Increased IL-17 production by peripheral T helper cells after tumour necrosis factor blockade in rheumatoid arthritis is accompanied by inhibition of migration-associated chemokine receptor expression

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Abstract

Objectives. The contribution of IL-17-producing Th17 cells to the pathogenesis of T-cell-mediated inflammatory disorders such as RA and atopic dermatitis (AD) has to be viewed in relation to the role of Th1/Th2 cells and long-recognized key cytokines like TNF. We aimed to study the frequency and migration-associated phenotype of peripheral Th17, Th1 and Th2 cells in healthy individuals, RA and AD patients, and to study the influence of anti-TNF therapy in RA.

Methods. Intracellular IL-17, IFN-γ and IL-4 production and CC-chemokine receptor CCR4 and CCR6 expression were analysed flow cytometrically in peripheral memory Th cells from healthy individuals, AD and RA patients. The latter were grouped by disease activity and presence or absence of adalimumab therapy. In RA patients initiating anti-TNF therapy, cytokine production by in vitro-stimulated peripheral mononuclear cells was measured by cytometric bead array.

Results. The peripheral Th17 cell frequency is elevated in AD but not in RA. In RA, Th17 cells and IL-17 production increase after anti-TNF therapy, irrespective of disease activity. Th1 cells and IFN-γ production are elevated in remission and under anti-TNF therapy. CCR6 expression is up-regulated in Th17 cells, but RA patients in remission under anti-TNF therapy have significantly lower expression than those with active disease.

Conclusions. The increase in peripheral Th17 cells in RA patients after anti-TNF therapy is accompanied by a decrease in Th17-specific CCR6 expression, which might prevent homing of these potentially pro-inflammatory cells to the synovium.

Key words: Rheumatoid arthritis, T-lymphocytes, Tumour necrosis factor, Interleukin-17, Chemokine receptors, Adalimumab.
Increased Th17 and Th1 after anti-TNF in RA

Patients and methods

Study populations

Forty-eight RA patients, fulfilling the 1987 ACR criteria for the classification of RA, were recruited from the outpatient clinic. Clinical parameters were recorded, including sex, age, disease duration, HAQ score, concomitant treatment and 28-joint DAS (DAS-28), which is based on the number of tender/swollen joints, a general health assessment and the ESR or the acute-phase reactant CRP [26]. For the cross-sectional part of the study, patients were divided into a group with moderately and highly active disease (DAS-28-ESR ≥3.2) and either receiving adalimumab (40mg s.c., every other week) for at least 3 months (n = 10) or not (n = 10), and a group in remission (DAS-28-ESR ≤2.6) receiving adalimumab (n = 10) or not (n = 10). Concomitant use of NSAIDs, MTX, LFN or oral glucocorticosteroids in a low and stable dose (≤7.5mg prednisone-equivalent per day) was allowed, but changes in DMARD therapy ≤3 months before inclusion or the introduction of oral/i.m. glucocorticosteroids ≤4 weeks before inclusion served as exclusion criteria, as well as age ≥75 years. Additionally, a group of 10 patients with moderate to severe AD [SCORing Atopic Dermatitis (SCORAD) index ≥25] [27] was studied. Exclusion criteria was the use of oral glucocorticosteroids or any immunosuppressive drug. For the longitudinal part of the study, a cohort of eight RA patients was included before the initiation of adalimumab therapy. The decision to refer patients for adalimumab therapy was made by the treating physician, based on the presence of eight or more inflamed joints and a HAQ of at least 15/60 despite adequate treatment with two or more DMARDS including MTX for at least 3 months. Exclusion criteria similar to those for the cross-sectional study set-up were used and the DAS-28-CRP was used to evaluate disease activity.

Sample collection and isolation of peripheral blood mononuclear cells

In the cross-sectional RA and AD patient groups and matched controls, heparinized peripheral whole-blood samples (Vacutainer; Becton Dickinson, Meylan, France) were processed within 3h of sampling. In the RA patient cohort and matched controls, peripheral blood samples were collected on EDTA just before the first administration of adalimumab and 12 weeks later, immediately preceding the seventh injection. Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque-1077 (Sigma Aldrich, Bornem, Belgium) density gradient centrifugation. The dilution and washing buffer was calcium/magnesium-free Dulbecco’s PBS (D-PBS; Gibco Invitrogen, Merelbeke, Belgium) containing 10 mM EDTA. Samples (Vacutainer; Becton Dickinson, Meylan, France) were collected on EDTA just before the first administration of adalimumab and 12 weeks later, immediately preceding the seventh injection. Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque-1077 (Sigma Aldrich, Bornem, Belgium) density gradient centrifugation. The dilution and washing buffer was calcium/magnesium-free Dulbecco’s PBS (D-PBS; Gibco Invitrogen, Merelbeke, Belgium) containing 10 mM EDTA.

Sample preparation for intracellular cytokine and surface epitope detection

One millilitre of whole blood was incubated with phorbol-12-myristate-13-acetate (PMA; Sigma, St Louis, MO, USA) and ionomycin (Sigma), at a final concentration of 10^{-6} M each, and brefeldin A (10 μg/ml) for 6h in a CO_2 incubator at 37°C. One-hundred microlitres of freshly collected and stimulated whole blood were stained with CD8-APC, CCR4-PE-Cy7, CCR6-PE, CD45RA-PE-Cy5, CD3 AmCyan (BD Biosciences, Erembodegem, Belgium) for 15 min at 4°C. Two millilitres of FACS Lysis solution (BD Biosciences) were added for 20 min at room temperature to lyse the red blood cells and fix the leucocytes. After washing in 500 μl PBS, cell membranes were made permeable with saponin 0.3% (Sigma Aldrich) and incubated with anti-cytokine antibodies [IL-4-AF488, IFN-γ-V450,
Cells were washed with 500 μl of saponin 0.3% and re-suspended in 300 μl of PBS for flow cytometric analysis.

Flow cytometric cellular analysis

Fifty thousand cells were analysed on a FACSCanto II flow cytometer (BD Biosciences) with FACSDIVA software (BD Biosciences). Analysis gates were set on lymphocytes according to forward and sideways scatter properties. CD3+CD8− lymphocyte populations were considered Th cells and further differentiated into memory (CD45RA+ and naive (CD45RA−). Because both CCR4 and CCR6 expression and cytokine production (IL-4, IFN-γ and IL-17) are largely confined to memory cells, results were expressed as the percentage of CCR-expressing or cytokine-producing CD3+CD8− CD45RA+ lymphocytes. Furthermore, cytokine expression in subgroups of CCR4+, CCR6+ and CCR4+CCR6+ cells was analysed.

Culture of PBMCs and measurement of extracellular cytokine production

Freshly isolated PBMCs were concentrated at 3 × 10⁶ cells/ml in X-VIVO 15 medium (Lonza, Verviers, Belgium) and cultured in 24-well plates for 24 h at 37 °C and 5% pCO₂, in the presence of soluble anti-CD3 (HIT3a; BD Biosciences) and anti-CD28 (CD28.2; BD Biosciences) 5 μg/ml each. Supernatant of cultured PBMCs was stored at −20 °C. All undiluted samples were measured simultaneously on a FACSCalibur flow cytometer (BD Biosciences) using Cytometric Bead Array Flex Sets (BD Biosciences) for IL-4, IFN-γ, IL-17 and TNF-α, according to the manufacturer’s instructions. Data were analysed using FCAP Array software version 1.0.1 and are expressed as pg/10⁶ PBMCs.

Statistical analysis

Results were expressed as mean and s.e.m. or as geometric mean with 95th percentile CI in case of logarithmic transformation. Student’s t-test and analysis of variance were used where appropriate with Tukey’s corrected post hoc testing for a significance level of 0.05. Statistical analysis was done with SPSS software version 16.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics and effect of adalimumab therapy on disease activity

The clinical parameters of each group of patients and healthy individuals are listed in Tables 1 and 2. In the cross-sectional part of the study (Table 1), age was not significantly different between the RA groups. Adalimumab-treated patients generally had long-standing disease, and those with high disease activity had significantly longer disease duration than patients in remission with adalimumab (P = 0.011). All AD patients had acute as well as chronic lesions. Six of eight patients in the RA cohort (Table 2) were good responders to adalimumab therapy according to the EULAR criteria [28] and achieved disease remission (DAS-28 <2.6). The other two patients, including one that had previously received another anti-TNF treatment, showed a moderate response.

| Table 1 Cross-sectional part of the study: patient characteristics and disease activity |
|---------------------------------|----------------|-----------------|-----------------|-----------------|----------------|
| Group description               | Age, years     | Disease duration, months | DAS-28-ESR | HAQ, %    | SCORAD       |
| Healthy controls (matched to RA) (n=10) | 54 (2)         | 82 (25)          | 4.6 (0.3)     | 33 (7)      |
| Active RA (n=10)                | 61 (4)         | 35 (9)           | 1.3 (0.2)     | 4 (3)       |
| RA remission (n=10)             | 60 (4)         | 233 (44)         | 3.8 (0.2)     | 28 (4)      |
| Active RA anti-TNF (n=10)       | 59 (3)         | 110 (25)         | 1.9 (0.2)     | 12 (5)      |
| RA remission anti-TNF (n=10)    | 63 (3)         | ND               | 1.9 (0.2)     | 12 (5)      |
| Healthy controls (matched to AD) (n=10) | 38 (4)         | ND               | 1.9 (0.2)     | 12 (5)      |
| AD (n=10)                       | 32 (3)         | ND               | 1.9 (0.2)     | 12 (5)      |

Data are represented as means (s.e.m.). ND: no data available.

| Table 2 Longitudinal part of the study: patient characteristics and disease activity |
|---------------------------------|----------------|-----------------|----------------|----------------|
| Group description               | Age, years     | Disease duration, months | DAS-28-CRP | HAQ, %    |
| Healthy controls (n=8)          | 57 (4)         | 90 (24)          | 5.4 (0.3)     | 64 (6)      |
| RA before anti-TNF (n=8)        | 56 (5)         | 2.7 (0.4)        | 24 (7)        |

Data are represented as means (s.e.m.).
Intracellular cytokine production in memory Th cells from RA patients, AD patients and healthy individuals

Production of IL-4, IFN-γ and IL-17 was mutually exclusive in individual memory Th cells after whole-blood PMA/ionomycin stimulation, since virtually no double-positive staining cells were detected (Fig. 1A). The production of IL-4 was not significantly different in RA or AD patients compared with healthy individuals, and in RA it was not influenced by disease activity or treatment (Fig. 1B). In contrast, IFN-γ production differed significantly between the RA groups (overall \( P = 0.018 \)) (Fig. 1C). It was particularly low in active RA patients compared with patients in remission under anti-TNF therapy, with a tendency towards higher values in remission (irrespective of anti-TNF therapy), under anti-TNF therapy (irrespective of disease activity) and in healthy individuals. AD patients had similar IFN-γ production to the healthy control group. IL-17 production was clearly higher in RA patients under anti-TNF therapy than in RA patients without such therapy or in healthy individuals, but there was no effect of disease activity (Fig. 1D, overall \( P < 0.001 \)). Significantly increased IL-17 production was also found in AD patients compared with healthy individuals.

Cytokine production by cultured PBMCs from healthy individuals and RA patients initiating anti-TNF therapy

To confirm the findings from the cross-sectional study, we used a different technique for cytokine detection to follow-up on RA patients initiating anti-TNF therapy. After stimulation of PBMCs with anti-CD3/anti-CD28 for 24 h, the levels of IL-4 in the supernatant were similar in healthy individuals (44 pg/10^6 PBMCs; 95th percentile CI: 22, 91) and RA patients (26 pg; CI: 14, 48) and were not influenced by adalimumab therapy (29 pg; CI: 17, 48) (Fig. 2A). Production of IFN-γ was significantly lower in RA patients (44 pg; CI: 21, 92) than in healthy individuals (178 pg; CI: 89, 353) and increased significantly after adalimumab therapy (79 pg; CI: 39, 158) (Fig. 2B). IL-17 production was equal in healthy individuals (83 pg; CI: 42, 166) and RA patients (115 pg; CI: 60, 218) and doubled after adalimumab therapy (212 pg; CI: 142, 315) (Fig. 2C). In individual RA patients, the level of IL-17 was not significantly correlated with the level of TNF-α \( (r = 0.31) \).

Chemokine receptor expression in memory Th cells from RA patients receiving anti-TNF therapy

To explore the relevance of the increased IL-17 production and Th17 cell frequency in anti-TNF-treated RA patients, expression of T-cell homing receptors CCR6 and CCR4 was determined in all memory Th cells and within the subpopulations of cytokine-producing cells from RA patients with active disease or in remission under anti-TNF therapy (Fig. 3). An overall significant difference in CCR6 expression was observed between these subpopulations (Fig. 3A), demonstrating enriched CCR6 expression in IL-17-producing cells and relatively low CCR6 expression in IFN-γ-producing cells compared with the whole of the memory Th cell population, in both RA patients (under anti-TNF therapy) with active disease and those in remission. However, patients in remission had significantly lower CCR6 expression than patients with active disease, not only in IL-17 but also in IFN-γ-producing cells (Fig. 3A). In contrast to CCR6, CCR4 expression decreased after PMA/ionomycin stimulation to values ~10% of memory Th cells in both RA groups. Although CCR4 was quite expectedly up-regulated on IL-4-producing cells (Fig. 3B), there was no enrichment of CCR4 expression in IL-17-producing cells and no effect of TNF inhibition. The fraction of CCR4/CCR6 double-positive cells was small and indiscriminate between the groups.

**Discussion**

While RA and AD were previously considered Th1- and Th2-mediated immune disorders, respectively, it is now increasingly being propagated that pro-inflammatory Th17 cells are recruited into the target tissues early in the immune cascade [29]. First of all, we found an elevated peripheral Th17 cell frequency in patients with acute and chronic AD lesions, which confirms an earlier report [13] and suggests a role for Th17 also at later time points in the relapsing-remitting process of AD. In contrast, patients with established RA did not display such elevation of peripheral Th17 cells, despite the presence of active inflammation. This might be explained by the fact that most of them had longstanding disease and that they were nearly invariably treated with DMARDs, since synovial IL-17 levels were shown to be elevated only in the earliest stage of RA, before the start of DMARD therapy [10]. Some authors have indeed also found similar peripheral IL-17 production to that in healthy individuals [11, 30, 31], while others reported increased values [17, 32–35].

An important issue remains the position of Th17 cells in the network of classical pro-inflammatory mediators such as TNF and IFN-γ. Th17 cells induce TNF production by macrophages [4], but TNF does not induce IL-17 production itself [36]. In mice, the pro-inflammatory effect of IL-17 depends on TNF, except during ongoing arthritis, where IL-17 gains partial TNF independence [37]. IFN-γ is a strong inhibitor of Th17 differentiation [38–40] and IFN-γ regulates susceptibility to IL-17-induced experimental arthritis [5, 41]. In humans, IFN-γ production has repeatedly been demonstrated to increase after TNF inhibition therapy [42–44]. Therefore, we hypothesized that peripheral Th17 cells would decrease after successful TNF inhibition therapy. Our data confirm that Th1 cells and IFN-γ production increase in RA patients under anti-TNF therapy, but we unexpectedly also found that the frequency of peripheral Th17 cells is dramatically lower under anti-TNF therapy, irrespective of disease activity. A study by Yue et al. [35] did not show an increase after starting adalimumab monotherapy for active RA, but an important difference with our study is that their patients were not on DMARDs before inclusion in the study. Other studies have only evaluated actual IL-17 production and
Fig. 1 Intracellular cytokine production in memory Th cells from RA patients, AD patients and healthy individuals. (A) Representative density plots of PMA/ionomycin-stimulated whole-blood sample from one patient with active RA under anti-TNF therapy, illustrating the mutual exclusivity of IL-4, IL-17 and IFN-γ production. (B) Intracellular IL-4 production, expressed as percentage of memory Th cells, in distinct groups of RA patients, AD patients and healthy individuals (control). (C) Intracellular IFN-γ production in memory Th cells. (D) Intracellular IL-17 production in memory Th cells. Statistical analysis included one-way analysis of variance for comparison of healthy individuals and RA patients, followed by Tukey's post hoc corrected tests, or Student's t-test for comparison of AD patients with healthy individuals. Bars represent the mean. *P < 0.001 for comparison of active RA under anti-TNF vs controls or RA not under anti-TNF. **P < 0.004 for comparison of RA in remission under anti-TNF vs controls or RA not under anti-TNF.
not circulating Th17 cells [32, 34, 45]. Interestingly, the increase in Th17 cells in anti-TNF-treated RA patients is in accordance with a recent observation by Notley et al. [46] in mice. They found that TNF blockade induces an increase in peripheral Th1 and Th17 cells in experimental arthritis, since TNF exerts a negative feedback on IFN-γ and IL-17 production through down-regulation of the common IL-12/IL-23 p40 unit, which is essential in the differentiation programme of Th1 and Th17 cells [46]. Down-regulation of p40 production by macrophages and dendritic cells in response to TNF has been described before [47]. Although the induced Th1 and Th17 cells are potentially pathogenic, they do not exacerbate arthritis since their accumulation in the joint is inhibited simultaneously [46]. The effect of in vivo TNF blockade on T-cell accumulation in the synovium [48] and expression of homing receptors [42, 43, 49, 50] has long been recognized, but it has never before been evaluated in Th17 cells. We demonstrated lower Th17-specific CCR6 expression in RA patients who were successfully treated with adalimumab, compared with those with persistently active disease. CCR6 plays an important role in the recruitment of Th17 cells to the inflamed synovium [18, 19, 21]. Therefore, our findings together with the reported decrease in serum levels of CCL20 after anti-TNF therapy [51] might explain why the increase in potentially pathogenic peripheral Th17 cells after TNF inhibition therapy does not cause increased disease activity. However, the high Th17-specific CCR6 expression in patients with persistently active RA under anti-TNF therapy might be a reflection of TNF-independent pro-inflammatory effects of IL-17, which have been observed in animal models.
of arthritis [37]. Notwithstanding potential safety issues, one could speculate that these patients might benefit from combined TNF and IL-17 inhibition therapy. The lower TNF levels in AD compared with RA explain why the TNF-induced negative feedback loop that allegedly opposes peripheral Th17 cell differentiation in RA is absent in AD. Consequently, higher numbers of circulating Th17 cells can be observed in active AD. A caveat is that in our study, although IL-17 production by in vitro-stimulated PBMCs was increased in RA patients under anti-TNF therapy, there was no inverse correlation between the levels of TNF and those of IL-17. This illustrates that the timing and localization of this presumed TNF-mediated regulatory process are still unknown.

In conclusion, we found a dramatic increase in peripheral Th17 cells in RA patients receiving TNF inhibition therapy, accompanied by a decrease in both Th17- and Th1-specific CCR6 expression. Further research is necessary in order to find out whether this might prevent homing of these potentially pathogenic cells to the joint and subsequent exacerbation of synovial inflammation.

**Rheumatology key messages**

- Anti-TNF therapy increases the number of peripheral Th17 and Th1 cells in RA.
- CCR6 expression, responsible for trafficking of Th17 to inflamed synovium, is low in anti-TNF-induced remission.

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