy to identify five FOXP3/CD45RA-defined CD4+ T cell subsets. (B) Representative flow cytometric staining and gating strategy for five FOXP3/CD45RA-defined subsets in peripheral blood of one healthy control and one RA patient, and in paired synovial fluid of the same RA patient. (C) Proportions of FOXP3/CD45RA defined subsets within total CD4+ T cells in peripheral blood of healthy controls (HC PB) and RA patients (RA PB) and in RA synovial fluid (RA SF). (D) Fold increase in proportions of FOXP3/CD45RA defined subsets within CD4+ T cells in synovial fluid compared to peripheral blood of RA patients. Statistical significance is indicated as * p < 0.05, ** p ≤ 0.01, *** p ≤ 0.001. Mem = memory. T_{reg} = regulatory T cells. T_{conv} = conventional CD4+ T cells.
Figure 2. Chemokine receptor expression by memory T\textsubscript{reg} cells. (A) Representative flow cytometric staining for CXCR3, CCR4 and CCR6 expression by CD4\textsuperscript{+} T cells in peripheral blood (PB) and synovial fluid (SF) of one RA patient. (B) Expression of CXCR3, CCR4 and CCR6 by memory T\textsubscript{reg} cells, memory FOXP3\textsuperscript{low} T\textsubscript{conv} cells and memory FOXP3\textsuperscript{-} T\textsubscript{conv} cells in peripheral blood and synovial fluid of 6 RA patients. Cells with a Th1-like chemokine receptor pattern (CXCR3\textsuperscript{+}), a Th2-like chemokine receptor pattern (CCR4\textsuperscript{+}CCR6\textsuperscript{-}CXCR3\textsuperscript{-}) and Th17/Th22-like chemokine receptor pattern (CCR4\textsuperscript{+}CCR6\textsuperscript{+}CXCR3\textsuperscript{-}) can be distinguished. Box and bars represent mean and SEM. Statistical significance is indicated as * p < 0.05. T\textsubscript{reg} = regulatory T cells. T\textsubscript{conv} = conventional CD4\textsuperscript{+} T cells.

High proportions of memory Treg cells associated with milder disease activity

As T\textsubscript{reg} cells typically ameliorate inflammation, we questioned whether lower T\textsubscript{reg} cell frequencies in peripheral blood or synovial fluid were associated with more inflammation or disease activity in RA patients. The proportion of circulating naive T\textsubscript{reg} cells did not correlate with inflammatory markers ESR and CRP, nor with the DAS28...
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Locally expanding Treg cells have a Th1 like chemokine receptor pattern in rheumatoid joints. In contrast, frequencies of circulating memory T\textsubscript{reg} cells correlated inversely with ESR (Spearman r = -0.40, p<0.05) and CRP (Spearman r=-0.53, p<0.01). Likewise, the proportions of memory T\textsubscript{reg} cells in RA synovial fluid correlated inversely with ESR (Spearman’s rho -0.79, p = 0.028) and DAS28 score (Spearman’s rho -0.79, p = 0.048). Thus, relatively high numbers of memory T\textsubscript{reg} cells, both in peripheral blood and synovial fluid, were associated with less severe disease activity in RA patients.

Memory Treg and T\textsubscript{conv} cells express similar chemokine receptors in RA synovial fluid

Since our data indicated that memory T\textsubscript{reg} cells home towards rheumatoid joints along with memory T\textsubscript{conv} cells, we questioned whether memory T\textsubscript{reg} cells in synovial fluid express a similar chemokine receptor pattern as memory T\textsubscript{conv} cells. Based on CXCR3, CCR4 and CCR6 expression (Fig.2A) we distinguished cells with a Th1-like pattern (CXCR3+), Th2-like pattern (CCR4+CCR6-CXCR3-) and a Th17/Th22-like pattern (CCR4+CCR6+CXCR3-) [14]. Overall, memory T\textsubscript{reg} cells in synovial fluid expressed the same chemokine receptors as memory T\textsubscript{conv} cells (Fig.2B), although the number of combinations used was more limited in the memory T\textsubscript{reg} cell fraction (Suppl.Fig.2A). Naive T\textsubscript{reg} cells and naive T\textsubscript{conv} cells hardly expressed CXCR3, CCR4 and CCR6 (Suppl. Fig.2B). Interestingly, cells with a Th17/Th22-like pattern were decreased in all three memory subsets (Fig. 2B). This decrease coincided with a general increase in cells with a Th1-like chemokine receptor pattern, albeit statistically significant in memory T\textsubscript{reg} cells only. In addition, a substantial part of all memory subsets had a Th2-like chemokine receptor pattern, which did not differ between periperhal blood and synovial fluid. Taken together, synovial fluid memory T\textsubscript{reg} cells expressed relatively similar chemokine receptors as memory T\textsubscript{conv} cells with predominance of a Th1-like chemokine pattern over a Th17/Th22 pattern.

Peripheral blood and synovial fluid memory Treg cells have a high proliferative activity

Since our data showed the most pronounced increase of memory T\textsubscript{reg} cells in rheumatoid joints, we compared the proliferative activity of memory T\textsubscript{reg} cells and memory T\textsubscript{conv} cells in the synovial fluid. The percentage of cells expressing the proliferation marker Ki-67 was the highest in memory T\textsubscript{reg} cells, both in the peripheral blood and synovial fluid (Fig.3A and B). Although FOXP3\textsubscript{low} and FOXP3- memory T\textsubscript{conv} cells showed a significant increase in Ki-67+ cells in the synovial fluid, the overall percentage of Ki-67+ was still lower as compared to memory T\textsubscript{reg} cells.
Figure 3. Proliferation and apoptosis of T<sub>reg</sub> cells in RA patients. (A) Representative flow cytometric staining for Ki-67 in peripheral blood (PB) and synovial fluid (SF) CD4 T cells of one RA patient. (B) Percentages of FOXP3/CD45RA defined CD4<sup>+</sup> T cell subsets expressing the proliferation marker Ki-67 in paired peripheral blood and synovial fluid samples of 6 RA patients. (C) Representative annexin-V staining in peripheral blood and synovial fluid CD4 T cells of one RA patient (D) Percentages of FOXP3/CD45RA defined CD4<sup>+</sup> T cell subsets binding the apoptosis marker annexin-V in paired peripheral blood and synovial fluid samples of 7 RA patients. No data are shown for synovial fluid naive T<sub>reg</sub> cells, as these cells were not detected. Statistical significance is indicated as * p < 0.05. Mem = memory. T<sub>reg</sub> = regulatory T cells. T<sub>conv</sub> = conventional CD4<sup>+</sup> T cells.

Synovial fluid memory Treg cells display limited apoptosis

The accumulation of memory T<sub>reg</sub> cells in rheumatoid joints is also influenced by local apoptosis. To analyze apoptosis of memory T<sub>reg</sub> cells in rheumatoid joints, we analyzed the proportions of annexin-V<sup>+</sup> cells in peripheral blood and synovial fluid of RA patients (Fig.3C). In essence, very few apoptotic cells were found in the peripheral blood and synovial fluid of RA patients. The proportion of apoptotic cells in peripheral blood tended to be the highest among memory T<sub>reg</sub> cells (Fig.3D). We observed slightly increased apoptosis of FOXP3<sup>+</sup> memory T<sub>conv</sub> cells and naive T<sub>conv</sub> cells in synovial fluid when compared to peripheral blood. Overall, apoptosis of memory T<sub>reg</sub> cells was limited and similar to apoptosis of memory T<sub>conv</sub> cells in the synovial fluid.

Synovial fluid Treg cells express high levels of Bcl-2 and microRNA-21

Since several lines of evidence have indicated that T<sub>reg</sub> cells are more susceptible to apoptosis than T<sub>conv</sub> cells in inflammatory environments [22, 23], we questioned why synovial fluid T<sub>reg</sub> cells are relatively refractory to apoptosis. Therefore, we assessed synovial fluid T<sub>reg</sub> cells for expression of the anti-apoptotic factors Bcl-2 and microRNA-21 [18, 19].
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Figure 4. Expression of Bcl-2 and microRNA-21 by synovial fluid Treg cells. (A) Relative expression of FOXP3, (B) microRNA-146a, (C) Bcl-2 and (D) microRNA-21 in CD25<sup>high</sup> T<sub>reg</sub> cells and CD25<sup>-</sup> T<sub>conv</sub> cells from synovial fluid of 6 RA patients, as determined by qRT-PCR. MicroRNAs and Bcl-2 expression was normalised to RNU48 and TBP endogenous controls respectively using the ΔΔC<sub>t</sub> method. Statistical significance is indicated as * p < 0.05. T<sub>reg</sub> = regulatory T cells. T<sub>conv</sub> = conventional CD4<sup>+</sup> T cells.

As intracellular FOXP3 detection requires fixation of the cells, sorting of T<sub>reg</sub> cells requires the use of T<sub>reg</sub> cell surface markers. As previously shown for peripheral blood [11], we found that memory T<sub>reg</sub> cells exhibited the highest expression levels of CD25 in synovial fluid of RA patients (Suppl.Fig.3). As naive T<sub>reg</sub> cells were absent from synovial fluid, we relied on high CD25 expression to discriminate memory T<sub>reg</sub> cells from T<sub>conv</sub> cells in synovial fluid. We confirmed that sorted CD25<sup>high</sup> T<sub>reg</sub> cells from synovial fluid expressed high levels of FOXP3 and microRNA-146a, a microRNA highly expressed by T<sub>reg</sub> cells [24] (Fig.4A-B). Interestingly, we observed that CD25<sup>high</sup> T<sub>reg</sub> cells in synovial fluid contained significantly higher levels of Bcl-2 and micro-RNA-21 than T<sub>conv</sub> cells (Fig. 4C and D).
DISCUSSION

In the current study, we investigated the mechanisms leading to accumulation of T<sub>reg</sub> cells in rheumatoid joints. We found that memory T<sub>reg</sub> cells and T<sub>conv</sub> cells share a similar chemokine receptor pattern and accumulate in RA SF. We also observed that memory T<sub>reg</sub> cells increase relatively more in RA synovial fluid than memory T<sub>conv</sub> cells. This enhanced accumulation of T<sub>reg</sub> cells in SF could be explained by their high proliferative activity and limited apoptosis, which were consistent with high expression levels of Bcl-2 and microRNA-21.

We observed decreased numbers of memory T<sub>reg</sub> cells in the peripheral blood of RA patients, whereas numbers of naive T<sub>reg</sub> cells were not modulated. In accordance with other reports, memory T<sub>reg</sub> cells were characterized by high CD25 expression. Consequently, our results were in agreement with previous studies on CD25<sup>high</sup> T<sub>reg</sub> cells in RA [6, 8, 9]. Recently, Kim et al. applied a similar gating strategy to ours and also found decreased numbers of memory T<sub>reg</sub> cells and normal numbers of naive T<sub>reg</sub> cells in peripheral blood of RA patients [13]. The decrease in memory T<sub>reg</sub> cells and memory T<sub>conv</sub> cells in peripheral blood of RA patients was paralleled by accumulation of these cells in the synovial fluid. Previously, Cao et al. have proposed that the decreased numbers of circulating T<sub>reg</sub> cells may result from their migration into rheumatoid joints [6]. Little is known, however, about the chemokine receptors that would direct T<sub>reg</sub> cells into rheumatoid joints. Ruth et al. have shown that CXCR3 and CCR4 may be important for the migration of T<sub>conv</sub> cell into rheumatoid joints [25]. We found that synovial fluid memory T<sub>reg</sub> cells express similar chemokine receptors as memory T<sub>conv</sub> cells. Our findings therefore support a recently reported concept that T<sub>reg</sub> cells follow T<sub>conv</sub> cells into the tissues by means of a similar chemokine receptor pattern [14].

Overall, we noted a preponderance of a Th1 chemokine receptor pattern over a Th17/Th22 pattern in all synovial fluid memory subsets. This finding could indicate that circulating memory T<sub>reg</sub> cells and T<sub>conv</sub> cells with a Th1-like chemokine receptor pattern are more likely to migrate into rheumatoid joints than cells with a Th17-like pattern. Indeed, previous studies indicate that IFN-γ expressing Th1 cells and not IL-17 expressing Th17 cells are decreased in the peripheral blood of RA patient, whereas Th1 cells are much more abundant than Th17 cells in RA synovial fluid [26, 27]. It is also possible that T<sub>conv</sub> cells in rheumatoid joints shift from a Th17 phenotype towards a Th1 phenotype, as previously reported by Cosmi et al. [28]. Whether memory T<sub>reg</sub> cells in rheumatoid joints can exchange a Th17/Th22-like chemokine receptor pattern for a Th1-like pattern remains to be elucidated.

Despite their expression of CXCR3 and CCR4, it is unlikely that T<sub>reg</sub> cells actually produce effector cytokines of T helper cells in rheumatoid joints. Although Walter et al.
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recently demonstrated that PB-derived Treg cells can produce effector cytokines following in vitro culture with monocytes, Herrath et al. did not observe significant production of effector cytokines by RA SF Treg cells of RA patients [4, 29]. Even if Treg cells would locally acquire the capacity to produce effector cytokines, such as IFN-γ or IL-17, recent data indicate that these Treg cells would still retain their suppressive function [29]. In accordance with the notion that Treg cells contribute to tolerance in RA patients, we found that relative high numbers of memory Treg cells numbers in PB and SF are associated with less severe inflammation/disease activity in RA patients.

Unexpectedly, we found that memory Treg cells are relatively more increased in RA synovial fluid than memory Tconv cells. This relatively outspoken enrichment of RA synovial fluid with memory Treg cells was not paralleled by a more pronounced decrease of memory Treg cells in peripheral blood. Although we cannot exclude that some locally-induced Treg cells are present in rheumatoid joints, our findings indicated that memory Treg cells in synovial fluid expand locally to a higher extent than the memory Tconv cells. Indeed, we observed that memory Treg cells have the highest proliferative activity of all memory subsets in the synovial fluid of RA patients. Our findings are in accordance with a recent study showing that synovial fluid FOXP3+ Treg cells have the highest proliferation rate among CD4+ T cells in rheumatoid joints [4].

We also found few apoptotic memory Treg cells in RA SF. Although resistance to apoptosis of Tconv cells in rheumatoid joints has been reported by others [16, 17, 30], little is known about apoptosis of Treg cells in rheumatoid joints. In one in vitro study, Treg cells derived from peripheral blood of RA patients demonstrated enhanced apoptosis in 48-hour cultures [31]. These in vitro apoptosis rates, however, do not necessarily correlate with the in vivo apoptosis rates of Treg cells in RA patients. In fact, our direct ex vivo data indicate that apoptosis of memory Treg cells in peripheral blood and joints of RA patients is very limited. This finding is in line with other studies showing a prominent role of resistance to apoptosis in the pathogenesis of RA [16].

We also showed that synovial fluid Treg cells express high levels of the anti-apoptotic factor Bcl-2. Previously, Firestein et al. found high expression of Bcl-2 in synovial T cells [15], but so far expression of this anti-apoptotic factor in synovial fluid Treg cells has not been studied. It is well established that Bcl-2 prevents T cell apoptosis by blocking the intrinsic apoptosis pathway [32, 33]. Bcl-2 is typically upregulated by common γ chain signalling cytokines, such as IL-2, IL-7 and IL-15 [34, 35]. IL-2 and IL-7 are unlikely to be important for Bcl-2 upregulation in synovial fluid Treg cells, as IL-2 is hardly present in synovial fluid and synovial fluid Treg cells lack the IL-7Ra chain [36, 37]. Soluble IL-15, however, is present in rheumatoid joints [37, 38] and fibroblast-like synoviocytes in rheumatoid joints express membrane-bound IL-15, which can stimulate Treg cells [39]. As Treg cells express the highest
levels of the IL-2/15Rβ chain and IL-15Rα chain, they are also more sensitive to IL-15 stimulation than other CD4+ T cells [39]. Taken together, it is likely that IL-15 contributes to the upregulation of bcl-2 in synovial fluid T\textsubscript{reg} cells.

Synovial fluid T\textsubscript{reg} cells also expressed high levels of microRNA-21. MicroRNAs provide a layer of post-transcriptional regulation of gene expression in almost every human cell type by preventing translation of their target messenger RNA [24, 40]. The oncogenic effect of microRNA-21 has been established in many types of cancer [41]. In addition, microRNA-21 can prevent apoptosis and promote proliferation in T cells [18-20]. These regulatory effects of microRNA-21 may result partly from its silencing of transcripts of the tumor suppressor gene PTEN and PDCD4 [42, 43]. The high expression of microRNA-21 by T\textsubscript{reg} cells in RA SF was consistent with the limited apoptosis and high proliferative activity of these cells.

As the data obtained here provide additional insight into the trafficking of T\textsubscript{reg} cells into rheumatoid joints, as well as the local survival of these cells, our study may be relevant for future studies on T\textsubscript{reg} cell therapy in RA patients. So far, T\textsubscript{reg} cell therapy has been successfully applied in animal models of arthritis [44, 45]. Our data suggest that T\textsubscript{reg} cells with a Th1-like chemokine receptor pattern are more likely to end up in inflamed joints than T\textsubscript{reg} cells with a Th17/Th22-like chemokine receptor pattern. Furthermore, local survival of therapeutic T\textsubscript{reg} cells is unlikely a major concern, as T\textsubscript{reg} cells are actually expanding in rheumatoid joints.

**METHODS**

**Study population**

Samples were obtained from 3 cohorts of RA patients fulfilling the ACR 1987 criteria. RA cohort 1 consisted of 29 Korean RA patients who donated peripheral blood samples that were compared to peripheral blood samples of 39 Korean healthy controls (Table 1). From 9 RA patients of cohort 1 we also collected paired synovial fluid samples. Samples of cohort 1 were used to enumerate the numbers of naive and memory T\textsubscript{reg} cells. RA cohort 2 consisted of 6 additional Korean RA patients who donated peripheral blood and synovial fluid samples (supplemental table 1). Samples of cohort 2 were used to assess the chemokine receptor expression of T\textsubscript{reg} cells. RA cohort 3 consisted of 11 Dutch RA patients donating peripheral blood and synovial fluid samples (supplemental table 1). Samples of cohort 3 were used to assess proliferation and apoptosis of T\textsubscript{reg} cells and to study Bcl-2 and microRNA expression by T\textsubscript{reg} cells. All patients studied had active disease according to DAS28 score (> 2.6) and/or clinical expertise of an experienced rheumatologist. Patients with active infection, cancer or other rheumatologic diseases were excluded.
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The study was approved by the institutional review boards of Seoul National University Hospital and the University Medical Center Groningen and informed consent was obtained from all subjects.

Table 1. Patients characteristics

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All study subjects mentioned here were recruited in Korea. Data obtained from these study subjects is depicted in figure 1 and figure 2. No. = number. ESR = erythrocyte sedimentation rate. CRP = C-reactive protein. Anti-CCP antibodies = anti-cyclic citrullinated peptide antibodies. RF = rheumatoid factor.

Antibodies

The following anti-human monoclonal antibodies were purchased from eBioscience (San Diego, USA): FOXP3-APC [PCH101], CD4-PE-Cy7, CD4-efluor450, CD25-PE, CD45RA-efluor605 and CD45RA-PerCP-Cy5.5. Although anti-FOXP3 clone PCH101 was initially suspected of non-specific binding to activated cells, this notion appeared to be incorrect and the validity of anti-FOXP3 clone PCH101 was confirmed [21]. Anti-human CD3-APC-Cy7, CD4-APC-H7, CD8-PE-Cy7, Ki-67-PerCP-Cy5.5 and active caspase-3-PE were obtained from BD Biosciences (San Diego, USA). CXCR3-PE, CCR4-PE-Cy7 and CCR6-AF488 were purchased from Biolegend (San Diego, USA). CD4-FITC and CD25-PE were purchased from IQ Products (Groningen, The Netherlands). Annexin-V-FITC was purchased from Immunotools (Friesoythe, Germany). Isotype controls served as negative controls.
Cell isolation and flow cytometry

Mononuclear cells were isolated from peripheral blood and synovial fluid with Ficoll-Paque Plus (GE Healthcare Biosciences AB, Uppsala, Sweden) or Lymphoprep (Axis-Shield, Oslo, Norway). After surface staining, cells were fixed and permeabilized with a FOXP3 Staining Buffer Set (eBioscience). In some experiments, annexin-V staining was performed prior to fixation, as previously described [22]. Following fixation and permeabilization, non-specific binding was blocked with normal rat serum (eBioscience) and the cells were stained for FOXP3 and Ki-67. Finally, cells were washed and immediately analyzed on a FACS Canto II or LSR II Flow Cytometer (BD Biosciences). Data were typically collected for at least 100,000 cells and plotted using Flowjo software 7.5 (Treestar, Ashland, USA) and Kaluza Flow Analysis Software (Beckman Coulter, Woerden, Netherlands). Absolute numbers of CD4+ T cells were calculated by using percentages of CD3+CD4+ T cells within the lymphocyte gate in addition to data from total and differential white blood cell counts.

RNA isolation and qRT-PCR

For qRT-PCR analysis, CD4+CD25$_{\text{high}}$ T$_{\text{reg}}$ cells and CD4+CD25- conventional T cells were sorted by fluorescence-activated cell sorting (FACS) (MoFlo, DaKo Cytomation). Cells were directly lysed with the Qiazol reagent and total cellular RNA was extracted using the miRNeasy Mini Kit (both from Qiagen, Venlo, The Netherlands) following manufacturer's instructions. The RNA quantity was measured on NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). For microRNA-specific cDNA synthesis, RNA was reverse transcribed using the Taqman MicroRNA Reverse Transcription kit in combination with multiplexed reverse transcription primers of TaqMan microRNA Assays (Life Technologies, Carlsbad, USA) for microRNA-21 (ID: 000397), microRNA-146a (ID: 000468) and RNU48 (ID: 001006). The qPCR reaction was performed using qPCR MasterMix Plus (Eurogentec, Liege, Belgium).

The cDNA synthesis for mRNA was performed using Superscript III RTase (Life Technologies). The qPCR detection of FOXP3, Bcl-2 and TBP was performed using the qPCR MasterMix Plus (Eurogentec), Taqman Gene expression assay for FOXP3 (ID: Hs 01085834_m1) (Life Technologies), and gene-specific primers and probe (Integrated DNA Technologies, Coralville, USA) for detection of TBP: forward 5’-GCCCGAAACGCGGAATAT-3’, reverse 5’-CCGTTGCTTGCTGCTCTCT-3’ and probe 5’-6-FAM-ATCCCAAGCGGTGCTGCTGCGG-BHQ-1-3’; or with SYBR green PCR MasterMix (Life Technologies), and gene specific primers, for detection of Bcl-2: 5’-TCGCCCTGTTGATGACTGA-3’.
Locally expanding Treg cells have a Th1 like chemokine receptor pattern in rheumatoid joints

(forward) and 5'-CAGAGACAGCCAGGAGAAATCA-3'(reverse), and TBP: 5’-GCCCGAAACGCCGAATAT-3’ (forward), 5’-CCGTGGTTCTGGCTCTCT-3’ (reverse). Mean cycle threshold (Ct) values for all genes were quantified with the Sequence Detection Software (SDS, version 2.3), using ABI7900HT thermo cycler (both from Life Technologies). All reactions were run in triplicate. RNU48 served as endogenous control for microRNA-21 and microRNA-146a, while TBP served as endogenous control for FOXP3 and Bcl-2. Relative expression levels were determined using the ΔΔCt method.

**Statistical analysis**

Statistics were done with Graphpad Prism 5.3 for Windows. Data are presented as median with range unless otherwise stated. Non-paired samples were compared with the Mann-Whitney U test and paired samples with the Wilcoxon signed-rank test. Correlations were determined with the Spearman rank test. Two-sided p-values of less than 0.05 were considered statistically significant.

**List of abbreviations**


**Acknowledgement**

We thank all the patients and healthy volunteers who participated in this study.
Supplemental data

Supplemental table 1. Characteristics of patients from RA cohort 2 and 3.

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<th>DAS28</th>
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Additional characteristics of RA patients who donated paired peripheral blood and synovial fluid samples are shown. Patients from RA cohort 2 were recruited in Korea, whereas patients from RA cohort 3 were recruited in the Netherlands. Data obtained from cohort 2 is depicted in figure 3, data from cohort 3 in figures 4 and 5. * Patient initially diagnosed with juvenile idiopathic arthritis, later progressed towards RA. ND = not determined.

Supplemental figure 1. Absolute numbers of FOXP3/CD45RA defined subsets in peripheral blood of healthy controls and RA patients. Statistical significance is indicated as ** p ≤ 0.01. Mem = memory. T_{reg} = regulatory T cells. T_{conv} = conventional CD4+ T cells.
Supplemental figure 2. (A) Pie charts showing expression of CXCR3, CCR4 and CCR6 by CD45RA/FOXp3 defined CD4+ T cell fractions in peripheral blood (PB) and synovial fluid (SF) of 6 RA patients. (B) Expression of CXCR3, CCR4 and CCR6 by naive regulatory T (T_{reg}) cells and naive conventional CD4+ T cells (T_{conv}) in peripheral blood (PB) and synovial fluid (SF) of 6 RA patients. Cells with a Th1-like homing potential (CXCR3+), a Th2-like homing potential (CCR4+CCR6-CXCR3-) and Th17/Th22-like homing potential (CCR4+CCR6+CXCR3-) can be distinguished. No data are shown for synovial fluid naive T_{reg} cells, as these cells were not detected. Box and bars represent mean and SEM. Statistical significance is indicated as * p < 0.05.
Supplemental figure 3. (A) Representative flow cytometric staining and gating strategy for five CD4+ T cells subsets defined by FOXP3 and CD45RA expression in RA peripheral blood and synovial fluid and their expression of CD25. (B) Mean fluorescence intensity (MFI) for CD25 in naive regulatory T (T_{reg}) cells (fraction I), memory T_{reg} cells (fraction II), memory FOXP3^{low} conventional T (T_{conv}) cells (fraction III), memory FOXP3^- T_{conv} cells (fraction IV) and naive T_{conv} cells. Data are representative for samples from peripheral blood of 39 healthy controls and 29 RA patients, and synovial fluid from 9 RA patients. No data are shown for synovial fluid fraction I naive T_{reg} cells, as these cells were not detected. Box and bars represent mean and SEM.
REFERENCES


36. van Roon JA, Hartgring SA, van der Wurff-Jacobs KM, Bijlsma JW, Lafeber FP: Numbers of CD25+Foxp3+ T cells that lack the IL-7 receptor are increased intra-articularly and have impaired suppressive function in RA patients. *Rheumatology (Oxford)* 2010, 49(11):2084-2089.
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