# INVESTIGATION OF THE ADAPTIVE IMMUNE RESPONSE IN IMMUNE-MEDIATED DISEASES

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# 1. Introduction

Rheumatoid arthritis (RA), ankylosing spondylitis (AS) and inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) are chronic immune-mediated diseases affecting large proportions of the population, and leading to serious acute inflammatory conditions and, if insufficiently controlled, to chronic irreversible organ damage and premature death. An important link among these diseases is the pivotal role of tumor necrosis factor-α (TNF-α) in the pathogenesis, and a consequent high benefit of biological therapies targeting TNF-α. The adaptive immune system, which is composed of several specialized subtypes of T- and B-lymphocytes, is also a very important regulator of the abnormal immune processes in all four diseases. In the present thesis, I would like to summarize my research on the alterations of the composition of T-cell subsets in patients with RA, AS and IBD treated with biological therapies. My aim was to translate the results to clinically relevant findings, such as to find determinants of a good therapeutic response to these agents, and to better understand the mechanism of action of biological therapies.

## 1.1 Overview of the examined diseases

### 1.1.1. Rheumatoid arthritis

RA is the most common inflammatory rheumatic disease, affecting at least 0.5% of the population. Its hallmark is a symmetric polyarthritis, involving virtually all joints with a predilection to small joints of the hand and foot. The synovial inflammatory infiltrate is characterized by activated fibroblast-like synoviocytes, macrophages, T- and B-cells, mast cells and fibroblasts, massive production of proinflammatory cytokines, chemokines, matrix metalloproteinases, as well as pro-angiogenic mediators. The resulting synovial, cartilage and bone degradation will lead to early irreversible articular structural destruction, and without effective therapy, progressive joint damage will lead to permanent disability. The chronic inflammatory state, together with the various extraarticular manifestations, and the premature and accelerated atherosclerosis will lead to high morbidity and shortened life expectancy (1). While conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), including

methotrexate, leflunomide, sulfasalazine, (hydroxy)chloroquine, azathioprin and cyclosporine, have been effective only in a subset of RA patients, even despite a concomitant long-term corticosteroid therapy in many patients, the introduction of targeted therapies, in particular biological DMARDs (bDMARD), have provided most of the traditional csDMARD-resistant patients with the possibility of long-term remission or low disease activity. Most of the potential adverse effects of bDMARDs can be prevented or counterbalanced with cautious patient follow-up, but infections and the (probably only slightly increased) risk of malignancies, as well as the high cost of these drugs, still remain concerns. It is to be emphasized, however, that only approximately 30-35% of patients achieved an ACR70 response after 3 months of therapy in the large randomized trials of all biologicals (1). Furthermore, primary or secondary inefficacy is frequently encountered resulting in an average 2-year persistence on anti-TNFs ranging from 55 to 70% (2). It can be concluded that long-term remission or low disease activity is achieved only in a minority of patients on bDMARD therapy. The prediction of a good clinical response to the particular classes of bDMARDs would therefore be very useful, but the possibilities are quite limited. Clinical predictors of a long-term favourable therapeutic effect of anti-TNFs include male gender, younger age, shorter disease duration, better functional status, non-smoker status, anti-citrullinated peptide antibody (ACPA)- or RF-negativity and concomitant methotrexate therapy (3-6). The value of biomarkers is much less established: low pre-treatment Th17 frequency in peripheral blood (7), lack of an early upregulation of interferon-regulated genes (interferon signature) (8), and high score in a multi-biomarker disease activity test (including serum levels of 12 biomarkers, such as matrix metalloproteinases, adipokines, adhesion molecules, etc.) (9) were found to predict a subsequent favourable therapeutic response (10), but none of these have become validated tools for clinical practice yet.

The immune pathogenesis of RA can be summarized as an uncontrolled proinflammatory process, arising in genetically susceptible subjects in response to various environmental factors. The genetics of RA is polygenic, HLA-DR4 shared epitope carrier persons being at the most significantly increased risk for the development of the disease. Cigarette smoking, potentially through the alteration of the periodontal and bronchial microbiome, and the induction of the production of citrullinated peptides at these mucosal surfaces is currently regarded as the key driver of the production of ACPA, which are not only diagnostic and prognostic markers of RA, but probably have a direct pathogenic role. The presentation of citrullinated peptides and further putative antigens leads to an aberrantly intense and permanent activation of CD4 T-

helper cells, differentiating predominantly to the Th1 and Th17 phenotypic lineage, and the activated effector cells are key drivers of the complex inflammatory pathways in RA (1).

*1.1.2. Ankylosing spondylitis*

AS belongs to the group of pathogenetically and clinically related diseases called spondylarthropathies, and involves 0.2% of the adult population. Its axial manifestation implies chronic inflammation of intervertebral small joints and ligamentous insertions, with new bone formation and calcification of ligaments, eventually leading to total spinal immobility due to ankylosis of the intervertebral junctions. Peripheral manifestations include oligo- or polyarthritis, enthesitis (inflammation of insertions of tendons and ligaments) and dactylitis (sausage-like swelling of toes or fingers due to confluent arthritis, tendinitis and enthesitis). Uveitis and gut inflammation constitute the most common extraskeletal manifestations.

TNF-α-blocking agents are the mainstay of therapy, as they are able to induce long-term remission in a substantial proportion of AS patients with an inadequate response to non-steroidal anti-inflammatory drugs (NSAIDs) in axial, and to csDMARDs (in AS methotrexate and sulfasalazine are widely accepted agents) or to local or systemic corticosteroids in peripheral manifestations. Partial remission, as defined by the Assessment of Spondyloarthritis (ASAS) − PR criterion, is achieved by about 75% of patients with AS (11). Predictors of an effective anti-TNF therapy include younger age, shorter disease duration, higher C-reactive protein (CRP) levels (12), male gender, higher disease activity (measured with ASDAS score), presence of peripheral arthritis and better functional state as assessed by the Schober test (13).

AS has a strong genetic predisposition, since 90% of the patients carry the HLA-B27 haplotype. Our current understanding suggests that the axial and peripheral inflammation is driven by T-cells that excessively produce Th17 and Th1 type cytokines, e.g. interleukin-17 (IL-17), TNF-α and interferon-γ (14,48). The primary sites inflammation in both axial and peripheral musculoskeletal manifestations are the entheses, where IL-23 induces IL-17 and TNF-α overproduction from resident classical and innate-like T-cells (15).

*1.1.3. Inflammatory bowel diseases*

Both CD and UC are characterized by severe, chronic gastrointestinal inflammation leading to diarrhea, hematochezia, abdominal pain and weight loss. However, important differences distinguish the two diseases: CD may arise at any site along the gastrointestinal tract, with a marked predominance of the terminal ileum, whereas 20% of the patients develop colonic involvement. In 20% of patients, perianal manifestations such as fistulas or abscesses develop. The inflammatory process is segmental and transmural, with severe destructive sequelae with two major patterns: penetrating forms lead to fistula formation, whereas stenosing forms cause bowel obstruction. In contrast, in UC the inflammatory process affects only the mucosa of the colon and rectum, albeit in a continuous manner, with ulceration, cryptitis and microabscesses detected in histology samples. Both diseases are frequently associated with extraintestinal manifestations such as arthritis, erythema nodosum, pyoderma gangrenosum, primary sclerosing cholangitis and uveitis. Anti-TNF therapy is also beneficial in both IBD-s, but the reported incidence of primary nonresponse of 10 to 30% (16), and the annual risk of secondary nonresponse from 13% to 20.3% for adalimumab (17) warn us that more data are required for a better, personalized choice of novel therapies (18).

The immune pathogenesis is also distinct in the two diseases, although the primary abnormalities in both illnesses are a defective mucosal barrier function and an aberrant inflammatory response to gut microbiota. The dysfunctional interaction between the mucosal immune system and the bacterial microflora in the gastrointestinal tract is featured by enhanced initiation of inflammatory immune response by pattern recognition receptors in mucosal dendritic cells and macrophages, and insufficient differentiation toward induced regulatory T-cells (Tregs) (19). While CD is characterized by a skewed differentiation of naive T-cells to the Th1 and Th17 phenotype, and increased amounts of IL-12, Il-15, IL-23, and consequently TNF-α, interferon-γ and IL-17 are detectable in the gut mucosa (20). In contrast, UC the overproduction of IL-4, IL-5 and IL-13 is observed, consistent with an increased Th2 activation (20).

**1.2. T-cell subset composition in the examined diseases, and the impact of biological therapies on the adaptive immune system phenotype**

*1.2.1. Rheumatoid arthritis*

A large number of studies investigated the distribution of various T-cell subsets in RA, most of them focusing on the major effector T-helper cell phenotypic subtypes. It can be concluded that the frequency of Th1, Th2 and Th17 cells is increased (21, 22), while that of regulatory T cells (Treg) is decreased in the peripheral blood of RA patients (23-27).Increased numbers of CD4CD45RO+ memory T-cells were demonstrated in the peripheral blood of RA patients (28), and higher proportions of CD4 or CD8 memory T-cells (CD45RO+) were found to correlate with disease activity and IgM rheumatoid factor levels (29). The number of studies on frequencies of activated T-cells as characterized by the specific early (CD69), intermediate (CD25), or late (HLA-DR) activation markers is very limited, and it can be concluded that the prevalence of these subtypes is higher in RA than in healthy or osteoarthritic controls (30-33).

However, much less is known about how anti-TNF and IL-6-receptor-blocking biological affect the T-cell subset composition. Few studies, including our previous examinations (26), followed T-cell subset prevalence changes during anti-TNF treatment, but in most of them, only short term follow-up was evaluated (34-41), or involved relatively low numbers of patients (34,35,38,39), and the majority were restricted to the determination of Treg and Th17 proportions. Although most of the studies describe an increase in Treg and a decrease in Th17 frequencies (34,37,38,40), opposing results have also been published (26,36,41). As concerns the possibilities of therapeutic response prediction, Chen *et al* found that Th17 cell counts decrease in anti-TNF-treated patients who had shown a good response to therapy, whereas in non-responders, Th17 cell percentages increased (40). The number of CD8 cells was found to be unchanged during infliximab, an anti-TNF agent, but only 7 patients were investigated (42). In our previous study involving 8 weeks of short term follow-up, we have demonstrated that Th1 and Treg frequencies increased compared with the values measured at the time of the initiation of anti-TNF therapy, but the values remained significantly different from those of healthy controls (26).

Data on the effects of IL-6R blocker therapy are especially limited (43-45). Tocilizumab was found to increase the number of Tregs (43-45), to correct the increased Th17/Treg ratio

observed in active RA (45) and this change correlated with clinical improvement (43). Pesce *et al* have reported that Treg prevalence increases during tocilizumab treatment, while Th1 and Th17 frequencies did not change in their cohort (44).

### 1.2.2. Ankylosing spondylitis

Analyses of the peripheral blood T-cell composition has revealed increased percentages of circulating Th17 helper lymphocytes (46) as well as increased numbers of Th2 cells (47), but increased Th1 frequencies have also been reported (48,49). On the other hand, regulatory T-cells (Treg) display decreased prevalence in the blood of AS patients suggesting that their lack may contribute to the pathogenesis of the disease (50). The balance of Th17 and Treg cells has a major impact on T cell activities during inflammatory rheumatic diseases, such as AS (51), and an increased Th17/Treg ratio was detected in most of the examinations.

There are only few studies addressing the question of how TNF-inhibitors influence the T-cell repertoire in AS (48, 52-55). Previous studies from our research group have revealed that the ratio of naïve CD4CD45RA cells decreased, and of memory CD4CD45RO cells increased after 6 weeks of infliximab therapy (54). In a large number of patients, Xiey *et al* have focused on Th17 and Treg cells, and demonstrated that the prevalence of Th17 decreased and that of Tregs increased in responders, and these values changed exactly in the opposite direction in non-responders (52). Th17 and Th1 cell subsets in anti-TNF-treated patients were lower than in anti-TNF-naive patients and were similar to healthy controls (48). In contrast, another study on 15 AS patients has reported on an increase in Th17 cell prevalence in anti-TNF-treated patients (53). In contradiction to the previous investigations, Liao *et al* have detected increased Treg frequencies in 15 AS patients, moreover adalimumab therapy has reduced the Treg prevalence (55). Important limitations are, however, that most of the existing examinations have examined only the role of Tregs and/orTh17 cells (52,53,55), and many of the available articles did not make a distinction between peripheral and/or axial AS, and they use non-radiographic spondyloarthritis as an early stage of AS (49,56) rather than as an individually existing entity. Importantly, only one study analyzed patients with relatively long-standing anti-TNF treatment (6 months) (52), whereas two further investigations, including our previous work, followed the T cell repertoire only for short-term (6 weeks) (53,54), although we have already demonstrated in our preceding study of RA patients that there are profound differences in the T cell subset distribution as measured after short-term or long-term follow-up (57).

*1.2.3. Inflammatory bowel diseases*

Due to its accessibility, much of the studies on the adaptive immune system in IBD took advantage of the possibility of examining the target tissue, i.e. the gastrointestinal mucosal processes. As mentioned before, the gut-associated lymphoid tissue (GALT) of CD patients contains more Th1 cells, predominantly secreting IFN-γ, as measured by ELISPOT assay, whereas that of UC patients display an abundance of Th2 cells (58). Later, the importance of Th17 cells has also emerged in both IBD-s. In a parallel analysis of intestinal and peripheral blood T-cell phenotype, upregulation of Th17 cells and Tregs in active inflammatory lesions in patients with both CD and UC was detected as compared with quiescent/mildly inflamed lesions and healthy tissue (59). However, more and more data are accumulating that, in addition to classical Th17 cells, the major producers of IL17 are in fact novel and unconventional T-cell subtypes, such as IFN-γ+ IL17+ coproducing CD4 T cells, that share the transcriptional and cytokine-producing phenotype of Th1 and Th17 cells. Furthermore, these cells may be specifically involved in intestinal inflammation in CD and UC (60). Other such non-conventional cells develop during the bidirectional transition of Th17 and Treg cells, a phenomenon that has excessively been described (61). The importance of this plasticity in IBD was confirmed by Li *et al*, who demonstrated high amounts of IL17+RORγ+Tbet+Gata3+FoxP3+ "crossover" T-cells (Treg cells that simultaneously carry the phenotype of other Th subtypes) in the lamina propria of IBD patients, and the prevalence of Treg/Th2 crossover subpopulations was associated with clinical disease scores of both IBD-s (62).

Deviations in the peripheral blood T-cell subtypes have also been addressed. Decreased Treg frequency has been demonstrated in several studies both in UC and CD (63-67). In 46 Chinese CD patients, Th1 and Th17 proportions were found to be higher, whereas that of Tregs was lower than in healthy controls (68). The percentages of circulating Th1 (CD3+CD8-IFN-γ+) and type-one cytotoxic T cell (Tc1) (CD3+CD8+IFN-γ+) cells were detected to be higher in patients with active UC than in patients with inactive UC and normal controls, although levels were lower than those in CD (60). HLA-DR+ CD4 and CD8 activated T-cells were found to be increased in the peripheral blood in active IBD (70). The IL17 and FoxP3 double-expressing T-cells, mentioned in the previous paragraph, were also demonstrated in the peripheral blood of both CD and UC patients, moreover, with higher prevalence compared with age- and gender-matched controls (71), and the ability of Treg cells to suppress autologous T-cell proliferation was also reduced in this study. Another investigation with potential clinical correlation has found that a decreased CD8CD28+/CD8CD28- ratio indicates a poor prognosis

in CD (72). A hypothetical explanation for this finding was that these CD8CD28- regulatory CD8 cells shift from the intestinal tissue to the circulation.

During infliximab therapy, Treg cell frequencies did not display any change in the 20 investigated CD patients during 42 days of follow-up (73) or in 26 CD patients after 26 weeks of therapy (74). Treg prevalence was similar to controls both at baseline and at the end of follow-up during anti-TNF therapy. In contrast, Di Sabatino *et al* have found in their measurements on 20 CD patients that Treg frequency at the start of anti-TNF therapy was significantly higher in CD patients who later proved to be nonresponders than in responder patients. Clinical improvement in 12 CD patients was associated with a significant increase of Treg frequency after 10 weeks (75). Another study also obtained similar results (76). In contrast, in the study of Dige *et al*, high baseline Treg levels were predictive of a good response to anti-TNF (74). In further contradiction, Li *et al* and also Guidi *et al* did find an increase of Treg prevalence during infliximab therapy, but this was significantly more pronounced in responders (77,78), or was detectable only in responders (79). Moreover, clinical response was associated with a parallel decrease of Tregs in the inflamed mucosa (77). Treg frequency also increased when it was measured 14 days after a single infliximab infusion (80). Importantly, all three groups analyzed CD and UC patients taken together. Grundström *et al* followed the levels of Th1, Th2, Th17, Treg (FoxP3+), CD69 and CD25 positive cells during adalimumab treatment in 20 IBD patients, and found no change over 6 weeks of therapy (81). Again, the results of CD and UC patients were not analyzed separately, and the authors reported that they detected no CD69 staining on peripheral blood lymphocytes, only in mucosal biopsies performed on 4 of the patients, in whom the proportion of this subset increased in the inflamed tissue by 6 weeks.

## 2. Aims

In view of the cardinal role of T cells in host defense, anti-tumor surveillance and immune homeostasis, we regarded it important to know how the composition of the T cell repertoire is skewed during TNF-blocker therapies, since these agents are usually applied for several years. The majority of studies on the effect of anti-TNF or – in RA – IL6R-blocker therapies on the T-cell homeostasis carry the limitations of low patient number, a restricted focus on selected T-cell subtypes – most often on effector T-helper cell phenotypic subsets – instead of a comprehensive overview, and of short follow-up– although changes in T-cell subpopulation proportions are expected to occur after longer time in view of the life-span of the cells. We

therefore aimed at the investigation of a wide scope of T-cell subtypes in anti-TNF-treated patients stratified as responders and non-responders, after a sufficiently long treatment duration. Our purpose was to gain a more precise insight into the mechanism of action of anti-TNF drugs, and, through the correlation of the laboratory data with the clinical characteristics and disease course, to find predictors to a favourable therapeutic response to these agents.

Specifically our aims were:

1. To recruit RA, AS, CD and UC patients with active disease before the initiation of anti-TNF – and in RA, IL6R-blocker – biological therapy

2. To measure the prevalences of total CD4 and CD8 cell proportions, and frequencies of naïve and memory, early (CD69+), intermediate (CD25+) and late (HLA-DR+) activated subsets within both CD4 and CD8 cells, and Th1, Th2, Th17 and Treg subtypes.

3. To perform repeat measurements after at least 3 months of biological therapy

4. To compare the baseline results with a) healthy controls, b) treatment-naïve newly-diagnosed cases with active disease

5. To compare the follow-up results a) between patient subgroups of responders and non-responders to biological therapy, b) with baseline values from the same patient, c) and with our previous results of short-term-treated patients (in RA and AS)

6. To correlate the results with the clinical parameters and disease course on biological therapy, and to seek for predictor biomarkers for therapeutic response

## 3. Patients and methods

### 3.1. Rheumatoid arthritis

In the rheumatoid arthritis study 92 patients were evaluated, the disease was classified according to the 2010 ACR/EULAR Classification Criteria for Rheumatoid arthritis (82). Forty-nine patients were treated with anti-TNF therapy (adalimumab: 14, certolizumab pegol: 14, etanercept: 11, infliximab: 6, golimumab: 4 patients), and 43 with the anti-IL-6R agent tocilizumab. Within the anti-TNF-treated patients, we distinguished two groups; anti-TNF-responder and anti-TNF-non-responder patients, in the tocilizumab group we only evaluated responder patients, because of the low number of non – responder ones. For define therapeutic response, we used the EULAR good response criteria (83), therefore in the responder group,

patients had a DAS28 score of ≤3.2, and the improvement was >1.2 in DAS28. The detailed clinical data and patient characteristics are presented in *Table1*. For the control group we included 30 age- and gender-matched healthy volunteers. Informed consent was signed by all participants, and the protocol had been approved by the Ethics Committee of the Ministry of Health of Hungary and ethical committee of the University of Szeged (ETT-TUKEB905/PI/09).

| | Anti-TNF responders (n=30) | Anti-TNF non-responders (n=19) | Anti-IL-6R responders (n=43) | Newly diagnosed untreated RA (n=19) |
|---|---|---|---|---|
| Mean age (range) | 57.2 (26-75) | 55.7 (29-71) | 57.5 (34-76) | 48.3 (22-67) # |
| Gender (female/male) | 19/11 | 12/7 | 28/15 | 11/8 |
| Anti-TNF treatments n(%) adalimumab | 30 (100.0) | 19 (100.0) | | |
| certolizumab | 10 (33.3) | 4 (21.1) | | |
| etanercept | 6 (20.0) | 8 (42.1) | | |
| infliximab | 7 (23.3) | 4 (21.1) | | |
| golimumab | 4 (13.3) | 2 (10,5) | | |
| | 3 (10.0) | 1 (5,3) | | |
| Mean disease duration (range) | 11.3 (2-33) yrs | 10.8 (4-44) yrs | 12.0 (2-34) yrs | 2.7 (1-3) months |
| Mean duration of current biologic therapy - months (range) | 29.8(6-52) | 30.95(6-50) | 33.0 (6-48) | - |
| Prior use of TNF inhibitors | 3/30 | 6/19 | 29/43 | - |
| No. of switching.n: 1 | 3/3 | 4/6 | 15/26 | |
| 2 | - | 2/6 | 9/26 | |
| 3 | - | - | 5/26 | |
| Patients on corticosteroid therapy n (%) | 8 (26.7) | 8 (42.1) | 15 (40.5) | - |
| Patients on conventional DMARD therapy n (%) | 26 (86.7) | 15 (79.0) | 23 (62.2)* | - |
| ACPA-positivity n (%) | 24 (85.4) | 14 (82.4) | 21 (41.4)* | 19 (100.0) |
| DAS28 score (mean±SD) | 2.2 ± 0.8 | 5.12 ±1.29+ | 1.89 ± 0.87 | 7.71±4.06# |

*Table 1. Clinical data and patient characteristics.*

*\* p<0.05 between anti-TNF-responders and IL6R-blocker responders. + p<0.05 between anti-TNF non-responders and IL6R-blocker responders, #p<0.05 between newly diagnosed untreated RA group and all other groups; DMARD: disease-modifying anti rheumatic drug,*

*ACPA: anti-citrullinated protein antibody, DAS28: Disease Activity Score with 28 joints, TNF: tumor necrosis factor- α, IL-6R: interleukin-6 receptor*

### 3.2. Ankylosing spondylitis

Twenty-two AS patients were enrolled in this study, who had been treated with anti TNF therapy for more than six months. AS was classified according to the Modified New York Criteria for AS (84). We distinguished anti-TNF-responder or anti-TNF-non-responder patients; and for the definition of the therapeutic response, we used the ASAS 5/6 improvement criteria (85) and for remission we followed the ASAS remission criteria set (86). Among these examined patients in the responder group, 6 received infliximab, 7 adaimumab, and 2 etanercept. In the non– responder group, there were 3 infliximab-, 4 adalimumab-, one etanercept- and one golimumab-treated patient. We recorded the HLA-B27 status, the presence of peripheral arthritis (in addition to the axial involvement), enthesitis, uveitis, inflammatory bowel disease (IBD) ever occurring during the course of the disease, the grade of sacroiliitis and the presence of AS-specific radiographic changes on the spine (syndesmophyte or bamboo-spine), and smoking ever in every patient. Parameters of disease activity (C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), BASDAI) and functional status (BASFI) were also registered. In the control group we enrolled 10 age- and gender-matched healthy volunteers as we did in our previous study. Informed consent was signed by all participants. Study protocol had been approved by the Ethics Committee of the Ministry of Health of Hungary and ethical committee of the University of Szeged (ETT-TUKEB905/PI/09). We compared the long term effects of biological treatment with treatment naive and short term treated AS patients using our previous work (54).Clinical characteristics are detailed in *Table 2*

| | anti-TNF treated responders (n=15) | anti-TNF treated non-responders (n=7) |
|---|---|---|
| **Age (yrs)** | 46 (31-63) | 41 (30-57) |
| **Disease duration (months)** | 15 (9-40) | 18 (940) |
| **anti-TNF therapy duration (months)** | 6 (6-12) | 6 (6-8) |
| **Smoking ever** | 7 (47) | 3 (43) |
| **HLA-B27 positivity** | 11 (73) | 5 (71) |
| **Presence of peripheral arthritis** | 10 (67) | 3 (43) |
| **Presence of enthesitis** | 2 (13) | 3 (43) |
| **Presence of uveitis** | 5 (33) | 1 (14) |
| **Presence of IBD** | 2 (13) | 1 (14) |
| **Radiographic SI index** | 2.67 (2-4) | 2.57 (2-4) |
| **Presence of radiographic abnormalities on the spine** | 10 (67) | 5 (71) |
| **CRP \*(mg/l)** | 7 (2-36) | 37 (2-100) |
| **ESR \*(mm/h)** | 12 (1-39) | 42 (5-67) |
| **BASDAI \*** | 1.45 (0,3-2.5) | 5.8 (4.1-6.8) |
| **BASFI \*** | 2.0 (0.5-2.8) | 5.67 (4.0-7.7) |

*Table 2. Clinical characteristics of long-term anti-TNF-treated AS patients*

*Numbers indicate mean (range) or absolute numbers (%). AS: ankylosing spondylitis IBD: inflammatory bowel disease, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, BASDAI: Bath Ankylosing Spondylitis Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index. * p<0.05*

*3.3 Inflammatory bowel disease*

We enrolled 114 IBD patients, who were all treated at First Department of Internal Medicine University of Szeged. The first part of this study was a prospective follow-up of CD and UC patients, in whom anti-TNF therapy was initiated. The second one was a cross-sectional comparison of the immune phenotype of IBD patients on established anti-TNF therapy, grouped as anti-TNF-responders and non-responders; these data were compared with results from active, therapy-naïve and inactive (treated) IBD patients.

In the prospective part CD and UC patients (n=16 for each disease) were enrolled, in whom the disease was active despite therapy with non-biologic immunosuppressive therapies, and in whom anti-TNF therapy was initiated after blood sampling (abbreviated as CDstart or UCstart group). All patients had a Crohn's Disease Activity Index (CDAI) (87) > 220 (88) or Mayo score> 6 (89). Patients were classified to disease subsets following the Montreal classification criteria (90).

During subsequent follow-up, treatment was conducted following the best clinical judgement of the treating physician, and data on the subsequent disease activity and therapy were recorded. Short-term (3 months) and long-term (12 months) response to anti-TNF therapy was assessed, with response defined as a reduction of > 50% in CDAI, or, for UC patients, in the Mayo score, or - when endoscopy was not performed at the time of assessment – in the partial Mayo (pMayo) score (91,92) without an increase in the anti-TNF dose above standard dose. Non-response (primary or relapse) was established if these activity index-based criteria were not fulfilled, or, based on the clinical assessment of the treating physician, corticosteroid had to be introduced, or its dose had to be increased, or anti-TNF dose had to be increased overstandard dose, or surgical intervention has become necessary, or anti-TNF therapy had to be switched to either another anti-TNF or to a biological of different class. The length of the response period was also recorded. For reimbursement requirements, in some patients at stable remission after 12 months of anti-TNF therapy, the drug was discontinued, and only non-biological maintenance therapy was continued. Disease course after such forced therapy withdrawal was also followed-up. In 6 patients from each disease group, control blood sampling was performed after at least 3 months of anti-TNF therapy, and the same laboratory examinations were repeated.

In the cross-sectional part, patients on established (> 3 months' duration) anti-TNF therapy were also involved, and their results were compared with those of the members of CDstart or UCstart groups. Thirty-one patients with CD and 16 with UC were included, and were

distinguished as responders (CDresp or UCresp) or non-responders (CDnonresp or UCnonresp) to anti-TNF therapy as defined following the response criteria described in the previous paragraph. The six patients in either the CDstart or UCstart groups, in whom repeated laboratory examinations were performed during anti-TNF treatment, were also involved in the responder or non-responder groups for comparison with the anti-TNF starter groups.

As controls, the following groups were formed and analyzed: 1) patients with newly diagnosed, active, untreated CD or UC: CDnew (n=7), UCnew (n=7); 2) patients with inactive disease controlled with non-biologic immunosuppressive drugs: CDinact (n=14), UCinact (n=7), and 3) age- and sex-matched healthy controls (n=30). All participants gave their informed consent, and the study was conducted in concert with the principles of the Helsinki declaration.

The demographic and most important clinical data of the patients in all subgroups are summarized in *Table 3*.Patient subgroups for both diagnoses were similar in terms of age, disease duration, frequency of extraintestinal manifestations, type of anti-TNF, other therapies ever used, or smoking status. Although there were numerical differences among the groups in terms of gender distribution, these were not statistically significant. However, CDinact patients less frequently had perianal manifestations than anti-TNF-treated CD patients (both responders and non-responders), and anti-TNF therapy duration was shorter in UCnonresp patients than in UCresp patients.

| | CDstart (n=16) | CDresp n=23 | CDnon resp n=8 | CDnew n=7 | CDinact n=14 | UCstart n=16 | UCresp n=10 | UCnon resp n=6 | UCnew n=7 | UCinact n=7 |
|---|---|---|---|---|---|---|---|---|---|---|
| Age | 30 (18-48) | 39 (20-66) | 31 (19-48) | 31 (25-44) | 37 (23-59) | 42 (20-65) | 40 (19-59) | 38 (21-53) | 36 (23-48) | 43 (26-61) |
| Female/male | 7/9 | 13/10 | 3/5 | 3/4 | 2/12 | 7/9 | 3/7 | 5/1 | 3/4 | 1/6 |
| Disease duration (month) | 82 (7-236) | 132 (8-410) | 121 (17-235) | 14 (0-72) | 77 (5-204) | 105 (6-389) | 117 (11-290) | 75 (13-180) | 3 (1-6) | 134 (35-288) |
| Any extra-intestinal manifest. | 1 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 1 | 1 |
| Smoking never past present | 5<br>1<br>8 | 9<br>2<br>9 | 4<br>1<br>3 | 5<br>0<br>1 | 9<br>1<br>3 | 8<br>6<br>1 | 5<br>2<br>1 | 3<br>1<br>1 | 5<br>1<br>1 | 0<br>3<br>2 |
| CD Montreal class. A1/2/3 L1/2/3/4 B1/2/3 Perianal Y/N | 2/12/2<br>2/2/10/2<br>5/6/5<br>8/8 | 3/16/4<br>2/3/16/2<br>6/9/8<br>16/7 | 1/7/0<br>1/0/7/0<br>3/2/3<br>6/2 | 0/7/0<br>3/2/2/0<br>3/2/2<br>3/4 | 3/8/3<br>2/3/8/1<br>6/6/2<br>1/13[+] | | | | | |
| UC Montreal class L1/2/3 | | | | | | 1/4/11 | 0/5/5 | 0/2/4 | 3/1/3 | 1/2/4 |
| Current anti-TNF: infliximab/ adalimumab | | 16/7 | 7/1 | | | | 8/2 | 6/0 | | |
| Duration of current anti-TNF | | 18 (4-57) | 26 (9-62) | | | | 11 (4-26) | 4 (3-6)* | | |
| Prior anti-TNF | 3 | 9 | 2 | | | 1 | 5 | 2 | | |
| Corticosteroid (ever) | 6 | 3 | 3 | 0 | 4 | 10 | 4 | 5 | 0 | 1 |
| 5-ASA/SSZ (ever) | 8 | 4 | 1 | 0 | 3 | 8 | 7 | 1 | 0 | 4 |
| AZA (ever) | 13 | 12 | 6 | 0 | 13 | 12 | 6 | 2 | 0 | 4 |
| Surgery | 7 | 17 | 5 | 0 | 6 | 1 | 1 | 0 | 0 | 0 |

*Table 3 .Demographic and clinical data of the different cohorts of IBD patients*

*Numbers indicate mean (range), or number of patients. Extraintestinal manifestation: musculoskeletal, cutaneous, ocular or hepatobiliary. Prior anti-TNF indicates the number of patients in whom anti-TNF therapy was already applied, but had been stopped at least 12 months before the current therapy was initiated. Smoking habit data were missing in 13patients. 5-ASA: 5-amino-salycylic acid, SSZ: sulfasalazine, AZA: azathioprine. For the explanation of the abbreviations of patient subgroups, please see the Patients section.[+]p<0.05 for comparison of CDinact with CDresp and CDnonresp;\* p<0.05 for comparison of UCresp vs UCnonresp*

## 3.4. Methods

### 3.4.1. Laboratory methods - Flow Cytometry

Anticoagulated blood samples were taken – fifteen milliliters – and peripheral blood mononuclear cells (PBMCs) were separated by centrifugation with Ficoll–Paque (GE Healthcare Life Sciences, Pittsburgh, PA, USA). PBMCs were frozen and kept at -80 °C until examination. After thawing, samples were washed twice with phosphate-buffered saline solution (pH 7.4). The following fluorescent antibodies (Becton Dickinson, San Diego, CA, USA) were applied for cell surface staining for T cell subtype differentiation: helper T cells (CD4+), Th1 cells (CD4+CXCR3+), Th2 cells (CD4+CCR4+), Th17 cells (CD4+CCR4+CCR6+), Tregs (CD4+CD25highCD127-), naive T cells (CD4+CD45RA+), or memory T cells (CD4+CD45RO+). The proportion of cells expressing the early (CD69), or the late (HLA-DR) activation markers were also determined within both the CD4+ and CD8+ subsets. An average of 200.000 cells was registered for each acquisition. All measurements were performed on a BD FACSAria flow cytometer (BectonDickinson, San Jose, CA, USA). Cell proportion values were allocated with conventional gating, through the use of FACSDiva software (BectonDickinson, San Jose, CA, USA).

### 3.4.2. Statistical methods

Clinical data were presented as mean (range), whereas cell subtype prevalence values as mean ± SD or median (25-75 percentile) depending on the distribution of the values. Cell subset percentage values were compared among groups with two-sample t-test or Mann-Whitney test if two groups were compared, or with analysis of variance or with Kruskal-Wallis test, with Bonferroni's or Dunn's tests for multiple comparisons, respectively, if more than two groups were analyzed. We have applied the ANOVA for the comparison of all the patient subgroups and healthy controls. Frequencies were compared with Fisher exact test or chi- squared test. Receiver-operated curve (ROC) analyses were performed to seek for values of baseline (i.e. the start of anti-TNF) cell percentages that would discriminate between patients in whom long-term remission was achieved in response to anti-TNF therapy, and Pearson's correlation analysis was performed to assess the correlation between cell frequency values at the start of anti-TNF and the length of a subsequent remission. A p value <0.05 was taken statistically significant in every study.

# 4. Results

## 4.1. Rheumatoid arthritis

### 4.1.1. T-cell subset distribution of patients with RA on long-standing anti-TNF therapy

The proportions and ratios of various T cell subsets are demonstrated in *Table 4* and *Figures 1* and *2*.
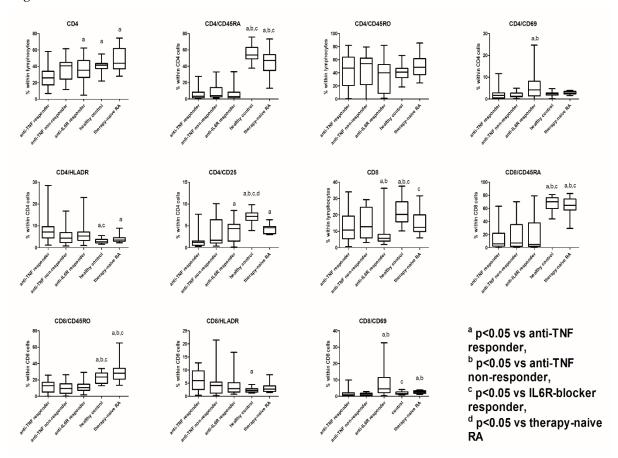


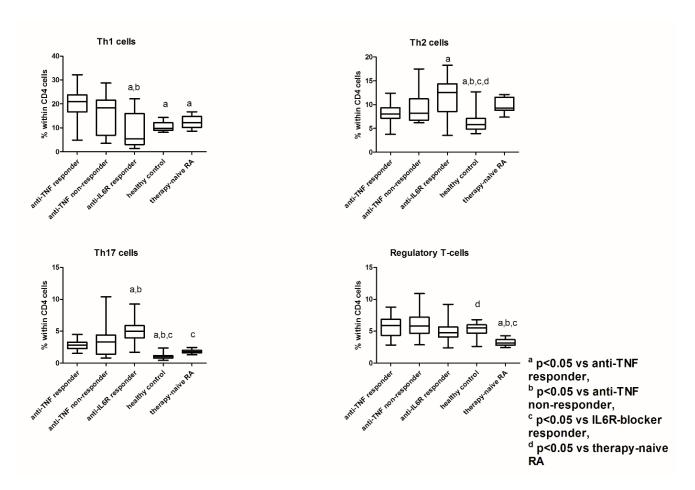*Figure 1. Prevalences of various T-cell subsets in rheumatoid arthritis patient groups of anti-TNF-responders (n=30), anti-TNF-non-responders (n=19) and IL-6R-blocker-responders (n=43), early, active, therapy-naïve RA patients (n=19), and in healthy controls (n=30).*

*Data are presented as median (horizontal line within boxes), 25 and 75 percentile (horizontal borders of the boxes), and minimum and maximum (whiskers).a p<0.05 vs anti-TNF responder, b p<0.05 vs anti-TNF non-responder, c p<0.05 vs IL6R-blocker responder, d p<0.05 vs early, untreated RA patients*

*Figure 2. Proportions of the major effector T-helper cell subsets in rheumatoid arthritis patient groups of anti-TNF-responders (n=30), anti-TNF-non-responders (n=19) and IL-6R-blocker-responders (n=43), early, active, therapy-naïve RA patients (n=19), and in healthy controls (n=30).*

*Data are presented as median (horizontal line within boxes), 25 and 75 percentile (horizontal borders of the boxes), and minimum and maximum (whiskers). a p<0.05 vs anti-TNF responder, b p<0.05 vs anti-TNF non-responder, c p<0.05 vs IL6R-blocker responder, dp<0.05 vs early, untreated RA patients*

| | | CD4 | CD4 CD45 RA | CD4 CD45 RO | CD4 CD69 | CD4 HLA DR | CD4 CD25 | CD8 | CD8 CD45 RA | CD8 CD45 RO | CD8 HLA DR | CD8 CD69 | Th1 | Th2 | Th17 | Treg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| anti- TNF responders | I | 25.90 | 3.370 | 47.15 | 1.635 | 7.180 | 1.165 | 10.70 | 3.690 | 12.85 | 6.020 | 0.9500 | 21.00 | 8.035 | 2.815 | 3.895 |
| | II | 17.43 | 1.54 | 20.68 | 0.4650 | 4.440 | 0.5600 | | 2.275 | 3.173 | 2.630 | 0.4650 | 16.70 | 7.143 | 2.250 | 4.345 |
| | III | 33.93 | 8.465 | 63.85 | 2.845 | 9.618 | 1.925 | 19.30 | 22.00 | 17.00 | 5.410 | 2.008 | 21.60 | 9.346 | 3.263 | 6.855 |
| anti- TNF non-responders | I | 40.8* | 4.13 | 53.90 | 1.310 | 4.330* | 1.710 | 12.80 | 7.180 | 9.480 | 4.100 | 1.270 | 18.40 | 8.190 | 3.290 | 5.800 |
| | II | 24.30 | 2.550 | 22.10 | 1.070 | 2.390 | 1.090 | 3.390 | 2.080 | 3.550 | 1.200 | 0.5950 | 6.900 | 6.740 | 1.410 | 4.640 |
| | III | 44.90 | 14.00 | 62.50 | 2.700 | 6.910 | 6.390 | 24.70 | 35.20 | 15.40 | 5.410 | 2.008 | 21.60 | 11.20 | 4.400 | 7.210 |
| aIL-6R-responders | I | 35.4* | 3.020 | 40.00 | 4.140^ab | 5.350 | 4.380* | 5.710^ab | 4.960 | 10.70 | 2.880 | 4.470^ab | 5.480^ab | 12.50* | 3.010^ab | 4.750 |
| | II | 26.6 | 1.515 | 8.620 | 1.460 | 3.330 | 1.500 | 4.000 | 2.010 | 7.370 | 1.640 | 2.250 | 3.040 | 8.540 | 3.950 | 4.080 |
| | III | 47.30 | 8.372 | 52.80 | 8.100 | 7.160 | 5.350 | 8.120 | 37.70 | 14.80 | 5.190 | 11.50 | 16.00 | 14.40 | 5.870 | 4.080 |
| Newly diagnosed untreated patients | I | 43.9* | 46.55^abc | 48.60 | 2.72 | 3.47* | 4.57* | 12.40* | 64.80^abc | 28.25^abc | 2.77 | 2.59^ab | 12.19 | 9.25 | 1.76* | 3.14^ab |
| | II | 37.05 | 34.90 | 37.25 | 2.36 | 2.97 | 3.19 | 9.78 | 57.48 | 21.05 | 1.84 | 1.77 | 10.20 | 8.84 | 1.61 | 2.81 |
| | III | 62.03 | 54.23 | 61.63 | 3.51 | 4.38 | 4.86 | 20.05 | 74.33 | 34.15 | 3.98 | 3.38 | 14.70 | 11.0 | 1.98 | 3.66 |
| Healthy controls | I | 41.59* | 52.65^abc | 41.35 | 2.4 | 2.8^d | 7.13^abcd | 19.64^abc | 69.2^abc | 23.65^abc | 2.3* | 1.88 | 10.79 | 5.79^abcd | 1.045^abc | 5.179^d |
| | II | 37.5 | 49.15 | 35.875 | 1.845 | 2.4775 | 6.1375 | 15.725 | 59.775 | 15.8 | 1.6525 | 1.375 | 9.2225 | 4.8525 | 0.7845 | 4.4425 |
| | III | 43.65 | 62.6375 | 49.4 | 2.855 | 3.8875 | 7.7925 | 24.125 | 73.775 | 28.2 | 2.9175 | 3.0775 | 12.375 | 7.095 | 1.1875 | 5.915 |

Table 4. Percentages of the various T-cell subsets in the examined patient groups and controls. Numbers indicate the median percentages and 25% and 75% percentile values within the whole T-cell population (CD4 and CD8 cells), or within CD4 cells (Th1, Th2, Th17, Treg and ratios), or for the other markers within the CD4 or CD8 cells, as indicated. I: median, II: 25th percentile, III: 75th percentile. [a] p<0.05 vs anti-TNF responder, [b] p<0.05 vs anti-TNF non-responder, [c] p<0.05 vs anti-IL6-receptor responder, [d] p<0.05 vs newly diagnosed, untreated RA patients.

*Anti-TNF-responders*

Anti-TNF-responders had lower proportions of CD4 cells, naïve CD4 and CD8 cells and memory CD8 cells, and higher percentages of activated CD4HLA-DR T-cells, but lower prevalences of activated CD4CD25 and of activated CD8CD69 T-cells than early, active, untreated RA patients. Anti-TNF responders were characterized by higher Th1 and Treg frequencies than early, active, untreated RA patients.

When compared with healthy controls, anti-TNF responders had lower prevalences of CD4 and CD8 T-cells. The frequencies of naive T-cells (both CD4CD45RA and CD8CD45RA cells) were lower than in controls, whereas those of the memory subtype (CD45RO) were similar among CD4 cells, and were also lower among CD8 cells in the anti-TNF responders than in healthy volunteers. The proportion of activated T-cells bearing the CD25 marker was lower and that of the HLA-DR-positive cells (both CD4 and CD8) was higher in anti-TNF-responders than in controls (*Figure 1*). In anti-TNF-responders, Th1, Th2 and Th17 proportion values were all higher than in healthy controls, but, importantly, Treg frequencies were not different (*Figure 2*).

*Anti-TNF-non-responders*

There are somewhat less differences between the T-cell composition of anti-TNF non-responders and early, untreated RA patients, but naïve T-cells (both CD4 and CD8) and also CD8 memory cells were less prevalent in anti-TNF non-responders, similarly to CD8CD69 activated cells. Comparison with healthy controls also revealed that anti-TNF non-responders had lower proportions of CD4 and CD8 naïve and CD8 memory T-cells, furthermore, CD4CD25 activated T-cells also occurred less frequently in the anti-TNF-non-responders than in the healthy subjects. The percentage of CD8 cells was lower in anti-TNF non-responders than in controls, but, in contrast with anti-TNF responders, CD4 cell prevalence was not different from controls (*Figure 1*). Similarly to the anti-TNF-responders, Th17 and Th2 percentages were also higher in non-responders than in the controls, and, again, Treg frequencies were equal to the healthy controls (*Figure 2*).

The differences between anti-TNF-responders and non-responders included significantly lower percentages of total CD4, and higher proportion of CD4HLA-DR T-cells in anti-TNF responders than in anti-TNF non-responders.

*The impact long term IL6-receptor blocker therapy on the immunophenotype of RA patients*

Comparing effective IL6R-blocker-treated and early, active, therapy-naïve RA patients, low number of CD8 cells, and the higher prevalence of Th17 and Treg cells in the IL6-R blocker subgroup are the most important differences. Naive T-cell (both CD4 and CD8) and CD8 memory cell proportions were lower in anti-IL6-R-treated RA patients (*Figures 1* and *2*). Some further differences can also be observed when comparing with healthy controls: higher prevalence of Th2 cells, of CD4HLA-DR and CD8CD69 activated T-cells in patients on tocilizumab.

Anti-IL6-R-responders differ from anti-TNF-responders in significantly higher CD4 and lower CD8 T-cell frequencies (*Figure 1*). Anti-IL6-R-responders had the lowest proportion of Th1 cells in all the examined groups, and this difference was significant from both anti-TNF-responders and anti-TNF–non-responders (*Figure 2*). On the contrary, the proportions of Th2 and Th17 cells were higher among anti-IL6-R-responders, than in anti-TNF-treated RA patients independent of the responder state. Importantly, similarly to anti-TNF-treated groups, Treg frequencies were normal (*Figure 2*). With regards to the activated T-cell subsets, anti-IL6-R-therapy was associated with higher percentages of CD69 T-cells, within both the CD4 and the CD8 subsets, than anti-TNF therapy, and CD4CD25 cells were also more prevalent than in anti-TNF-responders (*Figure 1*).

*Time-course of the changes in the T-cell subset distribution in RA patients on long-standing anti-TNF therapy*

We compared the T-cell subset proportion values from the beginning of the disease in 13 patients (7 anti-TNF-responders and 6 anti-TNF-non-responders). As compared with the baseline values (at disease onset, before anti-TNF therapy initiation), percentages of total CD4 T-cells, CD4 and CD8 naive T-cells decreased (*Figure 3*), but those of Tregs increased over time in both anti-TNF-responders and anti-TNF-non-responders (*Figure 4*). Th1 and Th17 proportion increased only in the anti-TNF-responder group, and Th2 cell frequencies increased only within the anti-TNF-non-responders (*Figure 4*). CD4CD69 cell proportion decreased in the anti-TNF-non-responders (p<0.05 with ANOVA, but no significant differences with Bonferroni's correction), and CD4HLA-DR cell percentages increased only in the anti-TNF-

responders (*Figure 4*). Among CD8 cells, memory T-cells became less prevalent during the course of the disease only in the anti-TNF-non-responders, while HLA-DR activated cell frequency was gradually rising in the anti-TNF-responder group only. As it can be seen in *Figure 3* and *4,* most of these changes have become evident only after long-term follow-up.
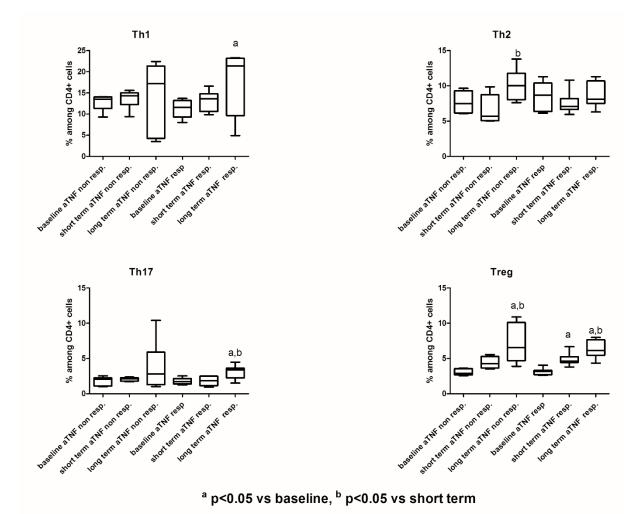


*Figure 3. Changes in the proportions of T-cell subsets in RA patients in whom long-term follow-up data from the initiation of anti-TNF therapy were available (n = 13).*

*Seven patients from the initial short-term cohort (26) proved to be long-term responders, whereas the other 6 patients lost the initial response, and therefore became part of the non-responder group. Measurement time-points: baseline: at anti-TNF initiation, short term: 8 weeks of anti-TNF treatment (previously published data (26)), long-term: current measurement results after long-standing anti-TNF treatment. Data are presented as median (horizontal line within boxes), 25 and 75 percentile (horizontal borders of the boxes), and minimum and maximum (whiskers). a p<0.05 vs baseline, b p<0.05 vs short term*

<sup>a</sup> p<0.05 vs baseline, <sup>b</sup> p<0.05 vs short term

*Figure 4. Changes in the proportions of the major effector T-helper cell subsets in RA patients in whom long-term follow-up data from the initiation of anti-TNF therapy were available (n = 13).*

*Seven patients from the initial short-term cohort (26) proved to be long-term responders, whereas the other 6 patients lost the initial response, and therefore became part of the non-responder group. Measurement time-points: baseline: at anti-TNF initiation, short term: 8 weeks of anti-TNF treatment (previously published data (26)), long-term: current measurement results after long-standing anti-TNF treatment. Data are presented as median (horizontal line within boxes), 25 and 75 percentile (horizontal borders of the boxes), and minimum and maximum (whiskers). a p<0.05 vs baseline, b p<0.05 vs short term*

*The connection of the length of biological therapy on T-cell subset proportions in long-term treated RA patients*

Since the duration of biological therapies was highly variable among long-term treated RA patients (ranging from 6 to 52 months) the question may arise whether this wide time-span could have an impact on the long-term effects of biologicals on the T-cell composition. We therefore further stratified the patients according to the duration of long-term biological therapy to "short" (6-12 months), "medium" (12-18 months) and "long" (> 18 months) duration of treatment. Comparison among these subgroups has revealed that only two of the examined 15 T-cell subtypes displayed a significant variability across these three subgroups: the prevalences of CD4+CD45RA+ and CD8+CD45RA+ naive cells decrease gradually among the three subgroups with longer treatment duration, and reaches a significance of $p<0.05$ in the comparison between „long" long-term duration *vs* the other two subgroups. All the other parameters have remained stable irrespective of the length of biological therapy *(Table 5).*

| | | | CD4 | CD4/ CD45 RA | CD4/ CD45 RO | CD4/ CD69 | CD4/ HLA DR | CD4/ CD25 | CD8 | CD8/ CD45 RA | CD8/ CD45 RO | CD8/ HLA DR | CD8/ CD69 | Th1 | Th2 | Th17 | Treg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| anti-TNF responders | „short" | I | 25,35 | 4,60 | 46,95[a] | 1,95 | 8,03 | 1,00 | 9,18 | 4,68[a] | 5,97 | 2,90 | 0,57 | 18,2 | 7,51 | 2,49 | 4,68 |
| | | II | 32,45 | 7,73 | 60,32 | 2,74 | 9,132 | 1,62 | 19,45 | 46,7 | 18,1 | 10,51 | 2,5 | 23,15 | 9,02 | 3,347 | 6,82 |
| | | III | 25,35 | 4,60 | 46,95 | 1,94 | 8,02 | 1,00 | 9,18 | 4,68 | 5,97 | 2,90 | 0,57 | 18,2 | 7,51 | 2,49 | 4,67 |
| | „mid" | I | 31,25 | 8,13 | 49,25[a] | 1,92 | 5,58 | 1,16 | 13,05 | 14,1[a] | 15,25 | 4,01 | 1,10 | 20,7 | 7,76 | 2,9 | 5,57 |
| | | II | 26,65 | 4,06 | 45,45 | 1,32 | 5,36 | 0,88 | 10,29 | 5,67 | 12,22 | 1,837 | 0,63 | 18,8 | 7,17 | 2,56 | 4,88 |
| | | III | 36,77 | 10,56 | 66,82 | 2,69 | 7,89 | 1,24 | 16,07 | 31,45 | 17,95 | 5,96 | 1,62 | 23,47 | 10,05 | 3,35 | 6,11 |
| | „long" | I | 22,2 | 1,79 | 37,3 | 0,845 | 6,02 | 1,10 | 8,57 | 3,97 | 10,85 | 6,82 | 0,78 | 19,9 | 8,06 | 2,38 | 6,43 |
| | | II | 16,7 | 1,28 | 13,69 | 0,232 | 4,35 | 0,51 | 4,63 | 1,07 | 4,357 | 3,027 | 0,49 | 16,65 | 6,99 | 2,17 | 4,3 |
| | | III | 32,87 | 2,91 | 56 | 2,597 | 12,57 | 1,77 | 17,97 | 6,32 | 14,2 | 9,74 | 1,09 | 23,77 | 9,00 | 3,04 | 6,86 |
| anti-TNF non-responders | „short" | I | 41 | 3,49 | 53,2 | 1,07 | 5,89 | 1,59 | 9,54 | 2,08 | 5,72 | 5,41 | 0,61 | 18,7 | 7,96 | 4,93 | 4,44 |
| | | II | 30,9 | 3,24 | 26,8 | 1,03 | 3,78 | 0,97 | 7,62 | 1,55 | 3,16 | 2,82 | 0,58 | 12,79 | 7,51 | 2,85 | 4,32 |
| | | III | 41,3 | 10,99 | 57,85 | 3,02 | 7,255 | 5,52 | 18,87 | 11,69 | 16,11 | 9,35 | 0,62 | 20,45 | 11,23 | 5,12 | 5,70 |
| | „long" | I | 40,15 | 4,52 | 55,7 | 1,37 | 4,20 | 2,32 | 13,35 | 7,45 | 11,19 | 4,07 | 1,36 | 18,25 | 8,7 | 2,71 | 5,86 |
| | | II | 25,62 | 2,47 | 22,92 | 1,15 | 2,6 | 1,16 | 6,33 | 2,40 | 6,86 | 1,33 | 1,05 | 11,92 | 6,65 | 1,45 | 5,44 |
| | | III | 44,92 | 13,17 | 62,67 | 2,63 | 6,28 | 5,64 | 20,57 | 36,12 | 14,87 | 4,67 | 2,07 | 21,5 | 11,1 | 4,20 | 7,81 |
| anti-IL6R responders | „short" | I | 31,7 | 3,96 | 28,7 | 5,19 | 4,17 | 4,28 | 5,79 | 3,47 | 8,52 | 2,88 | 3,68 | 4,83 | 13,0 | 5,19 | 4,69 |
| | | II | 25,3 | 1,81 | 17,7 | 1,85 | 2,52 | 1,5 | 3,73 | 1,71 | 7,37 | 1,46 | 1,76 | 3,4 | 9,31 | 3,09 | 4,01 |
| | | III | 42,4 | 8,03 | 42,7 | 8,1 | 5,83 | 4,78 | 6,96 | 9,96 | 15,2 | 4,23 | 11,5 | 13,6 | 14,3 | 6,19 | 5,83 |
| | „mid" | I | 27,05 | 3,21 | 47,75 | 3,63 | 5,65 | 4,30 | 5,41 | 4,54 | 9,7 | 2,92 | 6,96 | 7,41 | 11,26 | 4,69 | 4,75 |
| | | II | 25,77 | 2,51 | 6,415 | 1,34 | 3,51 | 1,076 | 4,04 | 2,89 | 8,15 | 1,605 | 3,37 | 3,44 | 6,74 | 3,27 | 4,59 |
| | | III | 36,25 | 10,04 | 64,6 | 6,4 | 10,47 | 6,02 | 8,82 | 32,48 | 12,3 | 8,21 | 10,29 | 17,82 | 16,4 | 5,38 | 5,0 |
| | „long" | I | 41,35 | 2,89 | 43,35 | 4,10 | 5,38 | 4,71 | 5,53 | 5,83 | 11,7 | 2,81 | 3,99 | 5,2 | 11,9 | 4,96 | 4,63 |
| | | II | 34,82 | 1,21 | 10,96 | 2,01 | 4,48 | 1,63 | 4,14 | 2,51 | 8,17 | 2,01 | 2,81 | 3,03 | 9,99 | 4,41 | 4,05 |
| | | III | 49,07 | 4,865 | 51,02 | 9,29 | 7,395 | 5,34 | 7,68 | 39,12 | 14,27 | 4,50 | 12,35 | 8,76 | 13,4 | 5,42 | 6,31 |

*Table 5. Subgroup analysis in the long-term treated RA patient groups according to the length of the biologic therapy.*

*Numbers indicate the median percentages and 25% and 75% percentile values within the whole T-cell population (CD4 and CD8 cells), or within CD4 cells (Th1, Th2, Th17, Treg and ratios), or for the other markers within the CD4 or CD8 cells, as indicated. I: median, II: 25th percentile, III: 75th percentile, [a] p<0.05 vs anti-TNF responders „long" long-term*

*The impact of previous switching of anti-TNF agents on T-cell subsets*

In some patients (especially in the IL6-R blocker-treated group) the biological agent applied at the time of sampling was not the first one, but there were previous switches from other anti-TNF drugs (*Table 1*). Although these switches were made more than 6 months before the blood sampling, we wanted to know whether the previous changes in therapy may have influenced the T-cell phenotype. We therefore compared the patient subgroups as defined by the number of previous switches in all therapeutic groups (*Table 6*). This analysis revealed that the proportions of CD4CD69 and of CD8CD69 cells were higher in IL6-R-responder patients who had experienced three switches before the current therapy than in those who had only one switch before (*Figure 5*). Opposite difference was observed with regard to Th1 percentage.

| | | | CD4 | CD4 CD45 RA | CD4 CD45 RO | CD4 CD69 | CD4 HLA DR | CD4 CD25 | CD8 | CD8 CD45 RA | CD8 CD45 RO | CD8 HLA DR | CD8 CD69 | Th1 | Th2 | Th17 | Treg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| anti-TNF-responders | „switched" | I | 33,4 | 14,1 | 40,3 | 1,47 | 5,36 | 0,96 | 14,2 | 20,2 | 14,1 | 5,51 | 0,96 | 16,7 | 8,95 | 2,67 | 6,84 |
| | | II | 33,05 | 8,08 | 40,1 | 1,145 | 4,575 | 0,695 | 12,4 | 12,18 | 9,265 | 4,07 | 0,575 | 15,95 | 8,36 | 2,305 | 5,515 |
| | | III | 41,75 | 18,55 | 44,8 | 2,02 | 5,44 | 1,21 | 16,5 | 25,6 | 15,25 | 5,755 | 1,49 | 17,55 | 9,47 | 2,92 | 6,87 |
| | „non-switched" | I | 24,4 | 3,17 | 47,3 | 1,8 | 7,92 | 1,2 | 9,77 | 5,41 | 12,6 | 6,19 | 0,86 | 21,7 | 8,02 | 2,82 | 5,85 |
| | | II | 17,25 | 1,505 | 19,65 | 0,46 | 4,49 | 0,58 | 5,34 | 2,19 | 5,60 | 2,89 | 0,48 | 17,2 | 7,13 | 2,25 | 4,44 |
| | | III | 33,15 | 7,77 | 64,8 | 2,91 | 9,84 | 1,57 | 19,25 | 15,5 | 17,2 | 9,74 | 2,09 | 23,85 | 9,24 | 3,30 | 6,63 |
| anti-TNF non-responders | „switched" | I | 30,15 | 2,77 | 38,55 | 1,425 | 3,59 | 1,54 | 24,75 | 8,55 | 11,02 | 2,86 | 1,85 | 20,9 | 8,81 | 1,4 | 7,09 |
| | | II | 19,07 | 2,11 | 6,30 | 1,28 | 1,85 | 0,94 | 11,11 | 2,21 | 2,77 | 0,96 | 1,31 | 15,4 | 8,02 | 1,11 | 6,19 |
| | | III | 41,37 | 11,25 | 58,7 | 2,21 | 6,38 | 3,53 | 27,35 | 29,35 | 13,58 | 4,95 | 2,53 | 21,9 | 10,31 | 3,48 | 9,19 |
| | „non-switched" | I | 41 | 4,92 | 57,5 | 1,25 | 4,33 | 2,94 | 9,96 | 7,18 | 9,48 | 4,31 | 1,05 | 18,1 | 7,07 | 4,11 | 5,65 |
| | | II | 29,8 | 3,49 | 49,8 | 1,07 | 2,69 | 1,19 | 6 | 2,17 | 5,72 | 1,38 | 0,535 | 6,9 | 6,39 | 2,08 | 4,64 |
| | | III | 45 | 12,9 | 62,5 | 2,7 | 6,08 | 6,39 | 17,3 | 33,8 | 15,4 | 5,41 | 1,49 | 21,5 | 12,9 | 4,4 | 6,14 |
| anti-IL6R responders | „non-switched" | I | 41,4 | 1,88 | 40 | 5,51 | 5,35 | 4,5 | 6,13 | 4,96 | 11,5 | 2,15 | 5,09 | 3,9 | 12,8 | 5,2 | 4,85 |
| | | II | 31,3 | 1,345 | 21,2 | 3,405 | 3,43 | 2,35 | 4,72 | 2,39 | 8,49 | 1,47 | 2,96 | 3,005 | 9,93 | 4,38 | 4,12 |
| | | III | 52,25 | 7,79 | 58,1 | 12,77 | 7,625 | 5,5 | 7,54 | 37,95 | 15,2 | 3,41 | 12,7 | 7,675 | 14,55 | 5,815 | 5,98 |
| | switching once | I | 25,7 | 5,435 | 28,7 | 1,44 | 5,55 | 1,5 | 7,54 | 3,37 | 8,2 | 4,23 | 2,45 | 16 | 10,7 | 3,95 | 4,69 |
| | | II | 22,9 | 1,60 | 9,04 | 0,78 | 3,98 | 0,66 | 3,87 | 1,87 | 5,975 | 2,30 | 0,92 | 6,12 | 7,35 | 3,07 | 4,39 |
| | | III | 33,65 | 10,05 | 43,45 | 1,85 | 7,16 | 3,94 | 10,24 | 33,9 | 11,75 | 5,68 | 5,24 | 17,85 | 12,2 | 4,895 | 6,17 |
| | switching twice | I | 35,4 | 4,12 | 12,5 | 4,6 | 5,83 | 5,14 | 6,36 | 6,64 | 12,5 | 4,28 | 4,11 | 5,65 | 13,3 | 5,41 | 4,45 |
| | | II | 26,6 | 3,02 | 10,7 | 3,13 | 5,77 | 2,47 | 4,17 | 2,47 | 10,7 | 2,88 | 3,12 | 4,92 | 7,85 | 3,73 | 4,21 |
| | | III | 48,9 | 10,6 | 13,3 | 10,6 | 8,88 | 6,57 | 9,65 | 37,7 | 13,3 | 9,37 | 9,97 | 17,3 | 15,8 | 6,45 | 4,75 |
| | switching three times | I | 34,3 | 2,58 | 11,8 | **5,0[a]** | 4,17 | 4,92 | 5,35 | 5,93 | 10,5 | 2,29 | **10,4[a]** | 3 | 12,7 | 5,07 | 4,81 |
| | | II | 27,5 | 2,38 | 3,29 | 4,14 | 3,11 | 4,78 | 5,02 | 5,63 | 6,46 | 1,98 | 5,04 | 2,72 | 12,5 | 5,01 | 3,82 |
| | | III | 42,4 | 2,89 | 50 | 15,8 | 4,68 | 5,35 | 5,74 | 9,96 | 14,8 | 3,8 | 16,3 | 4,33 | 13 | 5,3 | 5,06 |

*Table 6. Subgroup analysis of cell prevalences according to the number of previous switches*

*Numbers indicate values within the whole T-cell population (CD4 and CD8 cells), or within CD4 cells (Th1, Th2, Th17, Treg), or for the other markers within the CD4 or CD8 cells, as indicated. I: median, II: 25th percentile, III: 75th percentile. [a] p<0.05 vs anti-IL6R responders, switching once*
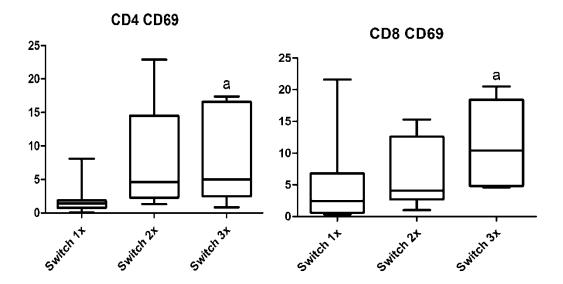
*Figure 5. The frequencies of CD4CD69 and of CD8CD69 cells in anti-IL6-R-responder RA patients grouped according to the number of previous switches in anti-TNF therapy before the initiation of the present tocilizumab treatment.*

*Numbers indicate percentages within the CD4 or CD8 T-cells, as applicable. [a]p<0.05 vs 1x switch.*

### Differences among the various anti-TNF agents

When we compared the T-cell subset proportions among the 5 anti-TNF agents individually (including adalimumab, etanercept, certolizumab, golimumab, infliximab) and between the particular anti-TNF drugs and anti-Il-6R-responders or healthy controls, only one significant difference was revealed in addition to the comparisons when the anti-TNF drugs were considered as one single group: the frequencies of CD4CD45RO cells were higher among etanercept-treated patients (responders and non-responders taken together) than among IL-6R-blocker-responders. The effect of the various anti-TNF agents on the immunophenotype of the RA-patients was not different (*Table 7*).

| | | CD4 | CD4/ CD45 RA | CD4/ CD45 RO | CD4/ CD69 | CD4/ HLA DR | CD8 | CD8/ CD45 RA | CD8/ CD45 RO | CD8/ HLA DR | CD8/ CD69 | CD4/ CD25 | Th1 | Th2 | Th17 | Treg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Infliximab** | I | 37.2 | 5.11 | 43.8 | 2.57 | 4.57 | 14.3 | 6.09 | 5.25 | 4.94 | 0.72 | 0.76 | 8.97 | 7.17 | 2.47 | 5.81 |
| | II | 26.5 | 1.83 | 22.0 | 0.85 | 3.18 | 8.89 | 1.98 | 4.21 | 3.65 | 1.00 | 0.97 | 18.4 | 8.22 | 1.83 | 4.59 |
| | III | 47.2 | 8.74 | 50.2 | 5.22 | 6.76 | 19.5 | 19.8 | 12.2 | 6.96 | 1.90 | 3.21 | 19.5 | 10.5 | 4.23 | 7.12 |
| **Adalimumab** | I | 29.4 | 3.24 | 51.1 | 1.50 | 6.02 | 14.1 | 4.78 | 11.3 | 5.20 | 0.46 | 0.50 | 16.6 | 7.34 | 3.01 | 5.66 |
| | II | 16.7 | 2.07 | 30.0 | 0.85 | 3.86 | 5.67 | 1.60 | 5.01 | 1.66 | 0.88 | 1.01 | 21.0 | 8.24 | 2.25 | 4.27 |
| | III | 37.0 | 10.4 | 62.3 | 2.37 | 10.0 | 23.5 | 6.44 | 15.7 | 9.78 | 1.75 | 2.07 | 23.4 | 9.72 | 4.17 | 7.31 |
| **Etanercept** | I | 32.7 | 4.20 | 67.2 | 1.88 | 7.86 | 9.36 | 11.8 | 13.8 | 6.19 | 0.68 | 1.19 | 5.25 | 6.34 | 3.29 | 4.96 |
| | II | 22.6 | 1.79 | 53.7 | 0.57 | 4.45 | 4.44 | 3.77 | 9.30 | 1.82 | 1.16 | 1.69 | 21.0 | 8.19 | 1.82 | 3.89 |
| | III | 40.1 | 7.75 | 77.1 | 3.04 | 13.3 | 24.8 | 36.3 | 21.9 | 10.6 | 1.42 | 5.49 | 23.0 | 11.3 | 3.98 | 5.93 |
| **Certolizumab** | I | 27.3 | 4.91 | 39.3 | 1.20 | 4.91 | 12.3 | 9.11 | 10.9 | 3.36 | 0.48 | 1.18 | 16.9 | 6.79 | 2.31 | 6.60 |
| | II | 16.3 | 2.04 | 0.79 | 0.57 | 1.61 | 7.19 | 1.16 | 0.89 | 1.05 | 2.04 | 1.50 | 19.7 | 8.03 | 1.40 | 5.64 |
| | III | 44.9 | 17.0 | 62.2 | 2.78 | 8.81 | 20 | 39.8 | 19.8 | 6.53 | 2.68 | 3.39 | 22.2 | 9.60 | 3.45 | 8.26 |
| **Golimumab** | I | 24.1 | 2.64 | 35.5 | 0.83 | 5.72 | 6.76 | 6.29 | 14.3 | 7.14 | 0.23 | 0.58 | 16.8 | 6.00 | 2.66 | 6.76 |
| | II | 18.7 | 0.62 | 5.71 | 0.16 | 4.65 | 2.12 | 1.72 | 3.79 | 3.91 | 0.68 | 1.12 | 23.5 | 6.68 | 1.67 | 6.01 |
| | III | 44.1 | 11.6 | 55.4 | 2.29 | 9.32 | 12.4 | 16.9 | 16.1 | 14.8 | 1.71 | 1.44 | 30.5 | 9.29 | 3.15 | 6.92 |

*Table 7. Prevalence values of the various T-cell subsets according to different anti-TNF therapies*

*Numbers indicate values within the whole T-cell population (CD4 and CD8 cells), or within CD4 cells (Th1, Th2, Th17, Treg and ratios), or for the other markers within the CD4 or CD8 cells, as indicated. . I: median, II: 25th percentile, III: 75th percentile.*

*Relationship between baseline T-cell subset prevalences and response to therapy*

Through the analysis of the prospective follow-up cohort, in which we compared the T-cell subset frequencies at baseline (i.e. at the start of the anti-TNF therapy), short-term (8 weeks) and long term therapy, we examined whether any baseline parameters are predictive of the long-term response to anti-TNF therapy. The proportion of CD4CD69 T-cells at baseline (2.16±0.12 vs 2.69±0.16, p=0.08) and at 8 weeks (2.01±0.20 vs 2.81±0.28, p=0.03) was lower in those who later belonged to anti-TNF-responders than in those who became anti-TNF-non-responders. ROC analysis revealed that a CD4CD69 T-cell percentage < 2.43 at baseline predicts a future response to anti-TNF therapy with a likelihood ratio of 4.29 (CI: 0.58-1.06), and discriminates between future anti-TNF-responders and non-responders with a sensitivity of 71.4% and a specificity of 83.3% (p=0.054*) (Figure 6).*
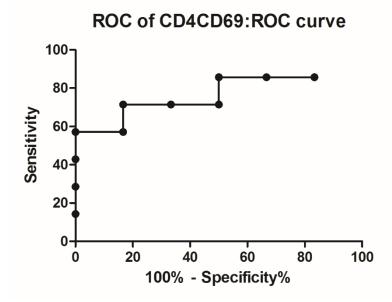


*Figure 6. Receiver-operator characteristic (ROC) curve of CD4CD69 positivity.*

*Predictive potential of the percentage of CD4CD69 positive cells at baseline (start of anti-TNF) to long-term response to therapy. CD4CD69 T-cell percentage < 2.43 has the highest likelihood ratio (4.29 [CI: 0.58-1.06] to discriminate between future anti-TNF-responders and non-responders (sensitivity: 71.4%, specificity: 83.3%, p=0.054).*

*4.2. Ankylosing spondylitis*

*4.2.1. T-cell repertoire composition of AS patients on long-term anti-TNF therapy*

T-cell phenotypes of AS patients on long-term anti-TNF therapies are presented in *Figures 7* and *8*, and numerically in *Table 8*. All anti-TNF-treated AS patients (both anti-TNF non-responders and anti-TNF responders) show a significantly lower prevalence of naive CD4 and CD8 cells (CD4CD45RA and CD8CD45RA), and higher frequencies of Th1 and Th17 cells, and Th17/Treg ratios than healthy controls. Treg proportion was similar to that in healthy subjects. The prevalence of T-cells with the late activation marker (CD4HLADR and CD8HLADR) was higher, and of T-cells with the intermediate activation marker (CD4CD25) was lower, and the Th1/Th2 ratio was higher only in the anti-TNF responders than in the controls. Differences between the two AS patients groups are as follows: in anti-TNF responders, the frequencies of naive CD4 and CD8 cells (CD4CD45RA and CD8CD45RA), and of early activated CD8CD69 cells were lower than in anti-TNF non-responders.

| | anti-TNF non-responders (n=7) | anti-TNF responders (n=15) | controls (n=10) |
|---|---|---|---|
| **CD4** | 39,39 ± 16,02 | 30,02 ± 17,23 | 40,56 ± 7,835 |
| **CD8** | 16,59 ± 13,73 | 16,63 1± 2,64 | 22,56 ± 8,073 |
| **CD4CD45RA** | 21,40 ± 16,50 | **6,484 ± 5,213[a]** | **56,76 1± 0,34[a,b]** |
| **CD4CD45RO** | 52,04 1± 8,71 | 49,87± 27,97 | 36,49 ± 10,05 |
| **CD4CD69** | 1,800 ± 0,6677 | 2,159 ± 2,514 | 2,109 ± 0,7517 |
| **CD4CD25** | 3,791 ± 2,766 | 2,420 ± 3,745 | **7,444 ± 0,8571 [b]** |
| **CD4HLADR** | 5,847± 2,449 | 14,33 ± 14,34 | **2,715 ± 0,7444 [b]** |
| **CD8CD45RA** | 36,81 ± 25,61 | **15,16 ± 18,93[a]** | **70,48 ± 8,991[a,b]** |
| **CD8CD45RO** | 21,66 ± 5,943 | 18,93 ± 9,592 | 22,31 ± 7,879 |
| **CD8CD69** | 10,55 ± 11,12 | **1,641 ± 2,855[a]** | 1,578 ± 0,5547 |
| **CD8HLADR** | 7,066 ± 3,303 | 10,41 ± 7,445 | **2,269 ± 0,9214 [b]** |
| **Th1** | 18,07 ± 6,070 | 21,39 ± 6,452 | **9,412 ± 0,8024[a,b]** |
| **Th2** | 9,766 ± 4,653 | 8,206 ± 1,326 | **5,937 ± 1,155[a]** |
| **Th1/Th2** | 2.435±1.526 | 2.686 ± 0.918 | **1.656 ± 0.437 [b]** |
| **Th17** | 3,736 ± 1,980 | 3,144 ± 1,066 | **1,001 ± 0,1746[a,b]** |
| **Treg** | 5,996 ± 2,131 | 5,575 ± 2,071 | 5,715 ± 0,7965 |
| **Th17/Treg** | 0.765 ± 0.599 | 0.697 ± 0.685 | **0.178 ± 0.036[a,b]** |

*Table 8. Prevalence and ratios of T-cell subsets in long term anti-TNF treated AS patient groups and controls.*

*Numbers indicate mean ± SD values within the whole T-cell population (CD4 and CD8) or within CD4 cells (Th1, Th2, Th17, Treg and ratios), or for the other markers within the CD4 or CD8 cells, as indicated. [a]p<0.05 vs anti-TNF non-responder, [b]p<0.05vs anti-TNF responder.*
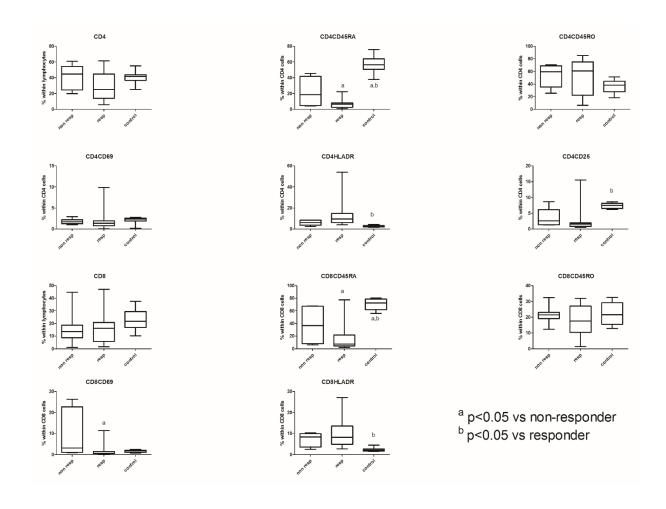
*Figure 7. Proportions of various T-cell subsets in ankylosing spondylitis patient groups of long-term anti-TNF-responders (n=15), long-term anti-TNF-non-responders (n=7) and in healthy controls (n=10).*

*Data are presented as median (horizontal line within boxes), 25 and 75 percentile (horizontal borders of the boxes), and minimum and maximum (whiskers). [a]p<0.05 vs anti-TNFnon-responder, [b] p<0.05 vs anti-TNFresponder, resp = responder.*
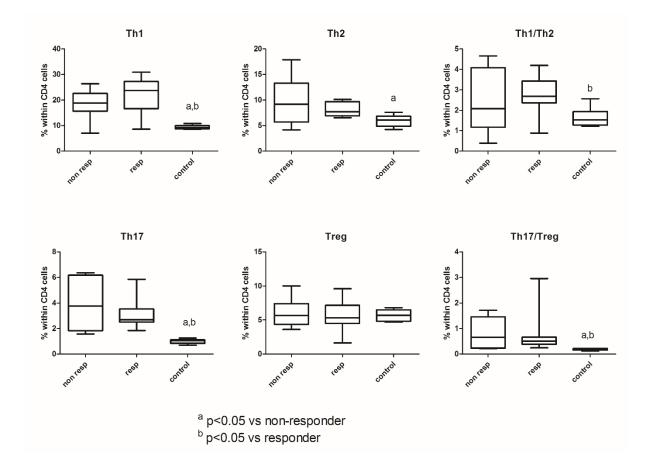
*Figure 8. Proportions of the major effectorT-helper cell subsets in ankylosing spondylitis patient groups of long-term anti-TNF-responders (n=15), long-term anti-TNF-non-responders (n=7) and in healthy controls (n=10).*

*Data are presented as median (horizontal line within boxes), 25 and 75 percentile (horizontal borders of the boxes), and minimum and maximum (whiskers). [a]p<0.05 vs anti-TNFnon-responder, [b] p<0.05 vs anti-TNFresponder, resp = responder.*

### 4.2.2. Comparison of the T-cell phenotype of AS patients on long-term and short-term anti-TNF therapy

The database of early alterations in the T-cell repertoire in a cohort of anti-TNF-treated AS patients was available for us from our previous study (54), so we could compare these values with our present results. T-cell subset prevalence in AS patients at the time of the initiation of anti-TNF therapy, 2 and 6 weeks thereafter (short-term) and after long-term follow-up (at least 6 months after initiation of anti-TNF therapy) can be seen in *Figures 9* and *10*. In summary, the decrease in the proportion of naive CD4 and CD8 cells (CD4CD45RA and CD8CD45RA), and

the increase in that of Th1, Th17 and Treg cells, and in Th1/Th2 ratios in the long-term anti-TNF treated AS patients (responders, non-responders and total) has proven to be significant not only when compared with the controls (see above) but also with therapy-naive and short-term anti-TNF treated AS patients. Alterations within the various activated T-cell subsets including – increase in CD4HLADR-positive cells in responders, and in CD8HLADR and of CD4CD25 positive cells in the whole long term treated AS group and in responders – have also become evident only after long-term anti-TNF therapy.
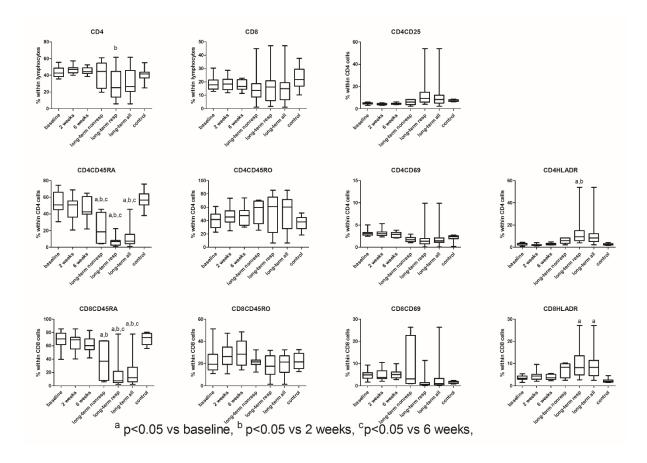


*Figure 9. Changes in the proportions of T-cell subsets in anyklosing spondylitis patient groups: comparison between short-term and long-term treatment*

*Data are presented from the initiation of anti-TNF therapy; baseline: at the start of anti-TNF therapy n=13; 2 weeks: after 2 weeks of anti-TNF therapy n=13; 6 weeks: after 6 weeks of anti-TNF therapy n=12 (54); long-term anti-TNF-responders (n=15); long-term anti-TNF-non-responders (n=7); long-term all: the two long-term subgroups together (n=22); and healthy controls (n=10). [a] p<0.05 vs baseline, [b] p<0.05 vs after 2 weeks, [c]p<0.05 vs after 6 weeks, [d]p<0.05 vs control.For CD4CD25 and CD4CD69, ANOVA revealed a significant (p=0.018)*

*difference among the groups, but the Bonferroni post hoc test did not identify any significant difference between the particular subgroups individually.*
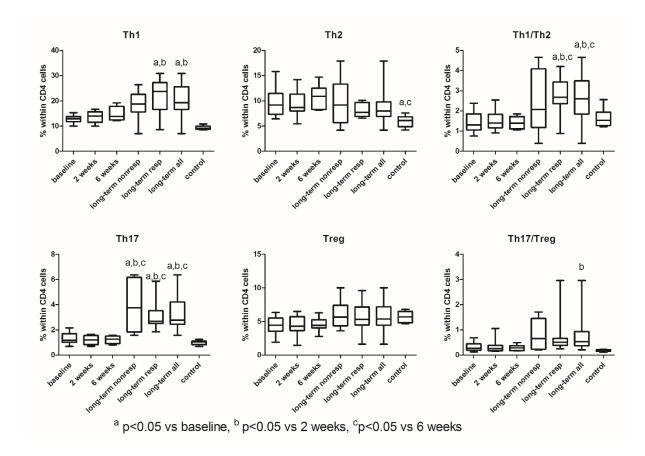


*Figure 10. Changes in the proportions of the major effector T-helper cell subsets in ankylosing spondylitis patient groups: comparison between short-term and long-term treatment.*

*Data are presented from the initiation of anti-TNF therapy; baseline: at the start of anti-TNF therapy n=13; 2 weeks: after 2 weeks of anti-TNF therapy n=13; 6 weeks: after 6 weeks of anti-TNFtherapy n=12 (54); long-term anti-TNF-responders (n=15); long-term anti-TNF-non-responders (n=7); long-term all: the two long-term subgroups together (n=22); and healthy controls (n=10). [a] p<0.05 vs baseline, [b] p<0.05 vs after 2 weeks, [c]p<0.05 vs after 6 weeks, [d]p<0.05 vs control*

*4.3 Inflammatory bowel diseases*

*4.3.1. Clinical follow-up*

The mean follow-up time of the patients after the blood sampling and the initiation of anti-TNF therapy was 35 months for CD and 24 months for UC patients. Follow-up data are summarized in *Table 9.* Long-term therapeutic response was observed in more than half of the patients (14 of 16 in CD and 9 of 16 in UC patients). In UC patients, primary non-response occurred more frequently than secondary inefficacy of anti-TNF therapy. The disease relapsed within 12 months in 9 of the 11patients in whom anti-TNF was stopped after one year of successful therapy, and in only 2 of them was a biologic-free remission observed, during a follow-up period of 12 and 36 months, respectively.

|  | **CD (n=16)** | **UC (n=16)** |
|---|:---:|:---:|
| **Duration of follow-up (months)** | 35 (16-47) | 24 (2-46) |
| **Short-term response achieved** | 15 | 12 |
| **Long-term response achieved** | 14 | 9 |
| **Duration of response (months)** | 27 (0-47) | 19 (0-46) |

*Table 9. Disease outcome after the initiation of anti-TNF therapy in IBD patients.*

*For 4 UC patients, follow-up was shorter than 12 months, long-term response was therefore evaluable in 20 patients. For the definition of short-term and long-term response, see Patients section*

*4.3.2. Lymphocyte subset distribution*

We have revealed a number of significant differences between healthy controls and IBD patients with respect of the prevalences of several T-cell subsets. ANOVA analyses have indicated that patients in every CD or UC subgroup had higher frequencies of Th2 and Th17 cells (*Figure 11*) than healthy controls.
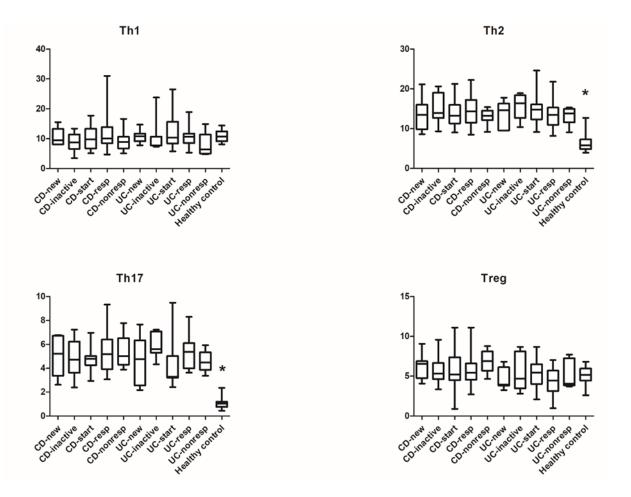
*Figure 11. Comparison of the frequencies of the major The effector cell subsets among the various patient groups and controls.*

*CDnew: new-onset Crohn's disease (CD) patients, CDinact: CD patients with inactive disease on immunosuppressive therapy, CDstart: patients with active CD in whom anti-TNF therapy is initiated, CDresp: anti-TNF-responder CD patients, CDnonresp: anti-TNF-nonresponder CD patients. Abbreviations are analogous for ulcerative colitis (UC) patients. Data are presented as median (horizontal line within boxes), 25 and 75 percentile (horizontal borders of the boxes), and minimum and maximum (whiskers). Numbers on the vertical axis indicate % within CD4 cells. *: p<0.05 for the comparison between healthy controls and all other patient subgroups with ANOVA and Bonferroni's correction*
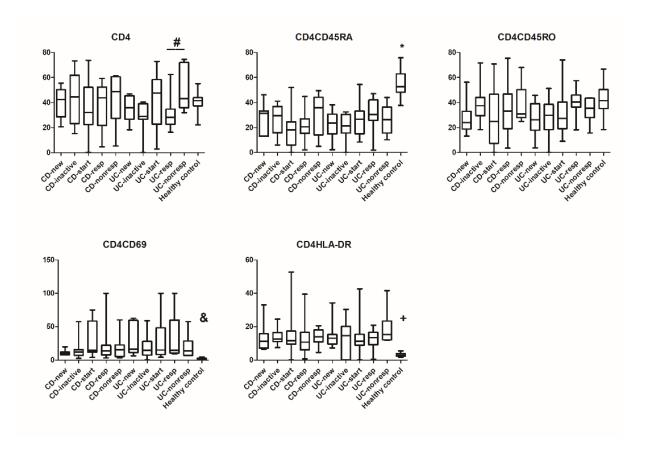
*Figure 12. Comparison of the frequencies of CD4 cell subsets among the various patient groups and controls.*

*For the explanation of the abbreviations of patient subgroups bars and whiskers, please see Legend to Figure 11. Numbers on the vertical axis indicate % within lymphocytes (for CD4) or % within CD4 cells (all other).*

*\*: p<0.05 for the comparison between healthy controls and all other patient subgroups with ANOVA and Bonferroni's correction*

*&: p<0.05 for the comparison between healthy controls and all other patient subgroups with the exception of CDnew, with ANOVA and Bonferroni's correction*

*+: p<0.05 for the comparison between healthy controls and CDnew, CDinact, CDstart, CDresp, UCstart and UCnonresp patients with ANOVA and Bonferroni's correction*

*#: p<0.05 for the comparison between UCresp and UCnonresp with Student's t-test*

Although the proportion of CD4 cells was comparable across all groups, patients in all IBD subgroups had less naive (CD45RA) T-cells than healthy controls, whereas the proportion of activated (CD69 and HLA-DR) T-cells was higher in most IBD subgroups, especially in CD patients, than in healthy controls (*Figure 12*). In the CD8 compartment, similar conclusions can be drawn, with the addition that the prevalence of memory T-cells (CD45RO) proved to be statistically significantly higher in 2 CD and 1 UC subgroup too (*Figure 13*).



*Figure 13. Comparison of the frequencies of CD8 cell subsets among the various patient groups and controls*

*For the explanation of the abbreviations of patient subgroups, and bars and whiskers, please see Legend to Figure 11.Numbers on the vertical axis indicate % within lymphocytes (CD8 cells) or % within CD8 cells (all other).*

*\*: p<0.05 for the comparison between healthy controls and all other patient subgroups with ANOVA and Bonferroni's correction*

*&: p<0.05 for the comparison between healthy controls and CDnew, CDresp and UCresp patients with ANOVA and Bonferroni's correction*

*+: p<0.05 for the comparison between healthy controls and CDnew, CDresp and UCstart patients with ANOVA and Bonferroni's correction*

*$: p<0.05 for the comparison between healthy controls and CDnew, CDstart, CDresp, CDnonresp and UCnew patients with ANOVA and Bonferroni's correction*

*#: p<0.05 for the comparison between UCstart and UCnonresp (CD8CD45RO), and between CDnew and CDinact (CD8CD69) with Student's t-test*

However, in contrast with our previous studies on RA and AS patients, relatively few differences could be demonstrated between newly diagnosed cases and patients in remission, and among patients before and after the initiation of anti-TNF therapy. Significant differences among the various patient subgroups in the cross- sectional part are as follows: CD4 proportion was lower in anti-TNF responder UC patients than in non-responders; CD8CD69 activated T-cells were observed more frequently in newly diagnosed CD patients than in those whose disease has become inactive with conventional immunosuppressive therapy; and CD8 memory cell prevalence was higher in anti-TNF non-responder UC patients than in UC patients at the time of anti-TNF-initiation.

We also attempted to compare the changes that occur in the cell subset distribution during anti-TNF therapy with the analysis of the data on patients in whom repeated measurements were performed, i.e. the T-cell profile at the initiation of anti-TNF could be compared with that after at least 3 months of therapy. Unfortunately, the numbers of eligible patients was fairly low as only 3 responder and non-responder patients could be compared in both IBD-s. These analyses could, therefore, not reveal any statistically evaluable difference, although the numbers indicating the changes in the cell prevalence values are strikingly different for many cell subtypes, in particular for Th2 in UC, and CD8CD69 and the naïve/memory composition in both diseases, therefore we have summarized these figures in *Table 10*. In contrast with our previous findings in RA and AS, Treg prevalence was not affected by anti-TNF therapy in either IBD.

| Cell subtypes | Responder vs Non-responder | Mean (UC patients) | Mean (CD patients) |
|---|---|---|---|
| Th1 | Non-responder | -4,513 | -6,043 |
| | Responder | -9,683 | -3,540 |
| Th2 | Non-responder | -7,167 | 2,607 |
| | Responder | -0,8700 | 0,2667 |
| Th17 | Non-responder | -0,6200 | 1,050 |
| | Responder | 0,2967 | 0,8467 |
| Treg | Non-responder | 1,123 | 0,7033 |
| | Responder | -0,6267 | 2,213 |
| CD4 | Non-responder | 10,14 | 4,290 |
| | Responder | 10,43 | 27,00 |
| CD4CD45RA | Non-responder | -3,433 | 0,1433 |
| | Responder | 4,467 | 27,64 |
| CD4CD45RO | Non-responder | 23,47 | 45,67 |
| | Responder | 7,733 | 11,87 |
| CD4CD69 | Non-responder | -16,97 | -30,78 |
| | Responder | -18,05 | -34,99 |
| CD4HLA-DR | Non-responder | 5,987 | -16,90 |
| | Responder | -9,533 | 6,590 |
| CD8 | Non-responder | 2,100 | 29,23 |
| | Responder | 20,18 | 24,10 |
| CD8CD45RA | Non-responder | 32,88 | 17,09 |
| | Responder | 13,89 | 14,24 |
| CD8CD45RO | Non-responder | 3,003 | -13,36 |
| | Responder | 15,43 | 0,1667 |
| CD8CD69 | Non-responder | -1,847 | 7,197 |
| | Responder | -24,98 | -9,097 |
| CD8HLA-DR | Non-responder | 10,42 | 17,80 |
| | Responder | 15,20 | 30,22 |

*Table 10. Changes in the cell subtype prevalence values during anti-TNF therapy in UC and CD patients, in whom repeated measurements were available.*

*Numbers indicate differences between percentage values during follow-up: % after at least 3 months of follow-up – % at start of anti-TNF. Six CD and 6 UC patients were analyzed (3each in both responder and non-responder groups).*

Finally, we analyzed if any of the cell prevalence values had a predictive value for the favourable clinical response to anti-TNF therapy. To answer that, we have performed ROC analyses in the CDstart and UCstart groups to search for frequency values that could discriminate between patients with and without long-term remission, and we carried out correlation analysis between the cell percentages and the duration of remission. In CD patients, CD4CD45RO cell prevalence of < 49.05% has predicted the long-term remission with a sensitivity of 100% (95% confidence interval:15.81% to 100.0%) and a specificity of 92.3% (95% confidence interval: 63.97 to 99.81%), and a likelihood ratio of 13.01. CD4HLADR cell frequencies showed a near-significant (p=0.058) correlation with the duration of remission (Pearson's r=0.50). In UC patients, there was a trend to significance (p=0.08) for a CD4 prevalence value of < 29.95 (sensitivity: 66.67% 95% CI: 9.43% to 99.16%, specificity 87.50% (95% CI 47.35% to 99.68%), likelihood ratio: 5.33), and Th2 and Th17 cell prevalence values showed a significant positive correlation with the duration of response (p=0.03, Pearson's r: 0.59, and p= 0.03, Pearson's r: 0.61, respectively), and a tendency with regard to a positive correlation with CD8HLADR (p=0.06, Pearson's r: 0.59).

## 5. Discussion

Our primary intention was to find laboratory parameters that would help clinicians to make scientifically established treatment decisions. In other words: to see if any of the 14 T-cell subtypes are useful in the decision if anti-TNF or – in RA – IL6R blocker therapy is expectably beneficial in the given patient, or conversely, the likelihood of refractoriness to these therapies is high. Therefore we highlight biomarkers that have predictive potential: in RA we found that CD4CD69 ratio is sensitive and specific to remission with anti-TNF agents, furthermore, CD8CD69 in both IBDs, and CD4CD45RO in CD are also remarkable. RA patients with high CD4CD69 or CD8CD69 probably benefit from switching to IL6R-blocking therapy after anti-TNF-failure instead of choosing a second anti-TNF. In CD patients, CD8CD69 can be regarded as an activity marker, furthermore, it is associated with treatment response to anti-TNF in UC. This suggests that T-cell typing by flow cytometry can be a potential diagnostic tool to select which class of targeted therapy is are a better choice.

Why it is important to know the T-cell composition during anti-TNF therapy? First, because the mechanism of action of these drugs is not fully understood. TNF-α impairs the activation of T-lymphocytes in response to antigen (93), and it induces the apoptosis of activated T-cells

by binding to TNF-receptor-I or –II (94). Anti-TNF agents reverse the activation-blocking effects of TNF-α (93), and thus could help activated cells to proliferate, but they can also induce apoptosis in T-cells, with differences among the particular drugs: infliximab, adalimumab and golimumab increase spontaneous apoptosis in these cells, while certolizumab pegol and etanercept do not (95). These effects may alter the composition of the T-cell subsets, but most of these results come from *in vitro* studies, and we do not know if they really exist in real life. Secondly, because most of the patients receive anti-TNF drugs for many years, and the long-term consequences of this treatment is not completely clear – for example, is there an increased risk of tumor development if certain T-cell subsets that are important in antitumor immune response decrease in number of function?

In anti-TNF-treated RA and AS, an important observation we made was that the main characteristics of T-cells reflect an activated state of the immune system, with one important exception: the normalization of Treg frequencies. We explain the first finding by the fact that both anti-TNF and anti-IL-6R therapies exert their action by the inhibition of the terminal phase of the effector arm of the autoimmune process (i.e. cytokine action), and the differentiation and the activation of naive T-cells by the permanent antigenic stimuli might be left unchanged. The normalization in Treg proportion starts soon after the initiation of anti-TNF-therapy, but the changes become statistically significant only after long-term treatment. Restoration of tolerance, as shown by the normalization of Treg numbers, seems to be a consistent phenomenon during the prolonged therapy of RA and also in AS. A further factor that should be considered is that despite Th17 prevalence remained increased, the ratio of Th17/Treg tended to approach the ratio observed in healthy controls in inflammatory rheumatic diseases. Analogously to our findings, Teniente-Serra *et al* have found that multiple sclerosis patients treated with natalizumab (monoclonal antibody to the integrin CD49d) had an increased percentage of early effector and central memory T cells, as well as of early thymic emigrant T-cells, as compared with untreated patients, indicating that T-cell activation may proceed despite the inflammatory process is blocked by the inhibition of the effector steps (96).The expansion of Th17 and Th1 cells has also been described by other authors (97,98).

A potential explanation was presented by Notley *et al*, who have shown that anti-TNF treatment of mice in the collagen-induced arthritis model lead to the up-regulation of the p40 subunit of IL12/23 receptor, and a resulting increase in both Th17 and Th1 cell frequencies (99). They explained the reduction in collagen-induced arthritis activity in anti-TNF-treated mice with their observation that the amount of Th1 and Th17 cells in the synovium markedly decreased

while they expanded in the blood and lymph nodes. In RA patients, Th1 and Th17 cells and IL-17 production was found to increase during anti-TNF therapy, and it was independent of the clinical response (97). However, CCR6 expression was significantly down-regulated in Th17 cells of RA patients in remission under anti-TNF therapy as compared with those with active disease (97). It was also found that TNF-α inhibits the expansion of effector CD4 and CD8 T-cells during viral infection by inducing apoptosis (100), so the blockade of TNF-α might release the activated T-cells from this negative regulation.

These findings suggest that, in spite of the increased amount of the circulating proinflammatory T-cells during anti-TNF therapy, the inactivity of the disease is maintained by alterations in various effector functions of these cells. It can also be concluded that the potential of the immune system to protect from tumor development and from infections persists during even several years of biological therapies, as the response to antigenic stimuli and the activation of T-cells seems to be preserved by the analysis of naive/memory and resting/activated T-lymphocyte subtypes.

In contrast to RA and AS, we have detected only subtle changes in IBD patients during anti-TNF therapy. Peripheral blood Treg frequency remained stable, and only total CD4 and CD8 memory cell percentages showed a difference in anti-TNF-treated UC patients. Our hypothesis for this discrepancy is that in IBDs the most relevant immunological events happen in the gut mucosa-associated immune system, and are reflected less in the circulating lymphocyte pool than in RA and AS. We propose therefore that studies on the pharmacodynamic immunological effects of anti-TNF, and possibly other novel targeted therapies are more appropriate from mucosal biopsies than from peripheral blood, or parallel samplings from endoscopic biopsies and peripheral blood should be performed. We note that peripheral blood T-cell composition did prove to be useful for the description of the adaptive immune system when CD and UC patients were compared with healthy individuals. It may be possible that an even longer follow-up would be needed until the putative mucosal immune alterations would proceed to the systemic immune system.

Our investigations involved several T cell subsets in all the diseases that have not been studied before. All untreated patient groups showed shifted immune cell balance from healthy controls, such as Th2 which was elevated in both RA and in IBDs compared to healthy controls. Th17 percentage was elevated in IBDs, but not in the inflammatory rheumatic diseases. Regulatory T cell frequencies were lower in RA and AS patients. Various activated T cell subtype

proportions were different in all examined diseases. CD4CD69, CD4HLA-DR, CD8-HLA-DR in RA, CD4HLA-DR, CD4CD25, CD8CD69 and CD8HLA-DR in AS, and CD4CD69, CD4HLA-DR, CD8CD69 and CD8HLA-DR in IBD all differed from healthy controls. Naïve T-cell frequency was lower in all diseases than in controls.

The proportions of various lymphocyte subsets during IL-6R-blocker therapy was previously investigated in detail in only one study (43). Our results add further knowledge to their results by including several T-cell subtypes not analyzed before, and the inclusion of a healthy control group. Common findings in their examinations and ours are the importance of the normalization of Treg counts, and the decrease in the frequencies of CD8 cells, naive T-cells, and an increase in that of CD69 activated T-cells. Although they did not find a significant change in Th1, Th2 or Th17 prevalences, their results also underline that activated cell subtype frequencies do not decrease during tocilizumab treatment. The importance of CD4CD69 as a predictive biomarker appears in IL6R-treated patients too: in those who had previously produced three times an incomplete response to anti-TNF therapy, the prevalences of CD4CD69 and CD8CD69 markers are significantly higher than in those who failed only one anti-TNF inhibitor before. It follows that, since all the above-mentioned anti-TNF non-responders with three switches (and with high CD4CD69 and CD8CD69 expression) became long-term anti-IL-6R responders, we propose that patients not responding to one anti-TNF, and having high CD4CD69 expression should be switched to anti-IL-6R therapy.

**Summary**

*Our key findings are:*

1. Long-term biological therapy normalizes the decreased Treg prevalence in RA and AS.

2. The T-cell phenotype of RA and AS patients on long-term anti-TNF therapy is featured by decreased naive CD4 and CD8 cell percentage, and many of the activated or effector (i.e. Th1, Th2 or Th17) subtypes were more prevalent, while some of the activated subtypes had lower frequency.

3. We have provided a detailed description of the T-cell composition in patients treated with the Il6R-blocker tocilizumab.

4. CD4CD69 T-cell percentage < 2.43 at the start of anti-TNF predicts a future response to this type of therapy in RA. Patients with more anti-TNF failures have higher CD4CD69 and CD8CD69 expression than those who only had one non-response to anti-TNF.

5. The immune phenotype after short-term and long-term anti-TNF therapy is markedly different in RA and AS; changes become obvious only after at least 3 months of anti-TNF treatment, but after 6 months of therapy, there are no further notable changes.

6. IBD (both UC and CD) patients have higher prevalence of Th2 and Th17 effector T-cells and lower frequency of CD45RA naive T-cells than healthy controls, whereas the proportion of activated (CD69 and HLA-DR) T-cells is higher in most IBD subgroups, especially in CD patients, than in healthy subjects.

7. In CD patients, CD8CD69 percentage is increased in active disease, and it decreases with treatment response to anti-TNF in UC.

8. In CD patients, CD4CD45RO cell prevalence of < 49.05% is a predictor of long-term remission. In UC patients, Th2 and Th17 cell prevalence values showed a significant positive correlation with the duration of treatment response.

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