



Interferon (IFN) Signals and Monocytic Sensitization of the IFN γ Signaling Pathway in Peripheral Blood of Rheumatoid Arthritis Patients

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**Interferon (IFN) Signals and Monocytic Sensitization of the IFN γ Signaling Pathway in
Peripheral Blood of Rheumatoid Arthritis Patients**

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ABSTRACT

Objective: Both, type I IFNs (IFN α , IFN β) and the type II IFN (IFN γ), signal via phosphorylating Stat1. Immunohistochemistry and gene expression signatures of rheumatoid arthritis (RA) synovial tissues suggest an activated IFN-Stat1-signaling-pathway. This study was performed to determine the systemic activity of the IFN-Stat1-signaling-pathway in RA peripheral blood cells.

Methods: Fluorocytometry or qPCR was used to measure the expression of Stat1, pStat1 and IFN-inducible genes (MIG, IP-10, OAS) in PBMC and purified CD14+ peripheral blood monocytes of RA patients and healthy individuals (HC). PBMC were also incubated for 48h with IFNs and several other cytokines to investigate influences on Stat1 levels. To examine the significance of Stat1 activation in RA monocytes after stimulation with IFN γ , the expression of pStat1 and of the IFN γ -inducible chemokine MIG were measured using fluorocytometry.

Results: Levels of Stat1 were significantly increased in RA peripheral lymphocytes and monocytes when compared to those of HC. Stat1 levels correlated well with RA disease activity, measured by DAS28 and CDAI. Further, Stat1 mRNA expression in RA CD14+ monocytes correlated with the expression of other IFN-target genes, such as IP-10, OAS, or MIG. In RA PBMC Stat1 was not only increased by IFNs, but also by TNF. RA monocytes demonstrated a considerably higher increase in pStat1 and MIG levels upon IFN γ stimulation when compared to monocytes from HC, indicating that RA monocytes are more sensitive to IFN γ stimulation.

Conclusion: Consistent with a systemic proinflammatory activity, these studies further suggest activation of the IFN γ -Stat1-signaling pathway, especially in RA monocytes.

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease of unknown etiology, which primarily attacks synovial joints. Rheumatoid synovial tissue is characterised by intimal lining layer hyperplasia and an infiltration of the sublining area by macrophages, lymphocytes and other cells that promote inflammation, leading to degradation of cartilage and bone. The overexpression of proinflammatory cytokines, such as TNF or IL-6, in cells of the synovial membrane is considered to drive the destructive process, since blocking these cytokines not only reduces disease activity, but also slows or even abrogates joint destruction in RA patients (1-3). In contrast, the role of type I interferons (IFN α and IFN β), and of IFN γ (type II IFN), in particular, in RA pathophysiology is still not sufficiently known.

All IFNs use signal transducer and activator of transcription 1 (Stat1) activation in their signaling pathways. In general, binding to the IFN receptor leads to phosphorylation of Stat1, mediated by Janus kinases (Jaks), which induces a change of conformation into its active form (4-6). Activated Stat1 homodimers (IFN γ) or heterotrimers (type I IFNs) then, amongst a variety of effects, are also able to induce Stat1 expression itself (7).

In RA synovial tissue, both IFN γ producing T cells (8-12), and elevated levels of Stat1 and of Stat1 target genes were found (13-19). Moreover, successful DMARD treatment decreased synovial Stat1 expression in RA (20). These data provide indirect evidence that the IFN-Stat1-signaling-pathway is functional and thus involved in rheumatoid synovial inflammation. Gene expression analyses of peripheral blood cells also showed overexpression of a plethora of genes associated with activities of IFNs, but this was rather related to type I IFNs than to IFN γ . Furthermore, the IFN-signature was found in a subset of RA patients only. Although, Stat1 is also inducible by IFNs, and hence part of the IFN-signature, the authors did not refer to Stat1 levels of peripheral blood cells (21). Nevertheless, gene expression analyses may not reveal the full degree of protein activation, which, as in the case of transcription factors like Stat1, is usually through phosphorylation and the functionally decisive state. Also, the use of

whole peripheral blood cells does not allow for distinguishing the cell population overexpressing the respective genes, be it neutrophils, lymphocytes or rather monocytes.

It was shown in vitro, that Stat1 expression is a highly dynamic process. Stat1 expression levels, especially in monocytes and macrophages, regulate the pattern of Stat activation by a cytokine over time. Thus, the exposure to low concentrations of IFNs results in increased or modified cellular responses to subsequent stimuli, such as microbial pathogens or cytokines, including IFNs. This phenomenon has been called priming. Importantly, the common theme in priming is an increased expression of Stat1, which fosters inflammation. (18, 22, 23). More recently, TNF was likewise described to prime macrophages for enhanced subsequent responses to cytokines. Subsequently, priming by TNF was shown to be mediated by small amounts of type I IFNs via the expression of interferon-inducible genes, such as Stat1 (16).

Synovial lymphocytes, and monocytes in particular, secrete proinflammatory cytokines (24, 25). It would be important to know if the synovial overexpression of Stat1 in RA occurs locally in these cells, or constitutes a systemic event. The latter would indicate that cells reactive to circulating IFNs enter the synovial membrane, thus being already prepared to deliver specific actions.

Given that RA is a systemic disease and in line with the IFN signature of unfractionated peripheral blood cells (21), we hypothesized that, similar to the situation in SLE (26), the Stat1 pathway might be systemically activated in lymphocytes and monocytes. In accordance with this hypothesis, we investigated the integrity and level of activation of the IFN-Stat1-signaling pathway and also related the findings to the clinical disease activity.

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METHODS

Study population

Peripheral venous blood was drawn from consenting patients suffering from RA classified according to the 1987 criteria of the American College of Rheumatology (27) and from consenting healthy donors (HC). In all RA patients, surrogates of inflammatory response (CRP and ESR) were determined routinely according to standard procedures. RA disease activity was measured with the Disease activity Score (DAS)-28 (28) and the Clinical Disease Activity Index (CDAI) (29).

Detailed demographic and clinical characteristics of RA patients and HC, whose PBMC were prepared to determine Stat1 by fluorocytometry, are provided in table 1. Subjects were defined as healthy by history and by review of their continuous medication. None of the healthy subjects had a history of cancer, of a rheumatic disease, or of a current infectious disease. Only two of the HC (the oldest) were on any medication, namely antihypertensives. Most of the RA patients and HC were Caucasians; one RA patient and one HC each were of Asian origin. Most of the RA patients received methotrexate (62.5%), while approximately 20% each were on leflunomide or TNF-blockers. The subgroups of subjects selected for the Q-PCR or the in-vitro assays also presented in this paper had comparable demographic and clinical characteristics. The local ethics committee approved the study.

Cell preparation and culture

Peripheral blood mononuclear cells (PBMC) were isolated over LSM 1077 gradients (PAA Laboratories, Pasching, Austria). For some experiments, purified monocytes were prepared from PBMC, using negative selection by magnetic-activated cell sorting (MACS; Monocyte Isolation kit II from Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were either cultured in complete RPMI (RPMI 1640 + L-Glutamine, Penicillin and Streptomycin, Hepes

Invitrogen Ltd., Paisley, UK) with 10% fetal calf serum (heat inactivated, PAA Laboratories) for long term kinetic experiments (e.g. Stat1 expression after 48 hours of incubation) or in pure RPMI 1640 (Invitrogen Ltd., Paisley, UK) for short term experiments (stimulation with IFN γ for 15 minutes). The following cytokines were used at indicated concentrations: IFN γ (100 U/ml; R&D Systems, Minneapolis, USA), IFN α (100U/ml), IL10 (10 ng/ml), IL18 (10ng/ml), IL6 (500 U/ml) (all Strathmann Biotech, Hamburg, Germany), and TNF (10ng/ml; R&D Systems).

Fluorocytometry

Intracellular staining of Stat1 and direct staining for Tyr-701-phosphorylated Stat1 (pStat1) were performed in PBMC as previously described (26), using monoclonal antibodies binding Stat1 (Cell Signaling Technology Inc., Beverly, USA), and pStat1 (BD Biosciences, San Jose, USA), respectively. Isotype matched antibodies (Beckman Coulter Inc, Fullerton, USA, or BD Biosciences, San Jose, USA, for Stat1 and pStat1, respectively), served as negative controls. PBMC were analyzed on a FACScan (BD Biosciences), and individual gates (by forward scatter/side scatter [FSC/SSC] profile derived in samples stained by specific markers) were used for analyses of lymphocytes and monocytes.

Staining of monokine induced by IFN γ (MIG) was performed using PE-conjugated monoclonal antibodies (R&D Systems) according to the manufacturer's protocols. In these experiments, monocytes were characterised not only by FSC/SSC profile, but also by simultaneous staining, using an allophycocyanin-conjugated anti-CD14 antibody (Beckmann Coulter). To inhibit cytokine secretion, GolgiStop (BD Biosciences) solution was used according to the manufacturer's protocol. Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences). All data were expressed as mean fluorescence intensity (MFI).

Western Blot

CD14⁺ monocytes isolated by magnetic cell sorting (MACS) were lysed in lysis buffer containing phosphatase inhibitor cocktail and sodium orthovanadate (both Sigma-Aldrich, St. Louis, USA). Protein extracts were separated by electrophoresis on a 7.5% acrylamide gel, followed by electrotransfer onto nitrocellulose membrane. After blocking for 24 hours at 4°C, the membranes were consecutively incubated with a monoclonal antibody against Stat1 (Cell Signaling) and a horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark). Specific bands were detected with the enhanced chemiluminescence (ECL) detection kit on Amersham Hyperfilm ECL (both from GE Healthcare Biosciences, Uppsala, Sweden). Protein expression was quantified using a Molecular Imager and Quantity One software (both from Bio-Rad, Hercules, USA).

Quantitative real-time polymerase chain reaction (qPCR)

For qPCR, the total RNA of CD14⁺ monocytes was extracted using the RNeasy Mini Kit (Qiagen, Valencia, USA) directly after purification by MACS or after incubation with various cytokines for 3 or 18 hours. Then, RNA was reverse-transcribed using the Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. Two microliters of complementary DNA was used for quantitative PCR, using the following primers: for Stat1: 5-GCCAAAGGAAGCACCAAGAGCCAAT-3 (forward) and 5-AGGAGACATGGGGAGCAGGTTGT-3 (reverse); for 2'5'-oligoadenylate synthetase (OAS): 5-TGCGCTCAGCTTCGTAAGTGA-3 (forward) and 5-GGTGGAGAACTCGCCCTCTT-3 (reverse); for IFN γ -inducible 10-kd protein (IP-10): 5-ATTTGCTGCCTTATCTTTCTG-3 (forward) and 5-TCTCACCTTCTTTTTCATTGTAG-3 (reverse); for MIG: 5-ATCAGCACCAACCAAGGGACT-3 (forward) and 5-GCTTTTCTTTTGGCTGACCTG-3 (reverse). Real-time PCR was performed on a LightCycler 480 using LightCycler 480 SYBR Green (Roche, Mannheim, Germany).

Expression of a tested gene was compared with that of the housekeeping gene (GAPDH: 5-TGATGACATCAAGAAGGTGGTGAAG-3 (forward) and 5-TCCTTGGAGGCCATGTGGGCCAT-3 (reverse)) to determine relative expression.

ELISA for TNF

TNF was measured in RA serum samples using a commercially available ELISA assay (Invitrogen). For this analysis, patients that received TNF-blockers were excluded.

Statistical analyses

Unpaired Student's t-tests and paired t-tests were employed for the comparison of groups, and of paired samples, provided that the data followed Gaussian distributions. These data are presented as mean \pm standard deviation (mean \pm SD). If data were not normally distributed, Mann-Whitney tests and Wilcoxon matched pairs nonparametric tests were used instead. For those measurements, median and quartiles are presented. Pearson and Spearman correlation coefficients, respectively, were calculated for investigating possible associations between variables that were or were not normally distributed. P-values <0.05 were considered statistically significant. The following categories were used to interpret r-values: 0.0 to 0.2: negligible correlation; 0.21 to 0.4: weak correlation; 0.41 to 0.7: moderate correlation; 0.71 to 0.9: high correlation; 0.91 to 1.0: very strong correlation.

RESULTS

Increased expression of Stat1 protein in RA PBMC

PBMC of 32 RA patients and 20 HC (detailed demographic and clinical characteristics are presented in Table 1) were analyzed for Stat1 contents by fluorocytometry. Individual gates (according to FSC/SSC profile) were used for analyses of lymphocytes and monocytes. RA peripheral monocytes contained significantly more Stat1 protein than HC monocytes (MFI 14.7 ± 8.1 vs. 8.0 ± 3.9 , $p=0.0002$, Figure 1A). In RA lymphocytes, Stat1 was also elevated, albeit to a lesser degree (MFI 7.9 ± 4.5 vs. 5.0 ± 2.1 , $p<0.005$, Figure 1A). To support this finding, Western blot analysis was performed on CD14⁺ monocytes, which were prepared by MACS. Again, monocytes of several RA patients, particularly those with high disease activity, contained more Stat1 protein when compared with HC (Figure 1B).

Correlation of Stat1 protein with RA disease activity

Next, we analyzed whether the increased expression levels of Stat1 were associated with RA disease activity. Indeed, a moderate correlation was found between Stat1 protein in RA lymphocytes and monocytes, as estimated by MFI, with RA disease activity, such as by DAS-28 ($r=0.43$, $p<0.02$ and $r=0.47$, $p<0.008$, respectively, Figure 2). Since DAS-28 prominently includes the acute phase response, and since the ESR also correlated with lymphocytic and monocytic Stat1 ($r=0.37$, $p<0.05$ and $r=0.4$, $p<0.05$, data not shown), we next analyzed the association with pure clinical disease activity as measured by CDAI. Again, the CDAI was well correlated with Stat1 expression (lymphocytes: $r=0.44$, $p<0.02$; monocytes: $r=0.51$, $p<0.003$, Figure 2). No correlations were observed between any type of treatment (including prednisolone) and Stat1 expression (data not shown). Importantly, while the healthy subjects were younger, no association of Stat1 expression was found with age.

IFNs are able to increase Stat1 protein contents in RA monocytes

IFNs are the classical inducers of Stat1 expression (5, 7). To exclude that cytokines other than IFNs would be responsible for increased Stat1 levels in RA, PBMC of HC (n=8) and RA (n=12) donors were incubated with IFN α , IFN γ , IL6, IL10, IL18, or TNF for 48 hours, which in preliminary experiments had been found to be the peak time of Stat1 expression, when PBMC were stimulated with either IFN α or IFN γ . After stimulation, PBMC were analyzed for Stat1 expression by fluorocytometry. Again, individual gates were used to separate lymphocytes and monocytes. Not surprisingly, both IFNs significantly induced Stat1 expression in HC and RA lymphocytes (data not shown) and monocytes (Figure 3A and 3B). On the other hand, neither IL6, nor IL10 or IL18 increased Stat1 in HC or RA cells.

TNF leads to increase in Stat1

When these results were analyzed in more detail, two interesting observations were made: First, RA lymphocytes and monocytes also increased their Stat1 expression when stimulated with TNF (RA lymphocytes: TNF: from MFI 5.1 ± 2.8 to 8.2 ± 6.1 , $p < 0.03$; RA monocytes: TNF: from MFI 17.9 ± 6.3 to 37.8 ± 31.4 , $p < 0.04$, Figure 3A). Second, RA monocytes displayed a much more pronounced increase of Stat1 after the addition of IFN γ when compared to HC (difference of the response to IFN γ : RA vs HC: MFI $+60.1 \pm 37.8$ vs. $+24.0 \pm 17.2$, $p < 0.03$, Figure 3B), indicating that RA monocytes are not only more sensitive to IFN γ but also that these cells could further be activated regarding the IFN-Stat1-signaling pathway. On the other hand, there was no significant difference between RA and HC lymphocytes when the increase of Stat1 after stimulation with IFN γ was compared (MFI $+6.1 \pm 3.5$ vs. $+4.0 \pm 2.7$, $p = 0.16$).

Stat1 in RA PBMC correlates with TNF serum levels

Given that TNF was able to increase Stat1 in RA PBMC we were also interested to know whether Stat1 expression would be associated with TNF serum levels. Therefore, TNF was measured in the sera of RA patients, for whom Stat1 expression had been determined by fluorocytometry. Of these 32 patients, 7 were excluded because they were on TNF inhibitors. As expected, TNF serum levels were associated with RA disease activity, such as by DAS28 ($r=0.58$, $p<0.03$) and CDAI ($r=0.72$, $p<0.0001$). Importantly, however, TNF serum levels also correlated with Stat1 protein contents in RA monocytes ($r=0.52$, $p=0.008$), and even lymphocytes ($r=0.51$, $p<0.008$). Although this correlation was moderate, these data – in line with the *in vitro* results shown in Figure 3 – suggest, that TNF may be involved in the increased Stat1 expression in RA monocytes and lymphocytes.

Association of Stat1 with the expression of other IFN-inducible genes

We were also interested to test whether Stat1, which is known to be inducible by IFNs, would be associated with the expression of other IFN-inducible genes, such as MIG, OAS or IP-10. Therefore, the expression of these IFN-inducible genes was assessed in MACS purified CD14⁺ monocytes from HC (n=15) and RA (n=22) patients by qPCR immediately after isolation. In conformity with the data collected by fluorocytometry, Stat1 mRNA expression (fold expression relative to GAPDH) was significantly increased in RA monocytes when compared with HC (39.2 ± 31.8 vs. 17.4 ± 5.6 , $p<0.02$, Figure 4A). While mRNA expression in RA monocytes showed a trend towards an increase of OAS (10.1 ± 8.7 vs. 7.1 ± 2.8 , $p=0.14$, *t*-test), IP-10 (1.4 ± 0.5 vs. 1.1 ± 0.8 , $p=0.47$), and MIG (19.0 ± 15.6 vs. 13.5 ± 6.4 , $p=0.16$), this did not reach statistical significance. Nevertheless, Stat1 mRNA expression in RA monocytes highly correlated with OAS ($r=0.78$, $p<0.0001$, Figure 4A), IP-10 ($r=0.86$, $p<0.0001$, Figure 4A), and MIG ($r=0.76$, $p<0.0001$, Figure 4A).

Since our data showed that Stat1 was increased in RA PBMC, while other IFN-inducible genes were not, and since the earlier results had also indicated that TNF might be involved in

Stat1 upregulation in RA (Figure 3A), we hypothesized that TNF would differently regulate these genes in RA monocytes. Therefore CD14⁺ monocytes (purified by MACS) of 12 RA patients were incubated with either IFN α , IFN γ or TNF for 3 and 18 hours and their mRNA contents of Stat1, OAS or IP-10 were assessed by qPCR. The expression of the indicated genes relative to their expression in unstimulated cultures was calculated. Within 18 hours, TNF not only induced the expression of Stat1, which is in line with the data obtained by fluorocytometry (Figure 3A), but also of the expression of OAS and IP-10.

Greater efficiency of RA monocytes to increase MIG after stimulation with IFN γ

So far, our ex vivo experiments revealed increased Stat1 in RA lymphocytes and especially in monocytes (Figure 1 and Figure 4A). Moreover, our in vitro stimulation studies suggested increased responsiveness of RA monocytes to IFN γ stimulation (Figure 3B). The latter finding would be of some importance, since RA monocytes migrate into the joints, where IFN γ is present in RA synovial tissues (8-12). We were next interested whether the higher sensitivity of RA monocytes to IFN γ would also affect the expression of other IFN-inducible proteins. Therefore, PBMC of HC (n=12) and RA (n=14) patients were stimulated with IFN γ for 4 hours. After stimulation, the extent of MIG was quantified by fluorocytometry. Monocytes were characterised not only by FSC/SSC profile but also by using an anti-CD14 antibody. RA CD14⁺ monocytes were more efficient than HC monocytes in increasing MIG expression upon stimulation with IFN γ (difference of the response to IFN γ : RA vs. HC: MFI +565 \pm 351 vs. +303 \pm 253 p<0.05, Figure 5A). Moreover, the increase in IFN-induced MIG expression after IFN γ correlated significantly with Stat1 ex vivo expression as estimated by MFI (r=0.64, p<0.02) and ESR (r=0.76, p<0.002) and showed a trend towards an association with RA disease activity (CDAI: r=0.44, p=0.1; DAS-28: r=0.63, p<0.02).

RA monocytes display more pronounced Stat1 phosphorylation after IFN γ stimulation

One mechanism underlying increased responsiveness to IFN γ appeared to involve increased phosphorylation of Stat1 after stimulation (22). Therefore, we next were interested in whether IFN γ -induced Stat1 phosphorylation would be increased in RA monocytes. PBMC of HC (n=14) and RA (n=14) patients were stimulated with IFN γ . After 15 minutes of stimulation, the degree of Stat1 phosphorylation was measured by fluorocytometry. Separate gates were set for lymphocytes and monocytes. Indeed, RA monocytes, despite already increased baseline levels (RA vs HC: MFI 5.1 ± 1.3 vs. 3.2 ± 0.7 , $p < 0.0001$), showed a more pronounced increase of pStat1 upon IFN γ stimulation than HC monocytes (difference of the response to IFN γ : RA vs HC: MFI $+3.0 \pm 1.7$ vs. $+1.5 \pm 1.1$, $p < 0.02$, Figure 5B). In contrast, lymphocytes of HC and RA patients neither differed in the baseline levels of pStat1 nor in the response to IFN γ (data not shown).

DISCUSSION

The data presented reveal that PBMC, especially monocytes, of RA patients overexpress Stat1. Moreover, the extent of Stat1 expression was significantly associated with clinical disease activity. It has been known for several years that, on the local level of RA synovial tissue, Stat1 is increased (13-16, 20). Our observations of Stat1 overexpression in PBMC not only confirm that RA is a systemic inflammatory disease, but suggest that cells in the synovial membrane may be recruited from a pool of pre-activated peripheral cells, rather than by, or in addition to, local proliferation.

These findings raise several questions. First, as to which cytokines were responsible for the increased Stat1 protein levels in RA? IFNs are known to be strong inducers (7) of Stat1 expression. In fact, even very low levels of both types of IFNs were shown to increase Stat1 protein contents. Unfortunately, these levels of IFNs are usually too low to measure. Moreover, while priming resulted in increased Stat1, the expression of other IFN-inducible proteins appeared unaffected (18, 22, 23). Thus, it appeared difficult to find direct evidence for a role of low levels of IFNs in increasing Stat1 levels in RA. In consequence, we tested the alternative hypothesis that other cytokines could effect the increased Stat1 in RA PBMC. Indeed, among a panel of cytokines, we found, that TNF increased Stat1 in RA PBMC. Moreover, TNF serum levels in RA patients were associated with Stat1 protein contents. Importantly, TNF also induced the expression of other IFN-inducible genes, such as OAS or IP-10 in vitro. These genes, however, were not increased in RA monocytes when the cells were analyzed immediately after isolation. Of note the TNF effect on "IFN-inducible" genes was observed at late time points only. Given the relatively short half-life of monocytes in peripheral blood (30), differences in kinetics in the regulation of OAS and IP-10 compared with Stat1 could help explain this discrepancy.

In this context, it is of interest, that TNF *in vitro* was shown to initiate an IFN β -mediated autocrine loop, leading to increased Stat1 expression (16). Interestingly, published studies have linked plasma type I IFN activity to the clinical response among RA patients treated with TNF inhibitors (31, 32). Unfortunately, in this regard, the number of patients on TNF-blocker in our study was too low.

Thus, the presented evidence supports the idea of a TNF-IFN network, which may, at least in part, be responsible for Stat1 overexpression in RA.

All these findings would be less relevant without functional consequences. Indeed, we also found a remarkable increase in the responsiveness of RA monocytes to IFN γ , as assessed by the increase of Stat1, pStat1 and MIG after stimulation. In line with previously reported *in vitro* data (22), we also noted an association among RA monocytes' of increased Stat1 expression with an increased response to IFN γ . The latter findings would be of some importance, since sensitized RA monocytes may move to joints, where IFN γ is present. Although, it has been argued that IFN γ levels in RA synovial tissue are too low to activate cells (1, 33), the increased Stat1, as seen in our study, might decrease this threshold.

Interestingly, RA lymphocytes share most, but not all of the features found in monocytes. Thus, lymphocytes showed increased Stat1 levels, which were also associated with RA disease activity, and increased responsiveness to TNF (with regard to Stat1 expression). On the other hand, as opposed to monocytes, for lymphocytes the susceptibility to IFN γ did not differ between RA and HC. Therefore, one could speculate that different mechanisms may be acting on monocytes and lymphocytes. Future analyses will have to focus on this aspect.

Some limitations of this study deserve specific comment. First, HC were younger compared with RA patients evaluated in this study. However, neither Stat1 nor the IFN-inducible genes OAS, MIG, or IP-10 or the response to IFN were associated with age. Second, the number of individuals studied was too low to perform subgroup analysis, which may have allowed for making observations beyond those reported here. Moreover, an effect of immunosuppressive therapies, which could still be active in cells at the time of isolation, but may be waning at later time points, e.g. by 48 hours, cannot formally be ruled out; reassuringly, however, some patients did not receive immunomodulating therapy at the time of the study, but also had increased Stat1 expression with their active disease.

Taken together, our data reveal an increased Stat1 expression in RA peripheral blood mononuclear cells, especially monocytes, and therefore allow to conclude on a systemic, rather than limited local induction of this transcription factor. This increase, which may be initiated by IFNs and/or TNF, in turn results in higher responsiveness to IFN γ , in line with the idea of priming for further cytokine exposure and perpetuation of an inflammatory response.

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	RA (n=32)	HC (n=20)
Age, mean±SD (range) years	58±12 (27-77)	41±14 (27-74)
Female, no. (%)	27 (84.4)	12 (60)
Prednisolone, %	18.75	
Prednisolone, mean±SD mg/d	5.7±3.8	
MTX, %	62.5	
Leflunomide, %	18.75	
TNF-blocker, %	21.9	
ESR, mm/h (range)	31.7±28.1 (5-105)	
CRP, mg/dl (range)	0.7±0.7 (0.02-3.0)	
CDAI, mean±SD (range)	10±9.6 (0-45.3)	
DAS28, mean±SD (range)	3.6±1.5 (1.2-7.75)	

Table 1. Demographic and clinical characteristics of RA patients and HC. MTX=methotrexat, SD=standard deviation

Figure 1. Increased Stat1 in RA PBMC. **A**, Stat1 expression was analyzed by fluorocytometry in peripheral blood mononuclear cells (PBMC) of rheumatoid arthritis (RA; n=32) patients and healthy individuals (HC; n=20). Individual gates (according to FSC/SSC profile) were used for analysing lymphocytes and monocytes. Lymphocytes and monocytes of RA patients contained significantly more Stat1 protein when compared with HC PBMC (t-test). The horizontal lines indicate means. **B**, Western blot analysis of CD14⁺ monocytes from 3 healthy controls and 6 RA patients (purified by magnetic cell sorting (MACS)) confirmed increased Stat1 protein expression in RA patients with high disease activity by DAS-28 and CDAI. The level of monocyte Stat1 expression was quantified using Molecular Imager and Quantity One software. The Stat1:Actin ratios are shown in the bar graph in the bottom panel. Disease activity measures and therapy are shown in the upper panel. MTX=methotrexate; LEF=leflunomide;

Figure 2. Correlation of Stat1 with RA disease activity. Stat1 protein expression in RA monocytes and lymphocytes, as estimated by fluorocytometry (see Figure 1), correlated well with the RA composite scores DAS-28 and CDAI (Pearson correlation).

Figure 3. TNF induced Stat1 expression in RA PBMC within 48 hours. **A**, PBMC were incubated with different cytokines for 48 hours. Stat1 was measured by fluorocytometry. Individual gates (according to FSC/SSC profile) were used for analyses of lymphocytes (data not shown) and monocytes. Incubation with either IFN α or IFN γ increased Stat1 in HC and RA. TNF significantly increased Stat1 expression in RA monocytes only. Bars and the table show Stat1 MFI (mean \pm SD) of 8 HC and 12 RA patients after 48 hours. The P values represent the comparison of unstimulated versus stimulated cells (paired t-test). **B**, In both, RA and HC monocytes, IFN γ stimulation for 48 hours resulted in the upregulation of Stat1 (p=0.0002 and p<0.006, respectively, paired t-test). The comparison of the increase in Stat1

MFI between RA and HC (bar graph), however, revealed increased responsiveness of RA monocytes to IFN γ as evidenced by unpaired t-test.

Figure 4. Correlation of Stat1 expression with MIG, IP-10 and OAS mRNA expression.

A, CD14⁺ monocytes of RA patients and HC were purified by magnetic cell sorting (MACS) and analyzed for Stat1, OAS-1, IP-10 and MIG mRNA expression by qPCR directly after purification. Stat1 mRNA expression was significantly increased in RA monocytes when compared to HC (t-test, horizontal lines indicate the mean). MIG, IP-10 and OAS mRNA expression, showed a strong correlation with Stat1 mRNA expression in RA monocyte.

B, CD14⁺ monocytes (purified by MACS) of RA patients were analyzed for Stat1, IP-10 or OAS after stimulation with either IFN α , IFN γ or TNF for time points indicated. The expression of the indicated genes relative to their expression in unstimulated cultures is shown. IFNs increased Stat1, IP-10, and OAS mRNA, as assessed by quantitative PCR. Interestingly, TNF not only increased the mRNA expression of Stat1, but also of IP-10 and OAS. Bars show mean \pm SEM.

Figure 5. Increased sensitivity of RA monocytes to IFN γ . **A,** PBMC of RA patients (n=14) and healthy donors (HC, n=12) were stimulated with IFN γ for 4 hours. Expression of MIG was measured by fluorocytometry. Monocytes were characterised by simultaneous staining, using an anti-CD14 antibody. After stimulation with IFN γ , MIG expression was significantly increased in both HC and RA monocytes, when compared to medium control (p<0.002 and p<0.0001, respectively, paired t test). RA monocytes produced significantly more MIG than HC monocytes, as per increase of MIG MFI (paired t test, bars show mean \pm SEM). **B,** PBMC of RA (n=14) and HC (n=14) were stimulated with IFN γ for 15 minutes and analyzed on a fluorocytometer immediately after staining. Separate gates were set for lymphocytes (data not shown) and monocytes. In both, HC and RA monocytes, Stat1 was activated by Tyr-701

phosphorylation after the addition of IFN γ ($p=0.0003$ and $p<0.0001$, respectively, paired t-test). The increase in pStat1 MFI was much more pronounced in RA than HC (t test). Bars show mean \pm SEM.

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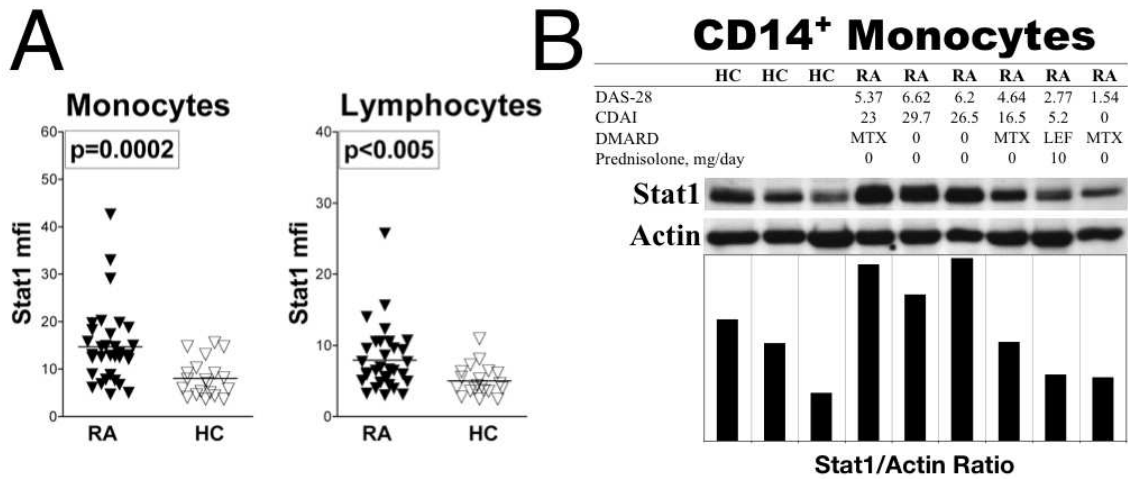


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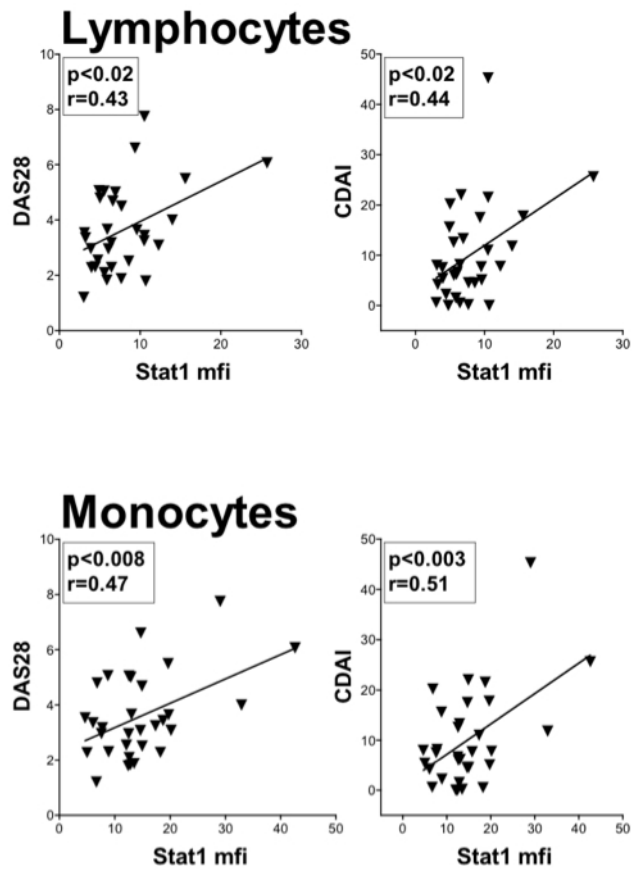


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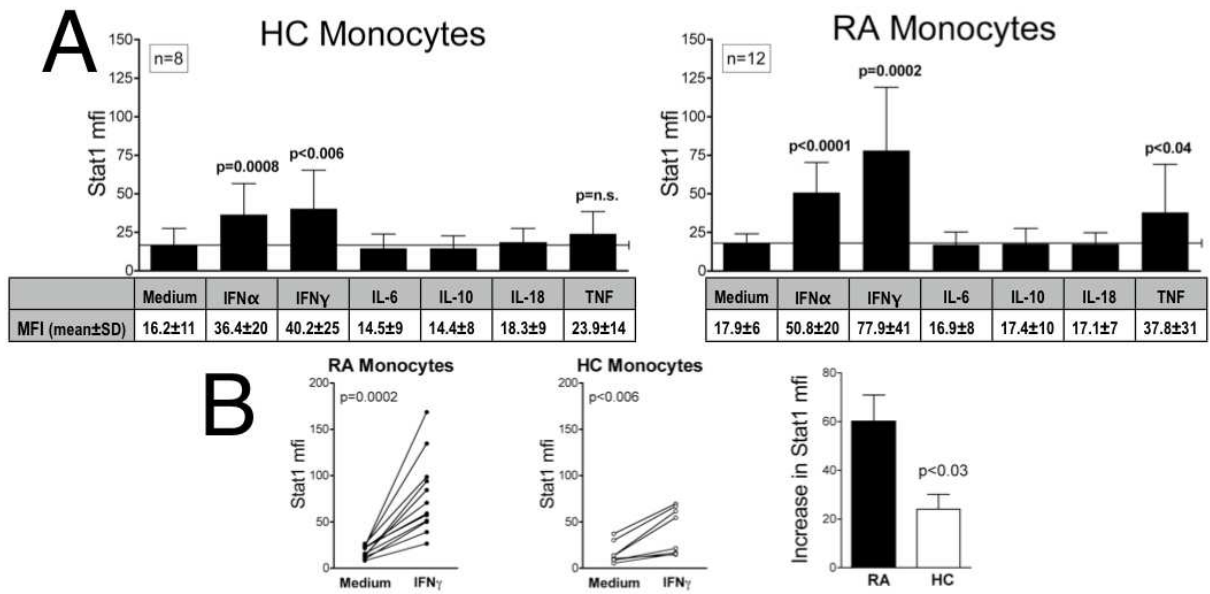


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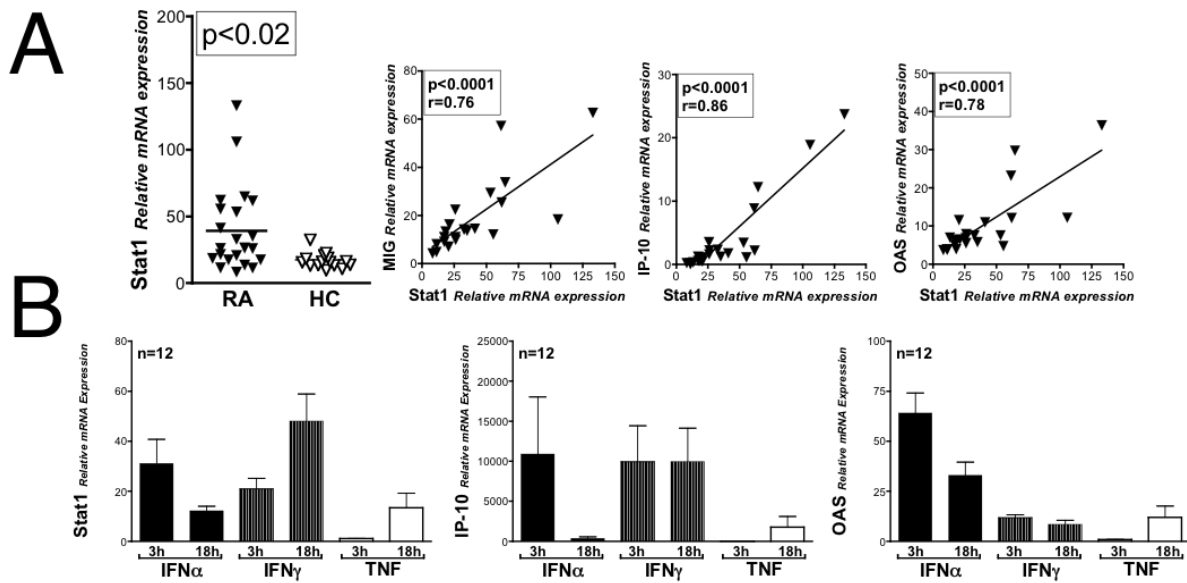


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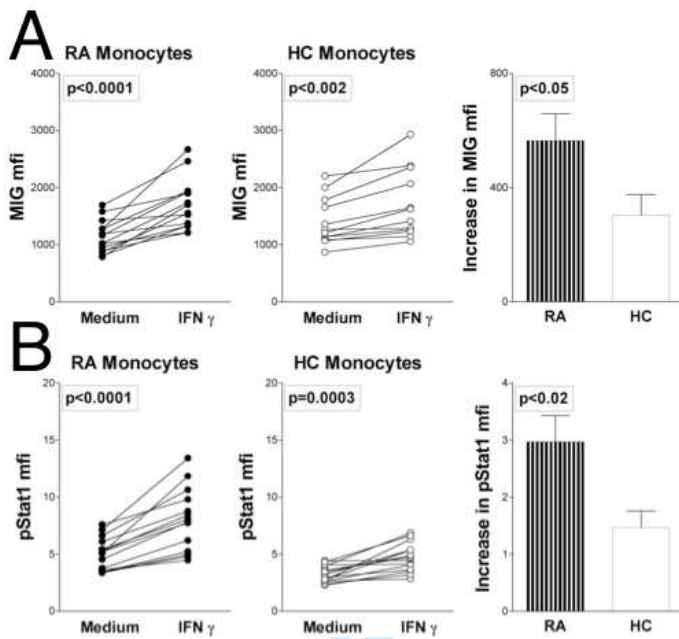
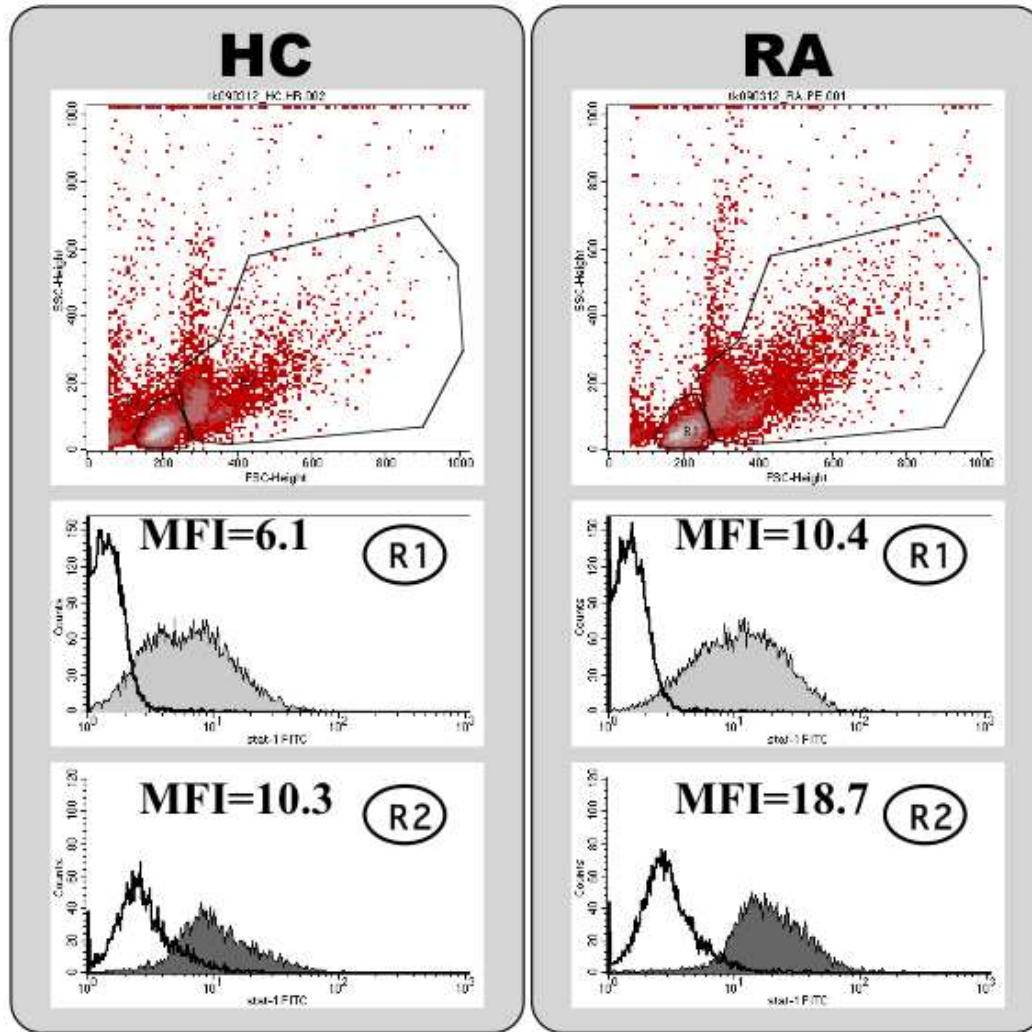
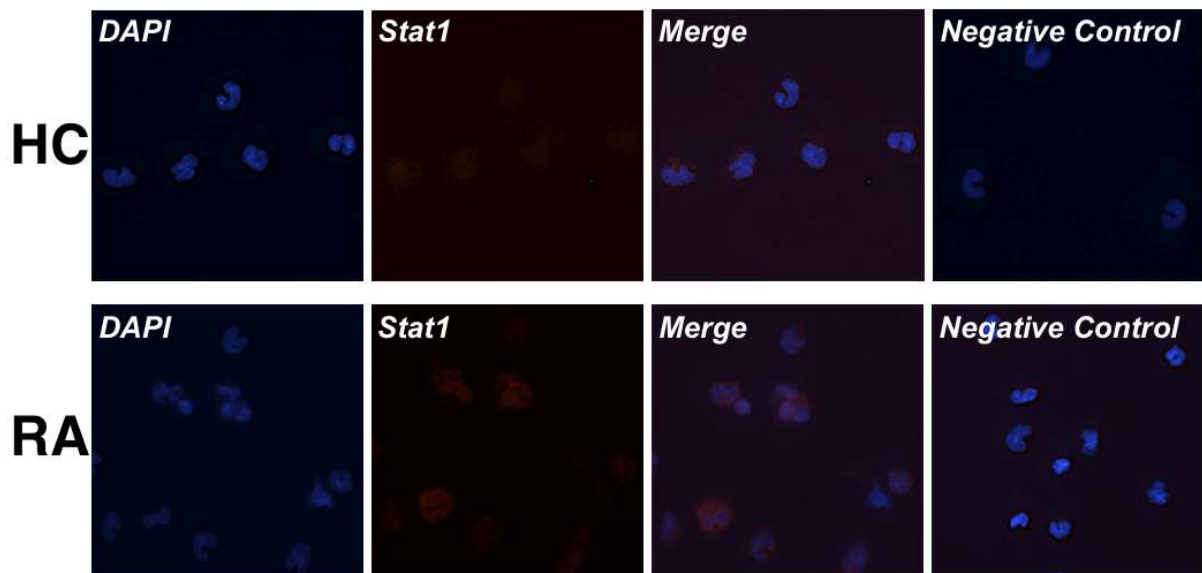


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Supplementary Figure 1. Representative histogram and dot plots for estimating Stat1 contents in PBMC of one HC (left) and one RA patient (right). PBMC were isolated over LSM 1077 gradients. Indirect intracellular staining of Stat1 was performed using either a monoclonal antibody binding Stat1 or an isotype control antibody in the first step, both followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody as a second-step reagent. PBMC were then analyzed on a FACScan. Individual gates were used for analyses of lymphocytes (R2) and monocytes (R1).



Supplementary Figure 2. PBMC of a patient with active RA (CDAI: 26.7 DAS-28: 5.41) and of a healthy person (HC) were isolated over LSM 1077 gradients and immediately placed on coverslips. After 30 minutes non-adherent cells were removed by washing. For adherent cells indirect intracellular staining of Stat1 was performed using the Fix&Perm assay and either a monoclonal antibody binding Stat1 or an isotype control antibody in the first step, both followed by an Alexa-Fluor-555-conjugated goat-anti-mouse secondary antibody as a second-step reagent. Nuclear counter staining was performed using DAPI. In line with data obtained by fluorocytometry and western-blot analysis Stat1 was increased in RA monocytes when compared to HC (original magnification x 400).