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CD8$^+$T CELL PROFILES IN PATIENTS WITH RHEUMATOID ARTHRITIS AND THEIR RELATIONSHIP TO DISEASE ACTIVITY

Helena Carvalheiro, MSc[1], Cátia Duarte, MD[2,3#], Sandra Silva-Cardoso, MSc[1#], José A P da Silva, MD/PhD[2, 3], M Margarida Souto-Carneiro, PhD[1*].

1 – *ImmunoMetabolic Pharmacology Group, Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal*

2-*Rheumatology Department, Centro Hospitalar Universitário de Coimbra, Coimbra, Portugal*

3-*Clínica Universitária de Reumatologia, Faculty of Medicine, University of Coimbra, Portugal*

#- Both authors contributed equally to this study.

Running Title: CD8$^+$T cells in RA

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Corresponding Author

M Margarida Souto-Carneiro, PhD

ImmunoMetabolic Pharmacology Group, Center for Neuroscience and Cell Biology, University of Coimbra, Largo Marquês de Pombal, 3004-517 Coimbra, Portugal

Tel.: (+351)239-104424; Email: margarida.carneiro@cnc.uc.pt

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ABSTRACT

Objective

$CD8^+$T cells are abundant in rheumatoid arthritis (RA). However, their role in the disease pathogenesis is poorly defined. Here we investigated the relationship between disease activity and $CD8^+$T cell phenotypes, production of cytokines and cytotoxic molecules in RA peripheral blood (PB) and synovial fluid (SF).

Methods

$CD8^+$T cell phenotypes were determined in 96 patients with RA (44 in remission, 34 with active disease) and in 64 gender and age-matched healthy controls (HC). Ten paired PB and SF samples from patients with active RA were analyzed. The expression of surface markers, cytokines and proteolytic enzymes in CD8+T cells was evaluated using flow-cytometry.

Results

The PB $CD8^+$T cells from RA patients with active disease exhibited an effector $CD27^-CD62L^-$ ($p=0.005$) phenotype with elevated proinflammatory cytokine expression (TNF-α, IFN-γ, IL-6, IL-17A) when compared to HC. The phenotype observed in patients with active disease persisted in remission, with a significant increase in the frequency of CD69 ($p<0.001$) and was associated with lower cytokine production. $CD8^+$T cells from SF expressed more robust effector memory ($CD27^+CD62L^-$) and activated ($CD69^+$) profiles compared with paired blood-derived subsets. Cytokine-production (IL-6, IL-17A, and IFN-γ) by $CD8^+$T cells from PB and SF was positively correlated within individual donors. The production of cytokines (TNF-α, IFN-γ, IL-17A) by $CD8^+$T cells in the PB from RA patients positively correlated with DAS28.

Conclusion

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Herein we characterize the activation status and proinflammatory potential of $CD8^+$T cells subsets in RA patients. This activation status strongly suggests a local and systemic effector cytotoxic role in the disease.

INTRODUCTION

Genome-wide association studies and long standing phenotypic and relevant murine model data strongly implicate T cells in the pathogenesis of rheumatoid arthritis (RA). $CD8^+$T cells comprise approximately 40% of all T cells infiltrating the rheumatoid synovial compartment (1), and they are detected in the pre-clinical stages of disease development(2). $CD8^+$T cells can be subdivided into different functional subsets that include a short-lived effector subset (with high migratory capacity and intense production of pro-inflammatory cytokines and cytotoxic molecules); an effector-memory subset (which accumulates in the peripheral organs, is apoptosis-resistant and becomes effector upon reencounter with antigen), a central memory subset (which offers rapid proliferation and cytokine production but little cytotoxicity upon reencounter with cognate antigen), and a suppressor subset (IL-10-producing cells which down-modulate the inflammatory response) (3-5).

One prior study found that peripheral blood (PB) central memory $CD8^+$T cells were more frequent in RA patients when compared to healthy controls (HC) whereas the opposite profile was seen with effector memory $CD8^+$T cells. (6). Recently, the frequency of effector memory but not central memory $CD8^+$T cells was reported to be elevated in the PB and synovial fluid (SF) of RA patients when compared to PB samples from HC (7). An accumulation of autoreactive, clonally-related memory $CD8^+$T cells was found in RA SF (8-11)

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and their frequency correlated with serum rheumatoid factor (RF) levels (12). RA patients with DAS28>3.2 appear to have a slight increase in the frequency of circulating IL-17A-producing $CD8^+$T cells (13). $CD8^+$T cells are crucial in maintaining synovial ectopic germinal centers, which are associated in turn with more aggressive disease (14-16). However, some studies indicate that a suppressor subset of $CD8^+$T cells associates with disease amelioration (17, 18). Key outstanding questions remain including the identity of an overarching phenotype and the production of cytokines and cytotoxic molecules by $CD8^+$T cells in peripheral blood and the synovial compartment and their relationship with RA disease activity. As SF is becoming harder to obtain, it must be established whether studies in blood samples provide a reliable representation of the biological events taking place at the inflammatory site, reflected by the SF. Herein we address these critical issues.

PATIENTS AND METHODS

Patients

96 RA patients from Rheumatology Department of Centro Hospitalar Universitário de Coimbra were enrolled for this study (Table 1). RA disease activity was assessed at the time of blood collection through tender and swollen joint counts, Erythrocyte Sedimentation Rate and C-reactive protein) levels. Disease activity groups were defined according to the DAS28-CRP (3 variables) score: <2.6= remission; ≥2.6 <3.2= low; >3.2= moderate to highly active disease(19). SF was collected from patients with active disease whenever possible (n=10). The use of different medications was very similar in the three disease-activity groups, with the exception of anti-TNF agents, used by five patients, all with active disease. On average,

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patients were receiving a combination of 3 different medications for RA, this number being similar in the different disease activity groups. A total of 64 gender and age-matched healthy individuals (HC) were recruited among family members of patients in the same Department. Exclusion criteria: known or suspected ongoing infections, or, for HC, any history of autoimmune disease or immunosupressive therapy.

The study was approved by the institutional ethics committee and performed according to the Helsinki declaration on studies with human subjects. All subjects signed an informed written consent prior to any study procedure. Table I summarizes the demographic, clinical and therapeutic data of all subjects.

Flow cytometric analysis

After red blood cell lysis using a hypotonic solution, unstimulated PB mononuclear cells were stained for cell surface markers using fluorochrome-conjugated mouse anti-human monoclonal antibodies against: CD8, CD25, CD27, CD4, CCR7, CD62L, CD69, CD3 and CXCR4 (all from Biolegend). For intracellular cytokine quantitation, after staining for the cell surface antigens, samples were formalin-fixed and permeabilized using a saponin-based buffer prior to incubation with fluorochrome-conjugated mouse anti-human monoclonal antibodies against: IFN-γ, Granzyme B, IL-17A, TNF-α, IL-6, IL-10 (all from Biolegend) and Perforin (Immunotools). Irrelevant, directly conjugated, murine IgG1 or IgG2 (Biolegend) were used to ascertain background staining. All samples were analyzed on a FACScalibur cytometer (BD), with 50000 events collected within the lymphocyte gate. After calibration with CST beads single-fluorochrome stained cells were used for instrument compensation and PMT-setup. Resulting data were quantified using FlowJo Software (Treestar). Analysis of $CD8^+$T

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cell subsets (Supplemental table I summarizes the markers profile for each subset) was performed on total $CD8^+$T cells in the lymphocyte gate.

Statistical analysis

SPSS v.20 (IBM) was used to analyze the results. We elected to compare cells obtained from people with active RA (DAS28>3.2), vs cells from RA patients in remission (DAS28<2.6) vs cells obtained from age and gender-matched HC. Differences between independent samples were assessed through one-way ANOVA followed by LSD post-hoc test. Paired PB and SF samples were compared through the Wilcoxon rank sum test. Correlation between PB and SF was analyzed using Spearman correlation coefficient. Correlation between DAS28 and PB $CD8^+$T cells was analyzed using the Pearson correlation including all RA patients. PB $CD8^+$T cells were also correlated with MTX and GC´s doses through Pearson Correlation. Correlation coefficients were considered weak for *R* above 0.1, moderate for *R* values above 0.3, strong above 0.5 and very strong above 0.75.

In order to explore whether the influence of therapy upon the changes in biological parameters significantly correlated with DAS in univariate analysis, we performed a multivariate linear regression analysis of these measures, including the doses of medications (methotrexate, antimalarials, glucocorticoids and sulfasalazine) and DAS28 as covariates.

Statistical significance was considered for $p<0.05$ in all analyses.

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RESULTS

Altered status of peripheral blood CD8$^+$T cell subsets in RA patients

The relative frequency of circulating CD8$^+$T cells within the total lymphocyte population was similar in all groups (Fig 1A). The absolute number of circulating CD8$^+$T cells was similar in RA patients with active disease and in controls but was significantly lower ($p<0.05$) in patients in remission (HC: 394.2±1.6cells/μl; Active RA: 400.0±3.7cells/μl; Remission RA: 351.7±1.6cells/μl). This apparently arises from generalized lymphopenia in RA patients in remission (HC: 2478.3±156.4 cells/μl; Active: 2185.7±266.8 cells/μl; Remission: 1825.0±159.6 cells/μl) and suggests that the latter status is not commensurate with normal immunologic homeostasis.

The relative frequencies of CD27$^+$CD62L$^+$CCR7$^+$ central memory CD8$^+$T cells was lower in active RA than in HC (Fig 1B). Remission was associated with accentuation of this difference (Fig 1B). The frequency of CD27$^+$CD62L$^-$ effector memory CD8$^+$T cells was similar in all three groups (data not shown). The frequency of the short-term effector CD27$^-$CD62L$^-$CD8$^+$T cell subset was significantly higher in the active disease group when compared to controls (Fig 1B). This difference persisted in patients in remission.

Both RA groups had lower relative frequencies of CD25$^+$CD8$^+$T cells compared with HC, although significant differences were observed only for those patients in remission (Fig 1C). The frequency of PB CD69$^+$CD8$^+$T cells in active disease was similar to that in HC. The remission group had significantly more circulating CD69$^+$CD8$^+$T cells than the active disease and the HC (Fig 1C). There was an accumulation of CD69-expressing CD8$^+$T cells within the

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total CD62L$^-$ effector compartment of both patient groups – especially in the remission group - when compared to HC (Fig 1C). The frequency of PB CD8$^+$T cells expressing CXCR4 was significantly lower in both patient groups than in controls (Fig 1D). When focusing the analysis on the activated total effector CD8$^+$T cell population, the significant reduction of the proportion of cells expressing CXCR4 was maintained in both patient groups when compared to HC (Fig 1D).

Cytokine and cytolytic enzyme expression by CD8$^+$T cells in RA

Patients with active RA had a significantly higher percentage of unstimulated CD8$^+$T cells expressing TNF-α, IL-17A, IL-10 and granzyme B than controls (Table II). Patients in remission exhibited a higher than normal percentage of IL-10$^+$CD8$^+$T cells, but not TNF-α, IL-17A or granzyme B expressing cells. The frequency of CD8$^+$T cells producing other cytokines was similar across groups. Intracellular expression of cytokines, granzyme B and perforin in unstimulated PB CD8$^+$T cells (Table II) was quantified by mean fluorescence intensity (MFI). CD8$^+$T cells from active RA expressed significantly more granzyme B, IL-6, IL-17A, TNF-α and IL-10 than cells from control donors. CD8$^+$T cells from remission RA patients expressed significantly less IL-6, IL-17A, TNF-α and IFN-γ than those obtained from active RA.

Functional CD8$^+$T cell subsets in paired blood and SF samples of RA patients

Cell phenotypes in RA SF were compared with paired PB. The frequency of effector memory CD8$^+$T cells was significantly higher in SF than in paired PB (Fig 2A). CD8$^+$T cells expressing

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CD25 and CD69 were significantly more frequent in the SF than in the PB (Fig 2B). Similarly, the frequency of $CD69^+CD62L^-$ activated effector $CD8^+$T cells was significantly higher in SF. There was a significant accumulation of $CXCR4^+CD62L^-$ and $CXCR4^+CD69^+$ $CD8^+$T cells in the SF (Fig 2C). The frequency of TNF-α-expressing and IL-6-expressing $CD8^+$T cells was significantly higher in the RA SF than in PB. However, no significant differences were observed in the frequency of $CD8^+$T cells expressing other cytokines or granzyme B (Table III). Finally, the intracellular production of all cytokines and granzyme B by SF $CD8^+$T cells was similar to that in PB (Table III).

Correlation of $CD8^+$T cell subsets in the PB and SF

The frequencies of total $CD8^+$T cells in SF and PB had a strong positive correlation (Fig 3A). The percentages of total activated $CD25^+CD8^+$T cells and the $CD25^+CD62L^+$ memory subset in PB presented a strong positive correlation to the percentage of the same subsets in the SF (Fig 3A). Strong positive correlations were found between the intracellular production of granzyme B, IFN-γ, IL-6, IL-17A by $CD8^+$T cells from PB and SF (Fig 3B).

Correlation of PB $CD8^+$T cell subsets with DAS28 and influence of therapies

The frequency of total $CD8^+CD69^+CXCR4^+$ and $CD8^+CXCR4^+CD62L^-$ T cells in PB exhibited a weak negative correlation with DAS28 (Fig 3C). Weak positive correlations with DAS28 were found for the intracellular production of TNF-α and IL-17A, whereas intracellular IFN-γ presented a strong positive correlation (Fig 3D).

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The correlations between the dose of medications (MTX, sulfasalazine, hydroxychloroquine and glucocorticoids) and $CD8^+$T cell subpopulations, as well as intracellular proinflammatory mediator production, assessed through multivariate analysis as described, failed to show statistically significant impact of medications after consideration of DAS28 (supplemental table II).

DISCUSSION

Herein we report that PB $CD8^+$T cells from active and remission RA present an activated phenotype with a marked pro-inflammatory profile. We show that the expression of pro-inflammatory cytokines by circulating $CD8^+$T cells is directly correlated with the DAS28 score. $CD8^+$T cells from the SF of active RA exhibit an exacerbated effector and activated phenotype compared to those in paired PB. Finally, we observed that the production of cytokines by SF $CD8^+$T cells is correlated with that in paired PB derived cells.

Contrasting to a previous report (7), we did not find any differences in the frequency of total $CD8^+$T cells in PB and SF of RA patients. We suggest that these contradictions arise from the fact that they compared SF data to blood data of the whole RA cohort regardless of disease activity, whereas we performed a paired analysis restricted to patients with active disease. The circulating $CD8^+$T cell compartment of RA patients, regardless of disease activity, had a skewed distribution of central memory and short-term effector $CD8^+$T cell subsets, with enrichment of the latter. Accumulation of effector memory $CD8^+$T cells in the SF compared to the paired blood was equally present. RA patients accumulate effector $CD8^+$T cells both in the blood and in the SF - and at the same time present a reduction in the central memory

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$CD8^+$T cell subset. These results partially mimic our previously reported observations in K/BxN mice (20).

Our data confirm previous observations that $CD8^+$T cells in RA frequently express the early activation marker CD69 (21-24). The increased frequency of effector $CD8^+$T cells expressing CD69 in RA patients' blood – independent of disease activity – and SF, suggests that these cells might be constantly stimulated by the presence of their cognate antigen(s). Also in the K/BxN model of arthritis, the expression of CD69 in $CD8^+$T cells is increased in both the PB and articular tissue of arthritic mice (20). These data, together with previous studies, indicate that activated $CD8^+$T cells are enriched in RA PB cells (25). Even though, the peripheral blood only represents a small fraction of the total T cell pool of an individual, we speculate that the accumulation of activated $CD8^+$T cells in the PB during remission rather than active disease suggests that these cells remain in circulation and might be recruited into the joint when the disease increases its activity. The surge in $CD8^+$T cells expressing CD69 as well as $CD69^+CXCR4^+$ in the SF of active RA patients when compared to parallel PB samples, also indicates that these cells are enriched in the joints during disease flares. This interpretation is supported by our finding of a weak negative correlation between the frequency of PB effector and activated $CXCR4^+CD8^+$T cells and the DAS28 score, since CXCR4 is responsible for cytotoxic T cell-homing into inflammatory sites. Clearly, the correlations are too weak to establish this functional link but they mirror our previous results in the K/BxN mouse (20).

We measured *ex vivo* cytokine, perforin and granzyme B production by PB and SF $CD8^+$T cells without *in vitro* stimulation, in order to assess whether these cells actively contribute to the pro-inflammatory environment in RA and consequent joint destruction. Our data show that

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regardless of the similar numbers of circulating effector $CD8^+$T cells, remission and active disease are associated with distinct production of cytokines and cytotoxic molecules, and that the production of pro-inflammatory cytokines in PB was directly correlated with the DAS28 score.

We show that the expression of granzyme B by $CD8^+$T cells from PB and SF from active RA patients is higher than in PB from controls. The difference between remission and control is not significant. We confirm previous observations that granzyme B^+CD8^+T cells are commonly found in the synovium of RA patients (16, 26). Given that we excluded patients with known ongoing infections, we suggest that the increased production of granzyme B and perforin by $CD8^+$T cells in active RA is stimulated by the presence of autologous antigens and the pro-inflammatory environment. It also shows that $CD8^+$T cells are actively involved in maintaining the chronic inflammatory process and that after medication-induced remission granzyme B and perforin production by $CD8^+$T cells returns to normal levels.

The positive correlations obtained between the intracellular production of Granzyme B, IL-17A, IL-6 and IFN-γ by $CD8^+$T cells in the PB and those in the SF indicate that variations observed in the patients' PB mirror those in the SF. Hence, we demonstrate, for the first time, that variations in the production of these cytokines on peripheral $CD8^+$T cells provide a good representation of similar processes taking place at the joint level. This is an important finding, given that synovial fluid is now rarely available for research. Contrasting to previous suggestions derived from studies with unpaired PB and SF samples (27, 28) we did not observe an enrichment of IFN-γ^+CD8^+T cells in the SF of RA patients.

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A higher expression of IL-6 and TNF-α by $CD8^+$T cells from the PB was present in patients with active disease, highlighting the contribution of $CD8^+$T cells to the generalized inflammatory processes underlying RA. We observed a tendency to an expanded IL-10^+CD8^+T cell pool in the SF when compared to the paired blood RA samples, which was accompanied by a tendency for more IL-10 production by these cells. These observations confirm previous reports (7, 27) and seem to represent a mechanism to control inflammation.

Our results show that increased IFN-γ-production by PB $CD8^+$T cells is directly correlated with DAS28. This directly implies these activated T cells in the autoimmune reaction. We have carefully scrutinized the potential relationship between medications and this observation, through multivariate analysis. No influence of any of the medications upon this parameter persisted significant after considering DAS28.

Overall, our observations support the following model: active RA disease is characterized by a marked enhancement of $CD8^+$T cells' effector properties, and homing of those subsets into the joints (see supplemental figure). The expression of pro-inflammatory cytokines by $CD8^+$T cells in the PB (and SF) is strongly correlated with disease activity, suggesting that these cells have a relevant contribution to the systemic inflammatory milieu. After therapy-induced remission, $CD8^+$T cells recover some characteristics typical of healthy individuals, with significant reduction of cytokine production. However, some significant alterations, such as increased effector and activated phenotype, still persist and may be capable of maintaining the disease in a new biological equilibrium, with the potential to relapse. Through multivariate analysis we could not find a significant impact of any of the medications used, upon the frequency of $CD8^+$T cell subpopulations and intracellular production of effector molecules, after considering DAS28. Despite this, we believe that the influence of

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medication cannot be securely ruled-out by our data, given the limited sample size and the multiple combinations of therapies used.

Our results suggest that $CD8^+$T cells play a bigger role in RA than recognized in current paradigms of the disease pathogenesis and maintenance, according to which pathogenic T cells are HLA class II-restricted, i.e. $CD4^+$. Further investigation is warranted to clarify their involvement in disease onset and course, joint destruction and response to therapy.

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Authors' Contributions

Patient recruitment and clinical data collection: CD and JAPS

Experimental design: HC, MMSC and JAPS

Experimental data collection and raw data analysis: HC, SSC and MMSC

Statistical Analysis: HC and CD

Manuscript Preparation: HC, CD, JAPS, MMSC

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Table I– Clinical characteristics of RA patients and healthy donors.

Therapy	Controls	Total RA	Active	Low	Remission
N (SF donors)	64	96 (10)	34 (10)	18 (0)	44 (0)
Gender (F:M)	55:19	77:19	27:7	14:4	36:8
Medication - N (Avg. Dose)					
MTX	-	82 (17.4 mg/wk)	27 (19.5 mg/wk)	15 (16.3 mg/wk)	39 (16.3 mg/wk)
Hydroxychloroquine	-	21 (366.7 mg/day)	9 (344.5 mg/day)	2 (400 mg/day)	10 (380 mg/day)
Sulfasalazine	-	20 (1800 mg/day)	6 (1916.7 mg/day)	3 (1833.3 mg/day)	11 1727.3 mg/day)
Prednisolone	-	55 (5.3 mg/day)	21 (6 mg/day)	10 (5.8 mg/day)	25 (4.4 mg/day)
Leflunomide	-	2 (15 mg/day)	1 (20 mg/day)	1 (10 mg/day)	0
Azathioprine	-	1 (20 mg/day)	0	0	1 (20 mg/day)
Folic Acid	-	56 (7,95 mg/wk)	18 (8.9 mg/wk)	8 (6.3 mg/wk)	29 (7.9 mg/wk)
NSAIDs	-	50	19	9	22
TNF inhibitors	-	5	5	0	0

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Table II– Frequency of intracellular cytokines expression and their respective MFI in peripheral blood CD8$^+$T cells from RA patients and healthy controls.

	Ctrl	**Active RA**	**Remission**	***One way ANOVA*†**		
	Mean ± SEM[a] (n=64)	Mean ± SEM (n=34)	Mean ± SEM (n=44)	*p (Active vs Ctrl)*	*p (Rem. vs Ctrl)*	*p (Active vs Rem)*
Intracellular cytokines (% from total CD8$^+$T cells)						
IL-6	**2.3 ± 0.5**	**1.6 ± 0.2**	**1.7 ± 0.2**	*NS*[b]	*NS*	*NS*[a]
TNF-α	**1.2 ± 0.2**	**2.2 ± 0.4**	**1.8 ± 0.2**	**0.016**	*NS*	*NS*
IFN-γ	**2.2 ± 0.7**	**3.9 ± 1.2**	**2.4 ± 0.4**	*NS*	*NS*	*NS*
IL-17A	**1.5 ± 0.2**	**3.6 ± 0.8**	**2.3 ± 0.5**	**0.004**	*NS*	*NS*
IL-10	**0.9 ± 0.1**	**1.5 ± 0.2**	**1.8 ± 0.5**	*0.051*	**0.007**	*NS*
GrzB[c]	**14.8 ± 2.0**	**23.5 ± 3.7**	**16.2 ± 3.0**	**0.028**	*NS*	*NS*
Perforin	**2.6 ± 0.7**	**4.9 ± 2.1**	**2.0 ± 0.6**	*NS*	*NS*	*NS*
MFI[d] (within the cytokine-positive CD8$^+$T cells)						
IL-6	**13.0 ± 1.2**	**22.5 ± 4.0**	**14.4 ± 1.2**	**0.003**	*NS*	**0.015**
TNF-α	**11.9 ± 0.5**	**18.2 ± 2.1**	**13.4 ± 0.7**	**>0.001**	*NS*	**0.006**
IFN-γ	**24.8 ± 1.6**	**26.1 ± 3.7**	**15.4 ± 1.2**	*NS*	**0.002**	**0.001**
IL-17A	**17.8 ± 3.4**	**28.6 ± 2.9**	**18.5 ± 1.6**	**0.011**	*NS*	**0.022**
IL-10	**11.1 ± 0.6**	**17.4 ± 1.4**	**19.5 ± 1.8**	**0.015**	*NS*	*NS*
GrzB	**29.7 ± 2.6**	**64.0 ± 15.3**	**45.7 ± 11.7**	**0.012**	*NS*	*NS*
Perforin	**12.5 ± 0.6**	**22.3 ± 6.5**	**15.6 ± 1.4**	**0.030**	*NS*	*NS*

a) SEM: standard error of mean; b) NS: non-significant: c) GrzB: Granzyme B, d) MFI: mean fluorescence intensity

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Table III- Frequency of intracellular expression of cytokines and their respective MFI in CD8$^+$T cells from PB and SF from RA patients.

	SF	PB	Wilcoxon
	Mean (n=10)	Mean (n=10)	*p*
Intracellular cytokines (% from total CD8$^+$T cells)			
IL-6	**5.1 ± 1.3**	**2.0 ± 0.4**	**0.047**
TNF-α	**8.7 ± 3.7**	**2.5 ± 0.7**	**0.008**
IFN-γ	**6.9 ± 2.2**	**4.5 ± 1.9**	*NS*[a]
IL-17A	**11.7 ± 6.0**	**7.5 ± 2.2**	*NS*
IL-10	**8.5 ± 6.7**	**2.0 ± 1.2**	*NS*
GrzB[b]	**23.6 ± 5.6**	**35.9 ± 9.3**	*NS*
Perforin	**4.3 ± 1.3**	**10.8 ± 6.9**	*NS*
MFI[c] (within the cytokine-positive CD8$^+$T cells)			
IL-6	**24.3 ± 7.8**	**34.1 ± 14.4**	*NS*
TNF-α	**23.3 ± 5.0**	**29.0 ± 10.8**	*NS*
IFN-γ	**28.2 ± 5.5**	**36.7 ± 11.9**	*NS*
IL-17A	**34.9 ± 9.0**	**45.1 ± 12.8**	*NS*
IL-10	**72.9 ± 56.1**	**39.9 ± 22.0**	*NS*
GrzB	**121.6 ± 90.1**	**127.7 ± 44.3**	*NS*
Perforin	**20.7 ± 4.8**	**39.0 ± 19.1**	*NS*

a) NS: non-significant: b) GrzB: Granzyme B, c) MFI: mean fluorescence intensity

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Figure Legends

Figure 1 – Functional phenotyping of peripheral blood CD8$^+$T cells shows altered frequencies of subsets expressing activation, homing, memory and effector molecules in active and remission RA patients when compared to controls. A: Boxplots representing the 90%, 75%, median, 25% and 10% ranges of the frequency of total circulating CD8$^+$T cells within the lymphocyte gate. **B:** Representative dot-plots of CD62L vs CD27 gated on CD8$^+$ T cells for each group. Boxplots representing the 90%, 75%, median, 25% and 10% ranges of the frequency of circulating CD8$^+$T cell subsets within the total CD8$^+$T cell pool: CD27$^+$CD62L$^+$CCR7$^+$, CD27$^-$CD62L$^-$, **C:** Boxplots representing the 90%, 75%, median, 25% and 10% ranges of the frequency of circulating activated CD8$^+$T cell subsets within the total CD8+T cell pool: CD25$^+$, CD69$^+$, CD69$^+$CD62L$^-$, **D:** Boxplots representing the 90%, 75%, median, 25% and 10% ranges of the frequency of circulating CD8$^+$T cell subsets expressing homing molecules within the total CD8$^+$T cell pool CXCR4$^+$, CXCR4$^+$CD69$^+$CD62L$^-$. P values calculated by one-way ANOVA followed by LSD *post-hoc* test. Control: N = 64; Active RA: N = 34; Remission RA: N = 44.

Figure 2 – Functional phenotyping of CD8$^+$T cells from paired peripheral blood and synovial fluid from RA patients shows increased frequencies of CD8$^+$T cells expressing effector, activation and homing molecules in the synovial fluid. Boxplots representing the 90%, 75%, median, 25% and 10% ranges of the frequency of circulating CD8$^+$T cell subsets within the total CD8$^+$T cell pool: **A:** CD27$^+$CD62L$^-$ and CD27$^-$CD62L$^-$CCR7$^-$, **B.** CD25$^+$, CD69$^+$ and CD69$^+$CD62L$^-$, **C.** CXCR4$^+$, CXCR4$^+$CD62L$^-$ and CXCR4$^+$CD69$^+$. P values calculated by Wilcoxon non parametric test. Synovial fluid (SF) and peripheral blood (PB): N = 10.

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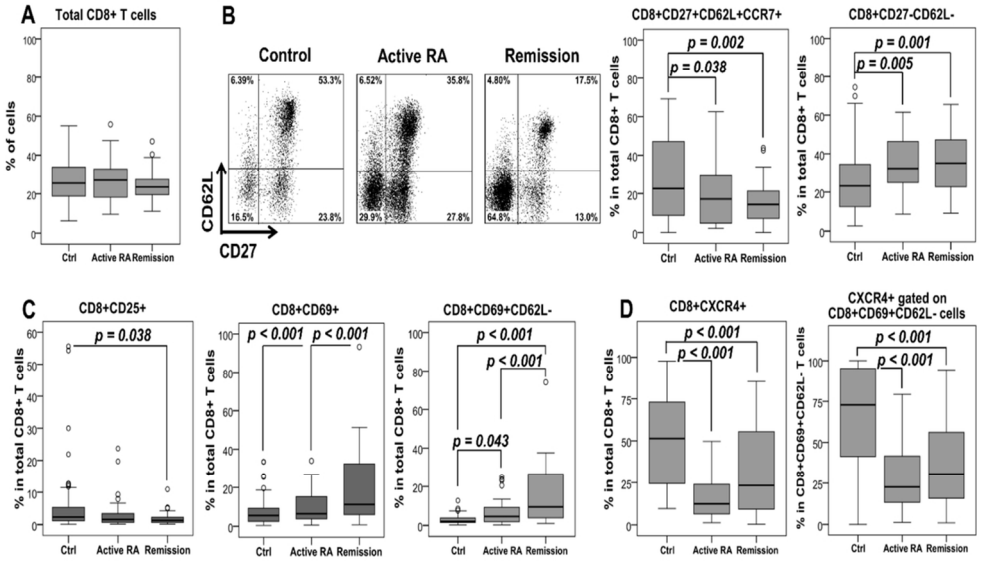
Figure 3 – Values observed in the patients' PB mirror those in the SF, and the percentage of $CD8^+$T cells with an inflammatory phenotype increase with the patients' DAS28 A: Correlation plots between $CD8^+$T cell subsets in the PB and SF of RA patients (N = 10). B: Correlation plots between cytokine production by total $CD8^+$T cells in the PB and SF of RA patients (N = 10). C: Correlation plots between PB $CD8^+$T cell subsets and DAS28 of RA patients (N = 96). D: Correlation plots between PB cytokine production by total $CD8^+$T cells and DAS28 of RA patients (N = 96). Correlations considered weak for *r>0.2,* moderate for *r>0.3,* strong for r >0.5 and very strong for r >0.75. Significance achieved for $p < 0.05$. A-G: Values obtained using the Spearman correlation. H-L: Values obtained using the Pearson correlation.

Supplemental Figure: The loss of circulating total $CD8^+$T cells, as well as activated ($CD69^+$) and effector ($CD62L^-$) $CD8^+$T cell subsets expressing the CXCR4 homing molecule in RA patients with active disease when comparing to healthy controls, seems to derive from their accumulation in the inflamed joints. The graph shows the mean frequency ±StdEr for each subset for healthy controls (HC, n=64) and paired peripheral blood (PB) and synovial fluid (SF) from patients with active RA (n=10).



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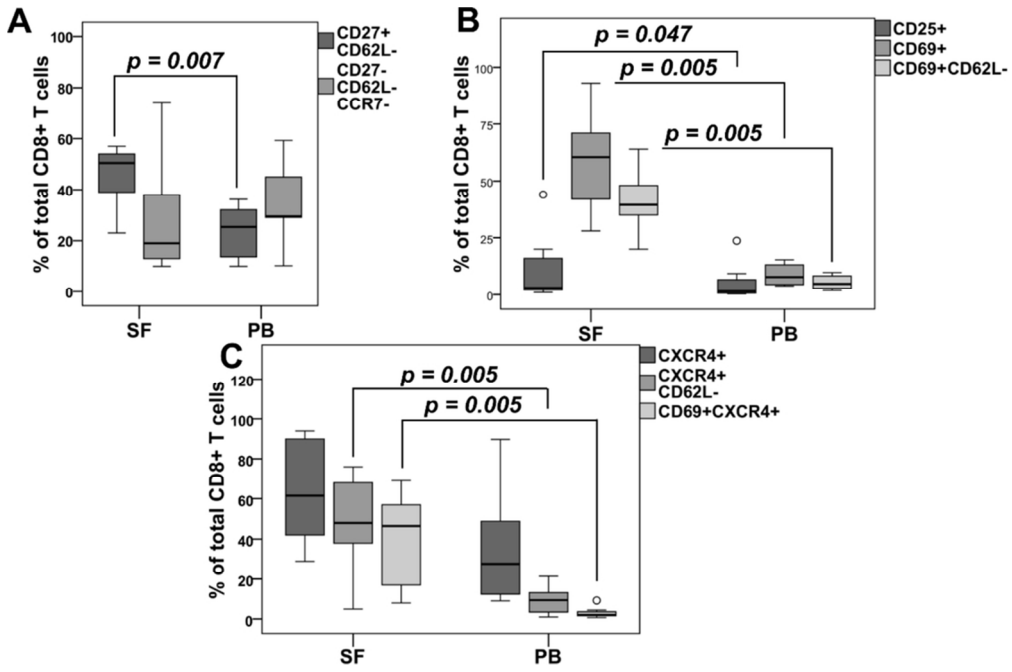

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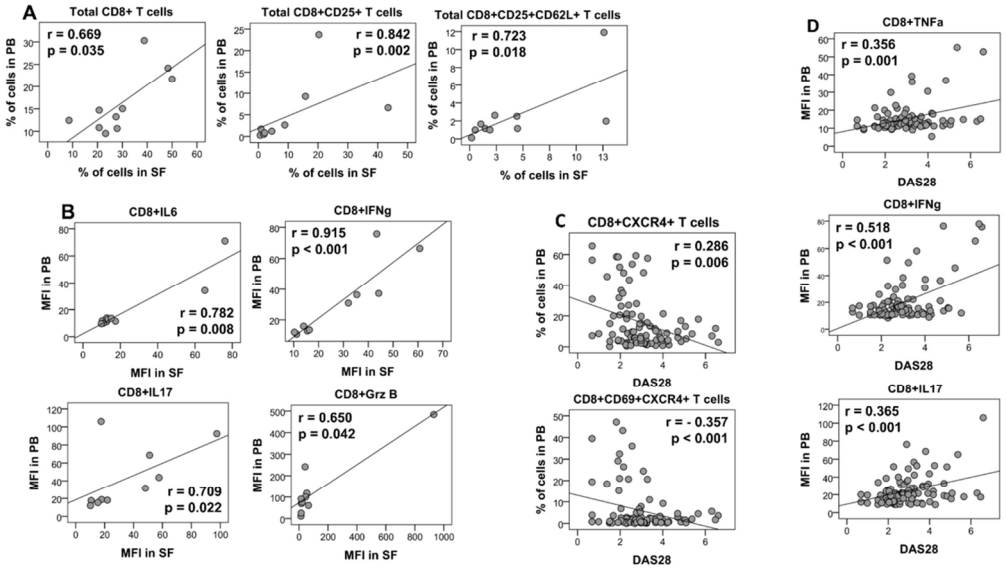

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