

T cell Signal Transducer and Activator of Transcription (STAT) 4 and 6 are affected by adalimumab therapy in rheumatoid arthritis

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Abstract

Objective

TNF- α inhibition therapy affects the systemic immune response in rheumatoid arthritis by influencing T cell subtypes (Th1, Th2, Treg), but its effect on the intracellular signal transduction in T cells remains largely unexplored. Here we studied the activation of Th1-associated signalling molecule STAT4 and Th2-associated STAT6 in CD4⁺ T cells.

Methods

Eight rheumatoid arthritis patients were studied before and after 12 weeks of adalimumab therapy and compared to 8 healthy individuals. Peripheral blood mononuclear cells (PBMC) were analysed flow cytometrically either directly after isolation or after 24 hours of anti-CD3/anti-CD28 stimulation, to determine spontaneous and IL-4/IL-12-induced STAT4 and STAT6 phosphorylation in CD4⁺ T cells. Cytokine production by stimulated PBMC was measured in the supernatant using a cytometric bead array. Non-parametric statistical tests were applied.

Results

After adalimumab therapy, phospho-STAT6 increased, both in freshly isolated and anti-CD3/anti-CD28-stimulated CD4⁺ T cells. The STAT6 response to brief IL-4 stimulation did not change. In healthy individuals and adalimumab-treated patients, anti-CD3/anti-CD28 induced the phosphorylation of STAT4, but not in untreated patients. IFN- γ production in untreated patients was significantly lower than in healthy individuals or adalimumab-treated patients. In contrast, the production of IL-4, IL-6 and IL-12 was not influenced.

Conclusion

Adalimumab therapy increases Th2-associated STAT6 phosphorylation and restores the activation-induced STAT4 phosphorylation to the levels in healthy individuals. This advocates against a pro-inflammatory effect of Th1-associated STAT4 and might provide an explanation for the influence of TNF inhibition therapy on the systemic T cell response in rheumatoid arthritis.

Key words

Rheumatoid arthritis, STAT transcription factors, T-lymphocytes, cytokines, cell differentiation, adalimumab.

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Introduction

The recognition that tumour necrosis factor alpha (TNF- α) is a key player in the pathogenesis of rheumatoid arthritis (RA) (1) has led to the development of a class of TNF- α inhibitor molecules, which have successfully made their way into the clinic (2). Nearly a decade later, their mode of action is still incompletely understood. In addition to its direct effects on synovial inflammation and joint destruction (3–5), TNF- α inhibition therapy reputedly affects the systemic immune response. In particular, it has been shown to influence cytokine production by peripheral blood monocytes (6–8) and T cells (6, 7, 9–13).

Differential cytokine production has provided a rationale for the categorisation of subsets of T helper cells into distinct lineages. Among these, the Th1 and Th2 have been extensively characterized as a pro- and anti-inflammatory lineage and linked to auto-immune and allergic diseases, respectively (14), but their pathogenetical contribution has not been fully elucidated. The differentiation program in T helper cells is initiated and consolidated by the activation of signal transducers and activators of transcription (STATs) and their interaction with other signalling molecules and transcription factors, ultimately leading to stable epigenetic alterations (15). Both *in vitro* and *in vivo*, interleukin (IL)-12-induced tyrosine phosphorylation and subsequent nuclear translocation of STAT4 is indispensable for Th1 differentiation, and Th2 differentiation is dependent on a similar activation of STAT6 by IL-4 (16).

While STAT4 might be absolutely required for Th1 differentiation, it obviously has also other functions in the immune system. STAT4 is equally expressed in other cells from hematopoietic origin, including monocytes, dendritic cells and other cells that potentially contribute to the immune response in RA (17, 18). Several groups have reported an association of single nucleotide polymorphisms in the STAT4 gene with RA (19–26), and animal models of arthritis have demonstrated that elimination of STAT4 protects against the induction of disease – in contrast to

worsening of symptoms in the absence of STAT6 (27, 28). However, it is not known if T cell STAT4 and STAT6 signalling is altered in rheumatoid arthritis, or if these molecules contribute to the effect of TNF- α inhibition therapy on the intrinsic mechanisms of T-cell differentiation.

We speculated that TNF- α inhibition therapy could exert an influence on T cell differentiation by inhibiting STAT4 phosphorylation and promoting the anti-inflammatory effect of STAT6. To test this hypothesis, we flow cytometrically studied the activation of STAT4 and STAT6 in peripheral blood CD4+ T cells as well as cytokine production, in rheumatoid arthritis patients who were initiating TNF- α inhibition therapy with the monoclonal antibody adalimumab.

Patients and methods

Study population

Eight rheumatoid arthritis (RA) patients, fulfilling the 1987 American College of Rheumatology criteria for the classification of rheumatoid arthritis, and 8 age- and sex-matched healthy volunteers were included. For preparative and complementary experiments, healthy individuals were recruited among hospital and laboratory workers. The decision to refer patients for adalimumab therapy (40 mg subcutaneously, every other week) was made by the treating physician, based on the presence of 8 or more inflamed joints and a health assessment questionnaire (HAQ) score of at least 15/60 despite adequate treatment with 2 or more disease-modifying anti-rheumatic drugs (DMARDs) including methotrexate for at least 3 months. Concomitant use of DMARDs or oral glucocorticosteroids in a low and stable dose (≤ 7.5 mg prednisone-equivalent per day), was allowed, but not changes in DMARD therapy ≤ 3 months before inclusion or the introduction of oral/intramuscular glucocorticosteroids ≤ 4 weeks before inclusion. At baseline and at 12 weeks of anti-TNF therapy, a set of clinical parameters was recorded, including sex, age, disease duration, morning stiffness, health assessment questionnaire (HAQ) and concomitant

Competing interests: none declared.

treatment. The disease activity score (DAS28-CRP) (29) was calculated, based on the number of tender/swollen joints, a general health assessment and the acute phase reactant CRP. Two patients previously receiving another TNF- α inhibition regimen, were included after a wash-out period of at least 2 months. Informed consent was obtained from all patients and healthy volunteers prior to sample collection. The study protocol was approved by the ethical review board of the University of Antwerp.

Sample collection and isolation and culture of PBMC

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 (PBMC) were isolated by Histopaque-1077 (Sigma Aldrich, Bornem, Belgium) density gradient centrifugation, using calcium/magnesium-free Dulbecco's phosphate-buffered saline (D-PBS) (GIBCO Invitrogen, Merelbeke, Belgium) containing 10 mM EDTA (PBS-EDTA). PBMC were concentrated at 20×10^6 cells/mL in RPMI 1640 (GIBCO Invitrogen) with 25 mM HEPES, L-glutamine and penicillin/streptomycin for immediate use or concentrated at 3×10^6 cells/mL in X-VIVO 15 medium (Lonza, Verviers, Belgium) and cultured for 24h at 37°C and 5% pCO₂ in 24-well plates in the presence of soluble anti-CD3 (HIT3a, BD Biosciences, Erembodegem, Belgium) and anti-CD28 (CD28.2, BD Biosciences) 5 μ g/mL each. TNF- α and IL-1 β (10 ng/mL each, Peprotech EC, London, UK) were added where indicated.

Cytokine stimulation, cell fixation/permeabilisation and staining of surface markers and phosphoproteins

One million freshly isolated or cultured PBMC were incubated in RPMI alone or stimulated with 50 μ L of IL-12 (100 ng/mL) and IL-4 (10 ng/mL) (Peprotech EC) for 15 minutes at 37°C. The cytokine dose and duration of stimulation were chosen because preparative experiments showed maximal phosphoryla-

tion of cytokine-induced STAT4/6 after 15 minutes. Subsequently, cells were fixed in 500 μ L of paraformaldehyde 4% in D-PBS for 20 minutes at room temperature. After washing in D-PBS, 500 μ L of ice-cold methanol-based BD Perm III buffer (BD Biosciences) was added slowly while vortexing at 4°C and cells were incubated for 30 minutes at 4°C, before washing in D-PBS, supplemented with 1% fetal bovine serum and 0.09% of sodium azide. Cells were stained with anti-CD4-PE, anti-CD3-PerCP, anti-phospho-STAT4-AF488 (pY693) and -STAT6-AF647 (pY641) (all from BD Biosciences) for 30 minutes at room temperature in the dark, before being washed and re-suspended in D-PBS with 0.09% sodium azide. Alternatively, cultured PBMC were stained with anti-CD4-FITC, anti-CD3-PerCP and anti-CD212-PE (BD Biosciences) for 15 minutes at 4°C in the dark, preceding fixation in 1 mL of FACS Lysis solution (BD Biosciences) for 20 minutes at room temperature, washing and re-suspension in D-PBS with 0.09% sodium azide.

Flow cytometric analysis

A total of 50.000 PBMC was counted per tube. Lymphocytes were gated morphologically and on CD3 and CD4 positivity. Phosphoprotein or membrane receptor positivity were expressed as the percentage of CD4+ T cells with a fluorescence intensity above the level of the 99th percentile of a fluorescence-minus-one (FMO)-sample (30). Alternatively, the mean fluorescence intensity of the gated population was standardized by the use of Dako Fluorospheres (Dako, Heverlee, Belgium). Data were collected on a FACSCalibur flow cytometer (BD Biosciences) and analysed using WinMDI software version 2.8.

Measurement of extracellular cytokine production

Supernatant of stimulated PBMC was stored at -20°C. All undiluted samples were measured simultaneously using Cytometric Bead Array Flex Sets (BD Biosciences) and analysed for IL-12, IL-1 β , IFN γ , TNF- α and IL-4, according to the manufacturer's instructions. Data were analysed using FCAP

Array software version 1.0.1 and are expressed as pg/10⁶ PBMC.

Statistics

Data are expressed as percentages and did not follow a normal distribution. They are reported as medians and range. The Friedman test, Mann-Whitney U-test, the Wilcoxon Signed Rank test and the Kruskal-Wallis test were used where appropriate, for a significance level of 0.05. Correlations are expressed as Spearman's rank coefficient. Statistical analysis was done with SPSS software version 14.0.

Results

Patient characteristics and effect of adalimumab therapy on disease activity

We included 6 male and 2 female patients, with a median age of 58 years (range 32-70) and a median disease duration of 78 months (14-204). The median HAQ decreased from 36/60 points (range 28-60) to 16/60 (0-29), and the DAS28 decreased from 5.2 (range 4.6-6.7) to 2.4 (1.6-5.1) after 12 weeks of adalimumab therapy. Six of eight patients were good responders according to the EULAR criteria (31) and achieved disease remission (DAS28 < 2.6). The two other patients, including one that had previously received another anti-TNF treatment, showed a moderate response.

Phosphorylation of STAT4 in freshly isolated and cultured CD4+ T cells and production of Th1-cytokines IL-12 and IFN γ

Spontaneous phosphorylation of STAT4 in CD4+ T cells was negligible in most patients and healthy individuals, and hardly increased after brief incubation of the freshly isolated cells with IL-12 (Fig. 1). Consequently, we did not find statistically significant differences in freshly isolated cells between the control and untreated/treated patient group (Fig. 2). As no IL-12R β 2 (CD212) was detectable flow cytometrically on the membrane of these freshly isolated cells, we incubated PBMC for 24 hours with anti-CD3/CD28, which significantly up-regulated IL-12R β 2 expression in a small fraction (<2%) of

CD4⁺ T cells (data not shown). Anti-CD3/CD28 stimulation of PBMC led to clearly significant STAT4 phosphorylation in healthy individuals (Fig. 1 and 2). In contrast, it had no effect in untreated RA patients. After adalimumab treatment however, STAT4 was activated in a small but significant proportion of cells by anti-CD3/CD28. The presence of TNF- α /IL-1 β during the 24 hour culture had no influence on STAT4 phosphorylation over anti-CD3/CD28 alone (Fig. 2). Probably, this was because anti-CD3 and anti-CD28 alone already induced similar levels of TNF- α and IL-1 β production by the PBMC (data not shown). On top of the effect of CD3/CD28, brief re-stimulation with IL-12 for 15 minutes clearly activated STAT4 in healthy individuals and treated RA patients (Fig. 2). Before adalimumab treatment, the IL-12-induced activation of STAT4 was also significant, but it tended to be less pronounced (median net IL-12 effect in untreated patients: 1.1% [0.3-4.5] versus healthy individuals: 5.4% [range 0-9.4], $p=0.08$). The level of IFN γ production in the supernatant was lower in untreated patients than in healthy individuals, and increased in patients under adalimumab therapy (Fig. 3). In contrast, IL-12 production was very low and not statistically different between the groups (data not shown). The influence of adalimumab on CD3/CD28-induced STAT4 phosphorylation was not significantly correlated with a change in disease activity in the individual patient. STAT4 phosphorylation and IFN γ production were not correlated in individual samples.

Phosphorylation of STAT6 in freshly isolated and cultured CD4⁺ T cells and production of IL-4

Unlike STAT4, STAT6 displayed a low but significant spontaneous phosphorylation level in most samples after the cell separation. It was not different between healthy individuals and untreated patients, but increased significantly after adalimumab therapy (Fig. 4). IL-4 stimulation additionally activated STAT6 within 15 minutes in a large proportion of the CD4⁺ T cells in healthy individuals, untreated and adalimumab-

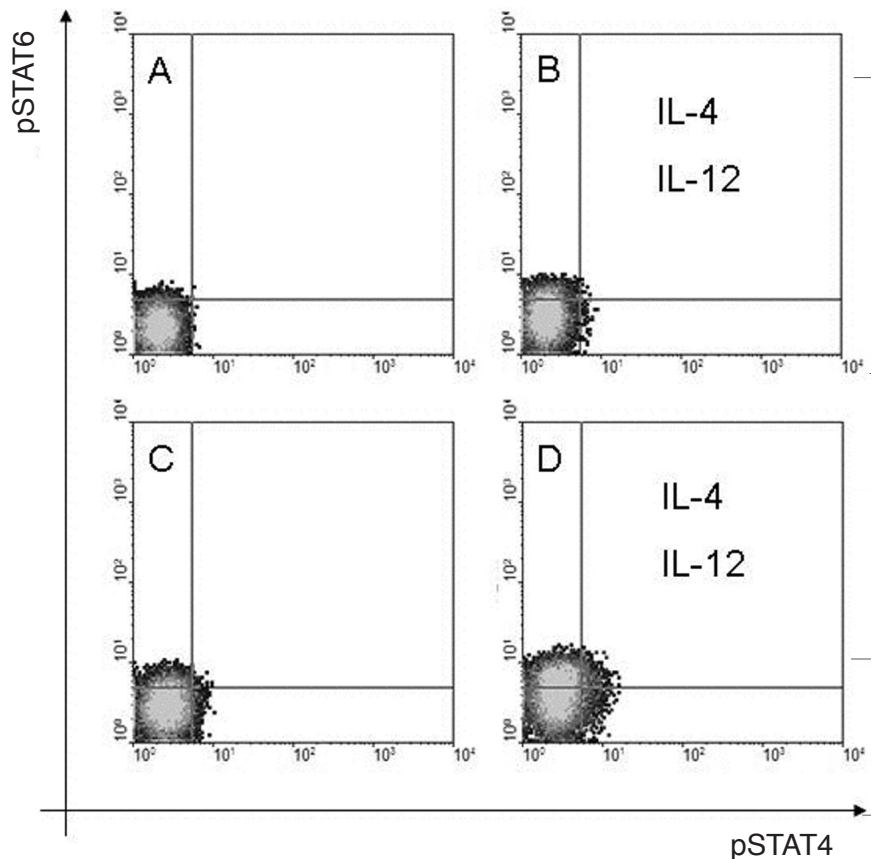


Fig. 1. Phosphorylation of STAT4 and STAT6 in flow cytometrically gated CD3⁺CD4⁺ lymphocytes from freshly isolated PBMC (A, B) and PBMC stimulated for 24h with anti-CD3/CD28 (C, D). Density plots from one representative sample depict phosphorylation after 15 minutes of incubation at 37°C in medium (A, C) or stimulation with IL-4 (10 ng/mL) and IL-12 (50 ng/mL) (B, D).

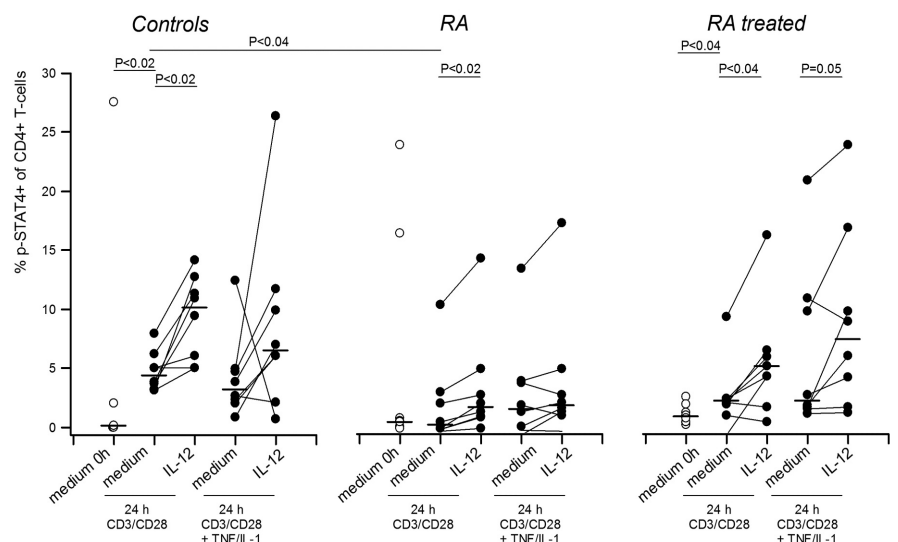


Fig. 2. Phosphorylation of STAT4 in freshly isolated and cultured CD4⁺ T cells from healthy individuals (controls) and rheumatoid arthritis patients before (RA) or during (RA treated) treatment with adalimumab. Phospho-STAT4 was determined in flow cytometrically gated CD3⁺CD4⁺ lymphocytes after 15 minutes of incubation of freshly isolated PBMC at 37°C in medium (0h, medium), or after 24 hours of stimulation with anti-CD3/anti-CD28 (1 μ g/mL each) with or without TNF- α /IL-1 β (10 ng/mL each), followed by incubation for 15 minutes in medium (24h, medium) or stimulation with IL-12 (50 ng/mL) (24h, IL-12). Phosphorylation was expressed as the percentage of phospho-STAT4-positive CD4⁺ T cells, based on an FMO-sample. Bars represent the median.

treated patients (Fig. 1 and 4), but quite similarly in the three groups. After 24 hours of anti-CD3/CD28-stimulation, STAT6 phosphorylation had increased significantly compared to freshly isolated cells (Fig. 4). There was no difference between healthy individuals and untreated patients, but this CD3/CD28-induced STAT6 phosphorylation increased significantly after adalimumab therapy. In individual patients, there was no significant correlation with disease activity parameters DAS28 or CRP. Short re-stimulation with IL-4 additionally activated STAT6, but did not discriminate between the control and untreated/treated patient group (data not shown). Again, the presence of TNF/IL-1 in the culture medium had no significant effect on STAT6 phosphorylation (Fig. 4). The production of IL-4 in the supernatant was similar in healthy individuals and patients before or after adalimumab (order of magnitude $10 \text{ pg}/10^6 \text{ PBMC}$).

Discussion

Despite ample evidence for the importance of STAT4 and STAT6 in the Th1/Th2 lineage decision of naïve CD4⁺ T cells (16), STAT4 and STAT6 have not been studied before in T cells in rheumatoid arthritis. We evaluated these signalling molecules on a single-cell level in rheumatoid arthritis patients and followed their activation status during adalimumab therapy.

We found that the normal phosphorylation of STAT4 during T cell receptor-mediated activation, as well as its subsequent response to IL-12, were absent or diminished in RA patients. However, treatment with adalimumab for 3 months seemed to restore the STAT4 response. This was fully in line with the level of extracellular IFN γ production, which is known to depend on STAT4 activation (16). We could not demonstrate a similar effect for IL-12. In parallel, adalimumab caused a substantial increase in spontaneous as well as activation-induced STAT6 phosphorylation in RA patients, unaccompanied by a rise in IL-4 production. Of note, IL-4 is both a powerful stimulus and a consequence of STAT6 phosphorylation (16).

Fig. 3. Production of IFN γ by PBMC from healthy individuals (controls) and rheumatoid arthritis patients before (RA) or during (RA treated) treatment with adalimumab. IFN γ was determined by cytometric bead array in the supernatant of PBMC stimulated for 24 hours with anti-CD3/anti-CD28 ($1 \mu\text{g}/\text{mL}$ each). Results are expressed as $\text{pg}/10^6 \text{ PBMC}$. Bars represent the median.

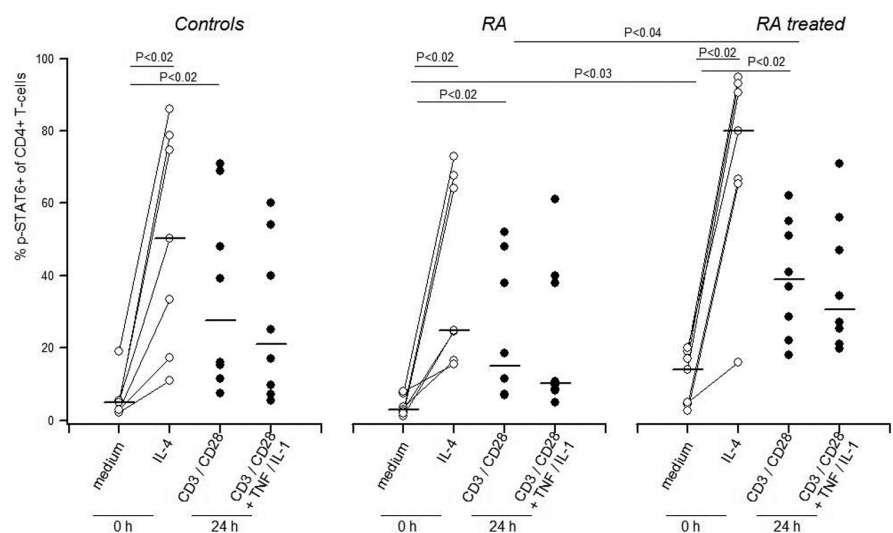
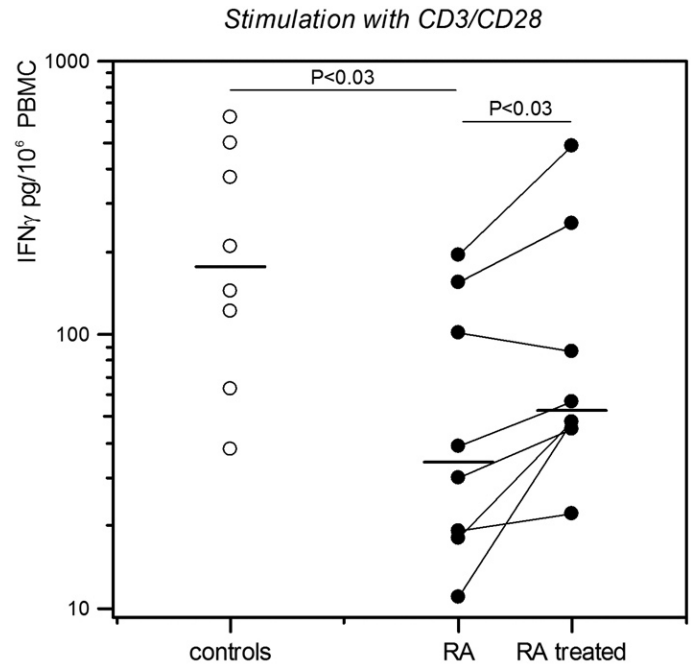


Fig. 4. Phosphorylation of STAT6 in freshly isolated and cultured CD4⁺ T cells from healthy individuals (controls) and rheumatoid arthritis patients before (RA) or during (RA treated) treatment with adalimumab. Phospho-STAT6 was determined in flow cytometrically gated CD3⁺CD4⁺ lymphocytes after 15 minutes of incubation of freshly isolated PBMC at 37°C in medium (0h, medium) or stimulation with IL-4 ($10 \text{ ng}/\text{mL}$) (0h, IL-4), or after 24 hours of stimulation with anti-CD3/anti-CD28 ($1 \mu\text{g}/\text{mL}$ each) with or without TNF- α /IL-1 β ($10 \text{ ng}/\text{mL}$ each) (24h, medium). Phosphorylation was expressed as the percentage of phospho-STAT6-positive CD4⁺ T cells, based on an FMO-sample. Bars represent the median.

The increase in activated STAT6 after adalimumab therapy fits with the generally accepted view that Th2 cells have anti-inflammatory effects in rheumatoid arthritis, and that successful therapy is often associated with a shift in the Th1/Th2 balance towards Th2 (for review: (32)). In contrast, the low STAT4 activation in active RA and the increase after TNF inhibition therapy are most in-

triguing and seemingly contradictory in light of the alleged Th1 predominance in RA. There are two major potential explanations for this. Firstly, most evidence for Th1 involvement in RA pathogenesis has been derived from synovial tissue studies, while the consequences of T cells homing to the joints on the cytokine profile of peripheral blood T cells are still the matter of intensive debate

(6, 7, 9, 10, 33-35). Secondly, the causative association of Th1 with RA has been challenged by slightly favourable effects of trials with recombinant IFN γ therapy in RA (36, 37) and more recently also by animal studies showing that arthritis is exacerbated in the absence of IL-12 or IFN γ signalling (38-40). Since the description of IL-17-producing T helper cells as a separate Th17 lineage (41), this has led to a new hypothesis that implies Th17 in the pathogenesis of RA and puts forward a mutual competition between the Th1, Th2 and Th17 lineages (42). Until now, evidence for Th1-independent, Th17-mediated inflammation in RA has mostly been derived from animal studies (43-46), but currently numerous studies are being conducted to further clarify the role of Th17 in human disease.

While the association of RA with a polymorphism in the STAT4 gene has received a lot of attention recently (19-26), it has never been demonstrated that inappropriate activation of STAT4 in T cells drives the inflammation in arthritis models or in RA. Indeed, our findings seem to indicate that T cell STAT4 is unlikely to contribute to RA disease activity, at least in patients from our cohort, who often had longstanding disease and were on concomitant DMARD treatment.

Conclusions

We demonstrate that adalimumab therapy modulates T-cell signalling in rheumatoid arthritis: it increases STAT6 phosphorylation and restores the activation-induced STAT4 phosphorylation to levels in healthy individuals. Given the crucial role of these signalling molecules in the T-cell differentiation program, this might provide an explanation for the influence of TNF inhibition on the systemic T cell response in rheumatoid arthritis.

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References

- BRENNAN FM, CHANTRY D, JACKSON A, MAINI R, FELDMANN M: Inhibitory effect of TNF alpha antibodies on synovial cell inter-

- leukin-1 production in rheumatoid arthritis. *Lancet* 1989; 2: 244-7.
- SCOTT DL, KINGSLEY GH: Tumor necrosis factor inhibitors for rheumatoid arthritis. *N Engl J Med* 2006; 355: 704-12.
- LIPSKY PE, VAN DER HEIJDE DM, ST CLAIR EW *et al.*: Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. *N Engl J Med* 2000; 343: 1594-602.
- SMEETS TJ, KRAAN MC, VAN LOON ME, TAK PP: Tumor necrosis factor alpha blockade reduces the synovial cell infiltrate early after initiation of treatment, but apparently not by induction of apoptosis in synovial tissue. *Arthritis Rheum* 2003; 48: 2155-62.
- ZWERINA J, HAYER S, TOHIDAST-AKRAD M *et al.*: Single and combined inhibition of tumor necrosis factor, interleukin-1, and RANKL pathways in tumor necrosis factor-induced arthritis: effects on synovial inflammation, bone erosion, and cartilage destruction. *Arthritis Rheum* 2004; 50: 277-90.
- SCHUERWEGH AJ, VAN OFFEL JF, STEVENS WJ, BRIDTS CH, DE CLERCK LS: Influence of therapy with chimeric monoclonal tumour necrosis factor-alpha antibodies on intracellular cytokine profiles of T lymphocytes and monocytes in rheumatoid arthritis patients. *Rheumatology (Oxford)* 2003; 42: 541-8.
- LORENZ HM, ANTONI C, VALERIUS T *et al.*: In vivo blockade of TNF-alpha by intravenous infusion of a chimeric monoclonal TNF-alpha antibody in patients with rheumatoid arthritis. Short term cellular and molecular effects. *J Immunol* 1996; 156: 1646-53.
- OHSHIMA S, SAEKI Y, MIMA T *et al.*: Long-term follow-up of the changes in circulating cytokines, soluble cytokine receptors, and white blood cell subset counts in patients with rheumatoid arthritis (RA) after monoclonal anti-TNF alpha antibody therapy. *J Clin Immunol* 1999; 19: 305-13.
- MAURICE MM, VAN DER GRAAFF WL, LEOW A, BREEDVELD FC, VAN LIER RA, VERWEIJ CL: Treatment with monoclonal anti-tumor necrosis factor alpha antibody results in an accumulation of Th1 CD4+ T cells in the peripheral blood of patients with rheumatoid arthritis. *Arthritis Rheum* 1999; 42: 2166-73.
- KAWASHIMA M, MIOSSEC P: mRNA quantification of T-bet, GATA-3, IFN-gamma, and IL-4 shows a defective Th1 immune response in the peripheral blood from rheumatoid arthritis patients: link with disease activity. *J Clin Immunol* 2005; 25: 209-14.
- DOMBRECHT EJ, AERTS NE, SCHUERWEGH AJ *et al.*: Influence of anti-tumor necrosis factor therapy (Adalimumab) on regulatory T cells and dendritic cells in rheumatoid arthritis. *Clin Exp Rheumatol* 2006; 24: 31-7.
- VALENCIA X, STEPHENS G, GOLDBACH-MANSKY R, WILSON M, SHEVACH EM, LIPSKY PE: TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood* 2006; 108: 253-61.
- NADKARNI S, MAURI C, EHRENSTEIN MR: Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *J Exp Med* 2007; 204: 33-9.
- DEL PG: The concept of type-1 and type-2 helper T cells and their cytokines in humans. *Int Rev Immunol* 1998; 16: 427-55.
- MURPHY KM, REINER SL: The lineage decisions of helper T cells. *Nat Rev Immunol* 2002; 2: 933-44.
- AGNELLO D, LANKFORD CS, BREAM J *et al.*: Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol* 2003; 23: 147-61.
- FRUCHT DM, ARINGER M, GALON J *et al.*: Stat4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. *J Immunol* 2000; 164: 4659-64.
- WALKER JG, AHERN MJ, COLEMAN M *et al.*: Characterisation of a dendritic cell subset in synovial tissue which strongly expresses Jak/STAT transcription factors from patients with rheumatoid arthritis. *Ann Rheum Dis* 2007; 66: 992-9.
- BARTON A, THOMSON W, KE X *et al.*: Re-evaluation of putative rheumatoid arthritis susceptibility genes in the post-genome wide association study era and hypothesis of a key pathway underlying susceptibility. *Hum Mol Genet* 2008; 17: 2274-9.
- KOBAYASHI S, IKARI K, KANEKO H *et al.*: Association of STAT4 with susceptibility to rheumatoid arthritis and systemic lupus erythematosus in the Japanese population. *Arthritis Rheum* 2008; 58: 1940-6.
- LEE HS, REMMERS EF, LE JM, KASTNER DL, BAE SC, GREGERSEN PK: Association of STAT4 with rheumatoid arthritis in the Korean population. *Mol Med* 2007; 13: 455-60.
- MARTINEZ A, VARADE J, MARQUEZ A *et al.*: Association of the STAT4 gene with increased susceptibility for some immune-mediated diseases. *Arthritis Rheum* 2008; 58: 2598-602.
- OROZCO G, ALIZADEH BZ, GADO-VEGA AM *et al.*: Association of STAT4 with rheumatoid arthritis: a replication study in three European populations. *Arthritis Rheum* 2008; 58: 1974-80.
- PALOMINO-MORALES RJ, ROJAS-VILLARAGA A, GONZALEZ CI, RAMIREZ G, ANAYA JM, MARTIN J: STAT4 but not TRAF1/C5 variants influence the risk of developing rheumatoid arthritis and systemic lupus erythematosus in Colombians. *Genes Immun* 2008; 9: 379-82.
- REMMERS EF, PLENGE RM, LEE AT *et al.*: STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. *N Engl J Med* 2007; 357: 977-86.
- ZERVOU MI, SIDIROPOULOS P, PETRAKI E *et al.*: Association of a TRAF1 and a STAT4 gene polymorphism with increased risk for rheumatoid arthritis in a genetically homogeneous population. *Hum Immunol* 2008; 69: 567-71.
- FINNEGAN A, GRUSBY MJ, KAPLAN CD *et al.*: IL-4 and IL-12 regulate proteoglycan-induced arthritis through Stat-dependent mechanisms. *J Immunol* 2002; 169: 3345-52.
- HILDNER KM, SCHIRMACHER P, ATREYA I *et al.*: Targeting of the transcription factor STAT4 by antisense phosphorothioate oligonucleotides suppresses collagen-induced

- arthritis. *J Immunol* 2007; 178: 3427-36.
29. PREVOO ML, VAN 'T HOF MA, KUPER HH, VAN LEEUWEN MA, VAN DE PUTTE LB, VAN RIEL PL: Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995; 38: 44-8.
 30. ROEDERER M: Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry* 2001; 45: 194-205.
 31. VAN GESTEL AM, PREVOO ML, VAN 'T HOF MA, VAN RIJSWIJK MH, VAN DE PUTTE LB, VAN RIEL PL: Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism Criteria. *Arthritis Rheum* 1996; 39: 34-40.
 32. SKAPENKO A, LEIPE J, LIPSKY PE, SCHULZE-KOOPS H: The role of the T cell in autoimmune inflammation. *Arthritis Res Ther* 2005; 7 (Suppl. 2): S4-14. Epub; 2005 Mar 16.:S4-14.
 33. MILTENBURG AM, VAN LAAR JM, DE KR, DAHA MR, BREEDVELD FC: T cells cloned from human rheumatoid synovial membrane functionally represent the Th1 subset. *Scand J Immunol* 1992; 35: 603-10.
 34. QUAYLE AJ, CHOMARAT P, MIOSSEC P, KJELDSEN-KRAGH J, FORRE O, NATVIG JB: Rheumatoid inflammatory T-cell clones express mostly Th1 but also Th2 and mixed (Th0-like) cytokine patterns. *Scand J Immunol* 1993; 38: 75-82.
 35. VAN DER GRAAFF WL, PRINS AP, NIERIS TM, DIJKMANS BA, VAN LIER RA: Quantitation of interferon gamma- and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients. *Rheumatology (Oxford)* 1999; 38: 214-20.
 36. VEYS EM, MENKES CJ, EMERY P: A randomized, double-blind study comparing twenty-four-week treatment with recombinant interferon-gamma versus placebo in the treatment of rheumatoid arthritis. *Arthritis Rheum* 1997; 40: 62-8.
 37. MACHOLD KP, NEUMANN K, SMOLEN JS: Recombinant human interferon gamma in the treatment of rheumatoid arthritis: double blind placebo controlled study. *Ann Rheum Dis* 1992; 51: 1039-43.
 38. VERMEIRE K, HEREMANS H, VANDEPUTTE M, HUANG S, BILLIAU A, MATTHYS P: Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol* 1997; 158: 5507-13.
 39. GUEDEZ YB, WHITTINGTON KB, CLAYTON JL et al.: Genetic ablation of interferon-gamma up-regulates interleukin-1beta expression and enables the elicitation of collagen-induced arthritis in a nonsusceptible mouse strain. *Arthritis Rheum* 2001; 44: 2413-24.
 40. MURPHY CA, LANGRISH CL, CHEN Y et al.: Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 2003; 198: 1951-7.
 41. KOENDERS MI, JOOSTEN LA, VAN DEN BERG WB: Potential new targets in arthritis therapy: interleukin (IL)-17 and its relation to tumour necrosis factor and IL-1 in experimental arthritis. *Ann Rheum Dis* 2006; 65 (Suppl. 3): iii29-33.
 42. TOH ML, MIOSSEC P: The role of T cells in rheumatoid arthritis: new subsets and new targets. *Curr Opin Rheumatol* 2007; 19: 284-8.
 43. HIROTA K, HASHIMOTO M, YOSHITOMI H et al.: T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis. *J Exp Med* 2007; 204: 41-7.
 44. UYTENHOVE C, VAN SJ: Development of an anti-IL-17A auto-vaccine that prevents experimental auto-immune encephalomyelitis. *Eur J Immunol* 2006; 36: 2868-74.
 45. ROHN TA, JENNINGS GT, HERNANDEZ M et al.: Vaccination against IL-17 suppresses autoimmune arthritis and encephalomyelitis. *Eur J Immunol* 2006; 36: 2857-67.
 46. KOENDERS MI, LUBBERTS E, OPPER-S-WAL-GREEN B et al.: Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. *Am J Pathol* 2005; 167: 141-9.