Concise report

Numbers of CD25⁺Foxp3⁺ T cells that lack the IL-7 receptor are increased intra-articularly and have impaired suppressive function in RA patients

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Abstract

Objectives. To investigate numbers and function of CD25⁺ regulatory T cells (Tregs) that lack IL-7 receptor (CD127) expression in RA.

Methods. Numbers of CD4 T cells expressing either CD25 or CD127, and those co-expressing or lacking both CD25 and CD127 were assessed in peripheral blood (PB) of RA patients and healthy controls, and in paired samples of SF and PB from RA patients. All T-cell subsets were analysed for FoxP3 expression. The anergic state and the capacity to suppress CD127⁺ proliferating responder T cells were determined.

Results. Numbers of CD127⁻ T cells and CD25⁺ Tregs in PB of RA patients were not different from controls but significantly increased in SF compared with PB. CD25⁺ and CD127⁻ T cells showed comparable FoxP3 expression. CD127⁺ T cells hardly expressed FoxP3. PB CD25⁺CD127⁻ T cells identified a subset that consisted for 75% of FoxP3⁺ cells. SF CD25⁺CD127⁻ T-cell number was increased; however, in SF fewer of these cells were FoxP3⁺. CD25⁺CD127⁻ T cells were anergic, and in controls potent suppressors of CD127⁺ proliferating T cells, but in RA patients these cells showed impaired suppression. In line with this, IL-7 had an increased capacity to activate total CD4 T cells from SF as compared with PB despite increased numbers of CD25⁺CD127⁻ in SF.

Conclusions. These data demonstrate improved identification of FoxP3⁺ T cells in RA patients by the absence of CD127 in addition to CD25 expression. Increased numbers of CD25⁺CD127⁻ T cells are found in inflamed RA joints, but they have an impaired suppressive function, which could contribute to the persistent arthritis in these patients.

Key words: Rheumatoid arthritis, IL-7 receptor (CD127), Foxp3, Regulatory T cells.

Introduction

IL-7 is a potent immunostimulatory cytokine and increased IL-7 levels are found in several inflammatory diseases, including RA. IL-7 effects are mediated through the high-affinity IL-7 receptor-α chain (IL-7R; CD127) in conjunction with the common-γ chain. IL-7 stimulates proliferation, survival and differentiation of T cells and induces T-cell-dependent monocyte and B-cell activation and osteoclast formation [1, 2]. We have described increased CD127 expression in the synovial tissue of RA patients and demonstrated that blockade of CD127 inhibited immune activation of cells from RA patients [3]. Blockade of the IL-7R and of IL-7 also inhibits several forms of experimental arthritis [4, 5, 6]. Hence, the IL-7R might be a potential therapeutic target in RA. Besides the interesting aspect of IL-7/IL-7R-driven arthritis, it has been shown that the lack of CD127 expression on CD25⁺ T cells could help to identify 'true' regulatory T cells (Tregs), expressing high FoxP3 levels [7].

CD25⁺ Tregs inhibit immune activation in experimental arthritis [8]. In addition, CD25⁺ Tregs in human *in vitro* conditions inhibit activation of CD25⁻ effector T cells

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and monocytes in patients with RA [9]. More recent studies indicated that the suppressive function of CD25-expressing CD4 T cells is confined to the CD25^{bright} population, supported by a high expression of FoxP3 that has been associated with their suppressive function and anergy in response to TCR/CD3 cross-linking. In contrast, CD25^{dim} CD4 T cells that express FoxP3 to a lesser extent, are not anergic and can be immunostimulatory [10]. This indicates that discrimination of Tregs on the basis of merely CD25 expression has its limitations and that there is a need for better definition of 'true' Tregs.

In the current study, definition of FoxP3-expressing CD4 T cells is also achieved in RA patients by the identification of CD25⁺ T cells that in combination lack the IL-7R. Increased numbers of these cells were found in RA patients but with impaired suppressive function.

Materials and methods

Patients

Peripheral blood (PB) from which mononuclear cells (MCs) were isolated was taken from 16 healthy controls [HCs; mean age 34.8 (2.8) years], and 25 patients with RA defined according to the ACR criteria [11] [mean age 54.9 (2.7) years and disease duration 6.6 (2.4) years]. SF was taken from 14 RA patients [mean age 49.9 (3.7) years and disease duration 8.3 (3.2) years]. Paired samples of PB and SF were obtained from 10 RA patients [age 50.0 (5.3) years, disease duration 10.8 (4.8) years]. For suppression experiments, PB samples from eight HCs [age 43.6 (5.0) years] and from eight patients with RA [age 57.5 (5.0) years, disease duration 8.1 (3.4) years] were collected. All patient material collection (SF and PB) was performed according to the medical ethical regulations of the UMC Utrecht and patients gave their written informed consent. The medical ethical committee from the UMC Utrecht approved this study.

PB and SF were diluted 1:1 with RPMI 1640 medium (Gibco BRL-Life Technologies, Merelbeke, Belgium) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM). Thereafter, MCs were isolated by density centrifugation (Ficoll-Isopaque; Pharmacia Biotech, Sweden).

Flow cytometry

IL-7Rα PE (CD127, clone R34.34; Immunotech, Marseilles, France) and CD25 FITC (clone ACT-1; DAKO) expression on CD4 T cells PE/Cy5 (clone MT310; DAKO, Glostrup, Denmark) isolated from SF and PB was analysed by flow cytometry. For control staining, IgG1/IgG1 FITC/PE-labelled (Immunotech) antibodies were used. To calculate percentages of CD25- and CD127-defined T cells quadrant markers were set based on these isotype control stainings. For intracellular Foxp3 staining, a rat anti-human Foxp3 allophycocyanin [antigen-presenting cell (APC), clone PCH101] staining set (eBioscience, San Diego, CA, USA) was used. For intracellular control staining, a rat isotype control APC-labelled antibody (eBioscience) was used.

Function of CD25⁺CD127⁻ CD4 T cells in vitro

To determine the functional ability of CD25⁺CD127⁻ CD4 T cells, cells were isolated from peripheral blood mononuclear cells (PBMCs) of RA patients and HCs by means of microbead-activated cell sorting. Untouched CD4 T cells were isolated using a CD4 isolation kit (Miltenyi Biotech, Amsterdam, The Netherlands). CD4 T cells expressing IL-7R-α (CD127) were selected by incubating CD4 T cells with a PE-labelled anti-CD127 antibody (R34.34, Immunotech), followed by incubation with anti-PE magnetic beads (Miltenyi Biotech) and separation of CD127⁺ and CD127⁻ T cells on a magnetic column. Subsequently, from CD127⁻ CD4 T cells, CD25⁺ cells were isolated using CD25 bead-labelled antibodies (previously described in [12]). For the comparison of CD4 T-cell activation by IL-7, CD14⁺ cells were isolated from the PB using a CD14 isolation kit (Miltenyi Biotech) and CD14⁺ monocytes were co-cultured with autologus CD4⁺ T cells from either PB or SF (n = 9, paired samples).

CD127⁺, CD25⁺CD127⁻ and CD25⁻CD127⁻ T cells (20 000 cells/well) were cultured in 96-well plates and activated with or without 2 μg/ml (coated) anti-CD3 mAb (clone OKT3, eBioscience), and irradiated autologous APCs (20 000 cells/well) for 3 days. To test the suppressive capacity of CD25⁺CD127⁻ and CD25⁻CD127⁻ T cells, these were co-cultured with CD127⁺ responder T cells in different ratios. Proliferation was measured using a [³H]thymidine incorporation assay.

Statistical analysis

Statistical analysis of percentages of T-cell subsets was done by using a non-parametric Mann–Whitney U-test for comparison of HCs and RA patients. Percentages of T-cell subsets in PB and SF from RA patients were analysed by a paired sample t-test. Number of FoxP3-expressing cells was analysed by a Kruskal–Wallis test for multiple group comparison followed by a Mann–Whitney U-test or independent sample t-test for parametric and non-parametric distributed data, respectively. Analysis of differences in proliferation of T cells from RA patients and HCs was done using an independent sample t-test.

Results

Percentages of circulating CD25⁺, CD127⁻ and CD25⁺CD127⁻ T cells (representative staining Fig. 1A) in RA patients were not significantly different from HCs (Fig. 1B). In RA patients, CD25⁺, CD127⁻ and CD25⁺CD127⁻ T cells were significantly increased in SF as compared with PB with the strongest increases in the latter two subsets (21.8 vs 13.5%, 39.7 vs 13.3% and 13.5 vs 3.4%; P < 0.05, 0.001 and 0.01, respectively, Fig. 1C).

Whereas CD25⁻, CD127⁺ and CD25⁻CD127⁺ T-cell populations hardly contained FoxP3-expressing cells, significant percentages of FoxP3-expressing cells were found among CD25⁺, CD127⁻ populations, and in particular in the population with combined CD25⁺ and CD127⁻ expression (representative staining in Fig. 1D). No

Fig. 1 CD25^{+}CD127^{−} CD4 T cells are increased in the SF of RA patients, and are largely FoxP3^{+}. Representative CD25/CD127 staining of CD4 T cells (**A**). Percentages of CD25/CD127^{−} defined CD4 T cells are not significantly different in patients with RA as compared with HCs (**B**). Percentages of CD25^{+}, CD127^{−}, CD25^{+}CD127^{−} CD4 T cells in SF were significantly increased compared with PB of RA patients (**C**). Representative FoxP3 staining (**D**). The CD25^{+}CD127^{−} phenotype identifies cells with high FoxP3 expression. The percentage of FoxP3^{+} cells in the CD25^{+}CD127^{−} T-cell subset is significantly reduced in SF as compared with PB of RA patients and as compared with PB of HCs (**E**). Statistically significant differences are indicated by *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. M1 indicates the marker that is set based on the autofluorescence control. M2 indicates the percentage of FoxP3-positive cells.
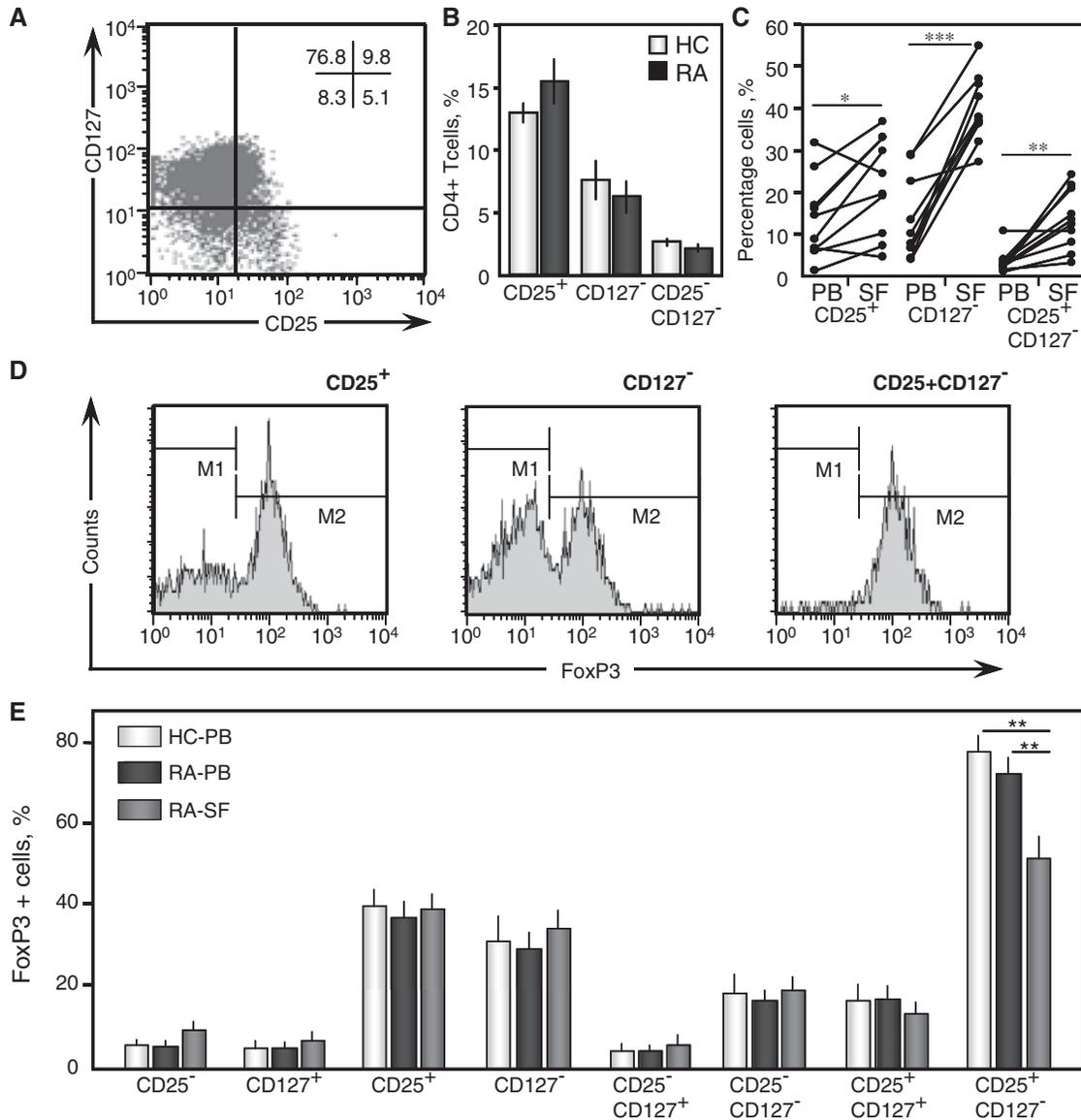
significant differences in of FoxP3 expression were found in the different T-cell populations in PB of HCs and RA patients. However, the percentage of FoxP3-expressing cells in SF CD25^{+}CD127^{−} CD4 T cells was significantly lower ($P < 0.001$) when compared with the same population in PB of RA patients as well as HCs (Fig. 1E).

To assess functional ability of CD127^{+} and CD25^{+}CD127^{−} CD4 T cells, these cells were isolated from PBMCs (Fig. 2A, representative CD127 staining of isolated CD127^{+} and CD25^{+}CD127^{−} T cells). Fig. 2B

shows proliferation (in absolute counts) of a representative healthy donor to demonstrate characterization of anergy (a proliferation of <20% as compared with responder CD127^{+} T cells, left panel), and suppression of CD25^{+}CD127^{−} T cells (percentage difference in proliferation compared with CD127^{+} T cells cultured alone, ratio 1 : 0, right panel). Anergy of CD25^{+}CD127^{−} T cells from RA patients was similar to HCs (Fig. 2C). CD25^{−}CD127^{−} displayed reduced proliferation in HCs, but these cells were not anergic. In RA patients, these T cells had a

FIG. 2 Suppressive capacity of CD25+CD127− Tregs is reduced in RA patients compared with HCs. CD127 expression of sorted CD127+ and CD127−CD25+ CD4 T cells (**A**, representative donor) CD3-restimulated *in vitro* in the presence of irradiated APCs. CD127+ T cells strongly proliferated, whereas CD25+CD127− were anergic to CD3 cross-linking (**B**, left, absolute counts of representative donor). CD25+ CD127− T cells added to responder CD127+ T cells strongly inhibited proliferation in HCs (**B**, right, absolute counts of representative donor). Similar to HCs, CD25+CD127− T cells from RA patients were anergic, in contrast to CD25− CD127− T cells (**C**, mean of eight RA patients and eight HCs). Function of CD25+CD127− T cells to suppress CD127+ T cells was impaired in RA as compared with HCs, in contrast to immuno-stimulatory effect of CD25−CD127− T cells on CD127+ T cells (**D**). CD4+ T cells from either PB or SF were cultured in the presence of CD14+ APCs from PB. IL-7-stimulated total CD4 T cells from SF showed a significantly higher proliferation compared with cells from PB (**E**). Statistically significant differences are indicated by *$P < 0.05$ and *$P < 0.01$ between HCs and RA patients or between CD4+ T cells from PB and SF (**E**).
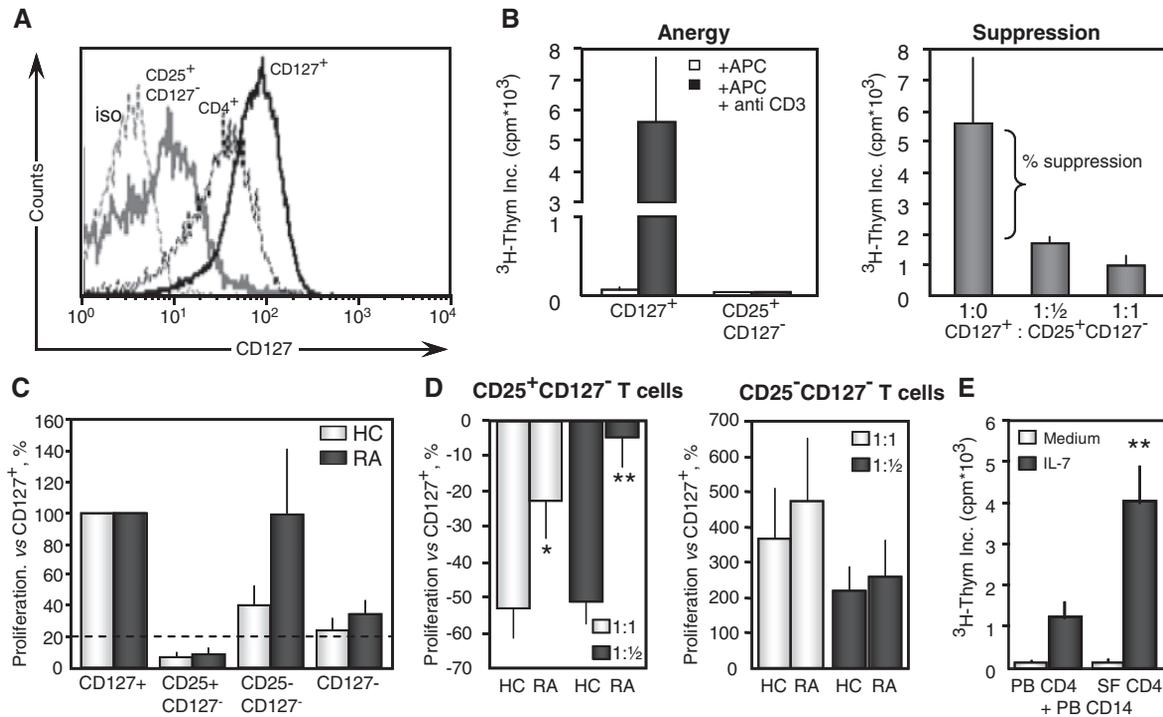
similar proliferative capacity to CD127+ responder T cells. The CD127− populations proliferated poorly, but were also not anergic (Fig. 2C). Surprisingly, CD25+CD127− T cells from RA patients displayed impaired suppressive capacity as compared with HCs, significant for both the 1:1 and the 2:1 ratio (Fig. 2D, left). Interestingly, CD25−CD127− T cells from both HCs and RA patients did not cause suppression, but strongly stimulated proliferation of CD127+ responder T cells when co-cultured (Fig. 2D, right).

To indicate whether the altered balance of CD127− and CD127+ T cells and impaired function of CD25+CD127− T cells was associated with altered immune activation, activation of CD4 T cells from PB and SF was compared. Since IL-7 is increased in RA joints, the capacity of IL-7 to activate T cells was assessed. IL-7-induced activation of SF CD4 T cells was significantly increased as compared with that of CD4 T cells from PB (Fig. 2E). The IL-7-induced fold increase *vs* medium cultures was 7.7 (2.4) *vs* 31.4 (8.2) (data not shown).

Discussion

In the present study, high FoxP3-expressing CD4 T cells can be identified in patients with RA, based on expression of CD25 and lack of IL-7R expression. However, the capacity of these cells to suppress proliferating responder IL-7R+ T cells is impaired in RA patients.

In the blood of RA patients, the majority of CD4 T cells express the IL-7R. We previously demonstrated that in RA joints T cells primarily express the IL-7R, and that IL-7R is over-expressed in RA synovial tissue [3]. IL-7, which is also over-expressed in the inflamed joints of patients with RA, has been shown to trigger IL-7R-mediated signalling inducing Th1 and Th17 activity, chemokine expression (including IP-10 and MIP1-α) and pro-inflammatory cytokines including TNF-α [13, 14]. Blockade of the IL-7R was found to reduce Th1 activity of RA mononuclear cells *in vitro* and to inhibit experimental arthritis that was associated with a reduction in T-cell activity [3, 4]. Together, this indicates that selective

inhibition of IL-7R-expressing arthritogenic T cells is a therapeutic option.

In the SF of RA patients, a significant reduction in the percentage of CD127^{+} was observed, indicated by a 3-fold increase in the percentage of CD127^{−} T cells. Of these CD127^{−} T cells, approximately one-third were CD25^{+} FoxP3-expressing T cells, whereas the other two-thirds were CD25^{−}CD127^{−} T cells. This latter subset comprised a low amount of FoxP3^{+} cells and were not suppressive *in vitro*, but strongly stimulated T-cell proliferation. This suggests that these T cells may represent *in vivo*-activated T cells. In support of this, T-cell receptor-mediated, as well as IL-7-induced T-cell activation can result in a loss of CD127 expression that at least to a considerable extent is transient [15].

The absence of CD127 expression in addition to CD25 expression adds to the identification of FoxP3-expressing anergic T cells in both HCs and RA patients. The impaired Treg function in RA found in this study is in line with previous observations demonstrating decreased suppressive capacity of CD25^{bright} CD4 T cells from RA patients as compared with HCs [16, 17]. The inflammatory microenvironment has been suggested to modulate the suppressive function of CD25^{+} Tregs. Treg-mediated suppression can be abrogated by CD28-mediated co-stimulation of T cells, which can be provided by ligand-expressing (CD80, CD86) APCs present in inflamed RA joints [12]. In addition, suppression by Tregs from RA patients can be inhibited by cytokines that are abundantly present at the site of inflammation. IL-7 and TNF are two cytokines known to activate responder T cells, making them unresponsive to suppression by Tregs [12, 16]. This may explain why despite an increased number of Tregs in RA patients, joint inflammation persists.

The results on the *in vitro* Treg function in RA are somewhat conflicting. The above-mentioned micro-environmental factors, as well as those that can promote suppressive function such as TGF-β and leptin [18, 19] may vary in experimental set-ups described in several reports. Most of these studies use human serum, which can contain varying amounts of these factors. In addition to this variation, inadequately defined Tregs may explain the conflicting data on the functional abilities of Tregs in RA.

The present study indicates that absence of CD127 can be helpful to select CD25^{+} T cells that are anergic and express high levels of FoxP3. However, our data also indicate that there remains a need to identify markers expressed by Tregs that, more clearly than FoxP3 expression, are associated with their functional (suppressive) ability. CD62L expression on CD25^{+} T cells may hold promise in this respect. Induction of CD62L-expressing Tregs, specifically induced upon anti-TNF-α (infliximab) therapy [20], were found to correlate with inhibition of disease activity. In addition to stimulation of Treg function or numbers, our data indicate that reducing effector T-cell activity through IL-7R blockade may be another promising and selective therapeutic option. The observation that patients who do not respond to anti-TNF-α therapy are characterized by persisting IL-7 levels [21] suggests that this may be relevant in particular for anti-TNF-α non-responders.

Conclusion

Improved identification of FoxP3^{+} T cells in RA patients by expression of CD25 and lack of IL-7R expression was shown. Increased numbers of CD25^{+}CD127^{−} CD4 T cells are found in inflamed RA joints. However, CD25^{+}CD127^{−} CD4 T cells from RA patients have a decreased capacity to suppress responder IL-7R^{+} T cells that have a pro-inflammatory character and proliferating activity. This was supported by the fact that IL-7 caused increased activation of CD4 T cells in SF as compared with cells from PB. This impaired suppressive function of CD25^{+}CD127^{−} CD4 T cells, and increased levels of IL-7 might contribute to the persistent arthritis in RA patients.

> ### Rheumatology key messages
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> - Increased numbers of (CD4^{+}CD25^{+}CD127^{−}) Tregs are present in inflamed RA joints.
> - CD25^{+}CD127^{−} Tregs from RA patients show decreased capacity to suppress IL-7R^{+} responder T cells.
> - IL-7 causes increased activation SF CD4 T cells as compared to cells from PB.

Acknowledgements

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