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BRIEF REPORT

Decreased Levels of STAT1 and Interferon-γ–Induced STAT1 Phosphorylation in Rheumatoid Arthritis CD4 and CD8 T Cells

Vishal Sharma, Brandon J. Pope, Nathan V. Santiago, Molly T. Boland, Dongmei Sun, Richard J. Reynolds, Alexander J. Szalai, S. Louis BridgesJr, and Chander Raman

Objective. We investigated whether a previously reported association of *IFNGR* expression with rheumatoid arthritis (RA) and its radiographic severity reflects differences in proximal interferon-γ (IFN-γ) signaling in T cells from patients with RA compared with healthy controls (HC).

Methods. Using phosphoflow cytometry, we compared IFN-γ–stimulated signal transducer and activator of transcription 1 (STAT1) activation in $CD4^+$ and $CD8^+$ T-cell populations from patients with RA and HC.

Results. Compared with controls, patients with RA had a higher proportion of $CD4^+$ T cells, associated with expansion of the $CD4^+$ effector memory subset. Several $CD4^+$ T-cell types exhibited reduced IFN-γ–induced phosphoSTAT1^{Y701} (pSTAT1^{Y701}) in patients with RA compared with HC. Engaging the T-cell receptor (TCR) complex on $CD4^+$ T cells during IFN-γ stimulation abrogated the reduction in STAT1 activation in patients with RA but had no effect in HC. The phosphorylation of STAT1^{S727} was similar in $CD4^+$ T cells from patients with RA and HC. In contrast to $CD4^+$ T cells, IFN-γ–induced pSTAT1^{Y701} levels in $CD8^+$ T cells were equivalent or higher in patients with RA compared with HC. Total STAT1 levels (phosphorylated + unphosphorylated) were lower in $CD4^+$ and $CD8^+$ T cells from patients with RA compared with HC.

Conclusion. We report diminished IFN-γ–induced pSTAT1^{Y701} levels in $CD4^+$ T cells in patients with RA, which were restored by TCR engagement. There were lower levels of total STAT1 in patients with RA compared with HC, but this likely does not explain diminished IFN-γ–induced pSTAT1^{Y701} levels in $CD4^+$ T cells because activation in $CD8^+$ T cells was higher or equivalent to that seen in HC. The enhanced *IFNGR* expression in patients with RA reported previously may reflect a compensatory mechanism to overcome deficiency in IFN-γ responsiveness.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory condition characterized by synovial inflammation and progressive joint destruction mediated by a variety of immune cells. RA affects 1% of the world's population and causes significant disability and mortality (1). The precise etiology of RA is not known, but many environmental and genetic factors contribute. This study focuses on T cells, whose activation, regulation, and function are associated with susceptibility to RA (2). Chemokines induced by interferon-γ (IFN-γ), such as CXCL9 and CXCL10, in synovial macrophages recruit T lymphocytes into the synovium and contribute to the differentiation of activated $CD4^+$ T cells into IFN-γ–producing T-helper (Th) 1 cells (3–6). These differentiated Th1 cells facilitate expansion of pro-inflammatory macrophages while suppressing regulatory T-cell function and interleukin 17–producing Th17 cells. This feed-forward effect, initiated by IFN-γ, is thought to play a role in the development of RA (7).IFN-γ–producing CD8 T cells

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No potential conflicts of interest relevant to this article were reported.

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are enriched in the RA synovium leading to the expression of IFN-γ–induced genes and proteins in fibroblasts and monocytes/macrophages (8).

Tang et al (9) showed that expression of *IFNGR1* and *IFNGR2* (genes encoding IFN-γ receptors) in whole blood from African Americans was highly associated with RA and its radiographic severity. In mouse models of arthritis, IFN-γ signaling has both pro-inflammatory and anti-inflammatory effects, indicating that increased *IFNGR* expression might contribute to a pathogenic role of IFN-γ in human RA. It is unknown whether *IFNGR* gene expression in T cells has biological consequences in RA. To address this, we studied IFN-γ signaling in peripheral blood T cells from patients with RA compared with healthy controls (HC) (10). $CD4^+$ T cells were expanded in patients with RA compared with HC, but their ability to phosphorylate the signal transducer and activator of transcription 1 (STAT1) in response to stimulation with IFN-γ was reduced. Costimulating RA $CD4^+$ T cells with αCD3 plus IFN-γ increased activation of STAT1 to the levels seen in HC cells receiving IFN-γ only, but costimulating $CD4^+$ T cells with αCD3 plus IFN-γ did not amplify IFN-γ–induced activation of STAT1 in HC cells. In contrast to $CD4^+$ T cells, IFN-γ–induced activation of STAT1 in RA $CD8^+$ T-cell populations was equivalent to or greater than that in HC $CD8^+$ T-cell populations. Notably, however, the total STAT1 protein (phosphorylated + unphosphorylated) was reduced in both $CD4^+$ and $CD8^+$ T cells from patients with RA.

PATIENTS AND METHODS

Study design. HC and patients with RA (Table 1) were enrolled in The University of Alabama at Birmingham Rheumatology Arthritis and Database Repository (RADAR) registry. RA was diagnosed by a rheumatologist, and all patients met 2010 American College of Rheumatology classification criteria for RA (11). Most patients were treated with methotrexate, biologic agents, and/or corticosteroids, but none received Janus kinase (JAK) inhibitors.

Table 1. Demographics of patients with RA and health controls

	Patients with RA (n = 17)	Controls (n = 11)
Female sex, %	77	36
Age at blood draw, mean ± SD, years	65 ± 10	42 ± 13
Disease duration, mean ± SD, years	25 ± 12	–
IgM RF-positive, %	65	–
Anti–CCP-positive, %	56	–
CRP, range mg/L	<1.0-239	–
Methotrexate (current), %	56	–
RA biologics (current), %	65	–
Corticosteroids (current), %	35	–
CDAI, mean ± SD	12 ± 9	–

Abbreviations: anti-CCP, anti–cyclic citrullinated peptide; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; IgM, immunoglobulin M; RA, rheumatoid arthritis; RF, rheumatoid factor.

Heparinized blood was collected and processed within 12 hours by centrifugation (2000 rpm). Plasma was decanted, and the buffy coat was resuspended in calcium- and magnesium-free phosphate-buffered saline (PBS) and subjected to gradient separation by using Lymphoprep (STEMCELL Technologies). The peripheral blood mononuclear cell (PBMC) band was collected, washed in PBS (calcium and magnesium free), and cryopreserved in freezing medium (90% fetal bovine serum and 10% dimethyl sulfoxide).

Cell cytometry. PBMCs were thawed quickly and brought to room temperature, washed, stained with fluorochrome-conjugated antibodies (Supplementary Table 1), and analyzed by using a Becton Dickinson FACSymphony instrument (BD Biosciences). After gating on lymphocytes and eliminating Zombie-NIR^+ (Biolegend) dead cells, we excluded $CD19^+$ B cells and then gated on $CD4^+$ and $CD8^+$ T cells and their naïve (T_N) ($CD45RA^+$, $CCR7^+$), central memory (T_{CM}) ($CD45RA^+$, $CCR7^-$), effector memory (T_{EM}) ($CD45RA^-$, $CCR7^+$), effector (T_E) ($CD45RA^-$, $CCR7^-$), and regulatory (Treg) ($CD127^{lo}$, $CD24^{hi}$) subpopulations (Supplementary Figure 1). Data were analyzed by using FlowJo v10 software (FlowJo, LLC).

Phosphoflow cytometry. Freshly thawed PBMCs were washed in RPMI, filtered, aliquoted into 96-well round bottom plates (8×10^6 cells per well), and allowed to rest (45 minutes at 37°C). PBMCs were then stimulated with 10 ng/ml of IFN-γ (BioLegend catalog No. 570206) and/or 1 ug/ml of αCD3 (BioLegend catalog No. 317347). The concentrations of IFN-γ and αCD3 used for stimulation and costimulation, respectively, and the time period for optimal IFN-γ–induced STAT1 were determined (Supplementary Figures 2, 3, and 4, respectively). After stimulation, the cells were partially fixed by incubating for 7.5 minutes with dilute (1:5) Fixation Buffer (BioLegend catalog No. 85450), resuspended in PBS containing 100 µl of Zombie Fixable Viability Stain (BioLegend catalog No. 423105) for 10 minutes at 4°C, and finally resuspended in a mixture of fluorochrome-conjugated antibodies for 15 minutes at 4°C (Supplementary Table 1). Surface-labeled cells were then completely fixed by incubating with Fixation Buffer (BioLegend catalog No. 85450) for10 minutes at real time in the dark. Thereafter, the cells were washed twice with PBS, permeabilized with 90% methanol (Perm Buffer III; BD Biosciences catalog No. 558050) for 20 minutes on ice in the dark, and washed with PBS. We probed the cells for $pSTAT1^{Y701}$, $pSTAT1^{S727}$, $pSTAT3^{Y705}$, $pSTAT5^{Y694}$, and total STAT1 in a separate acquisition.

Statistical analysis. Summary statistics (percentage of cells, mean fluorescence intensity) are presented as mean ± SEM. All statistical analyses were done by using Prism (GraphPad Software, LLC). When appropriate, comparisons were performed by using either paired two-tail Student's *t* tests, unpaired two-tail Student's *t* tests, one-way analysis of variance (ANOVA) by using post hoc Fisher's least significant difference tests, or two-way

repeated measures ANOVA by using post hoc Bonferroni-Holm's correction. An α value of $P < 0.05$ was considered significant.

RESULTS

CD4+ T cells are expanded in patients with RA but show decreased IFN-γ–induced activation of STAT1 (pSTAT1Y701). In the peripheral blood, the proportion of $CD4^+$ T cells was elevated approximately 1.5-fold in patients with RA compared with HC, and the analysis of T-cell subpopulations revealed that only the T_{EM} population was significantly greater in patients with RA (Figure 1A). Basal tyrosine phosphorylation of STAT1 ($pSTAT1^{Y701}$) in RA and HC $CD4^+$ T cells and T-cell subpopulations were similar (Figure 1B). All $CD4^+$ T-cell subsets responded to IFN-γ in patients with RA and HC (Supplementary Figure 5). However, the levels of IFN-γ–induced $pSTAT1^{Y701}$ in patients with RA were significantly lower than that in HC in all $CD4^+$ T-cell subsets, except T_{CM} cells (Figure 1B). IFN-γ–induced $pSTAT1^{Y701}$ was also significantly lower in Treg cells in patients with RA (Figure 1B).

JAKs are primarily responsible for phosphorylating STAT1 on Tyr^{701}, a step required for dimerization of STAT1 and its translocation to the nucleus. STAT1 can also be phosphorylated on Ser^{727}, independent of JAKs, which promotes its transcriptional activity (12,13). We found that the basal levels were higher in $CD4^+$ T cells from patients with RA compared with HC, as expected for a JAK independent event; $pSTAT^{S727}$ was not further increased by IFN-γ

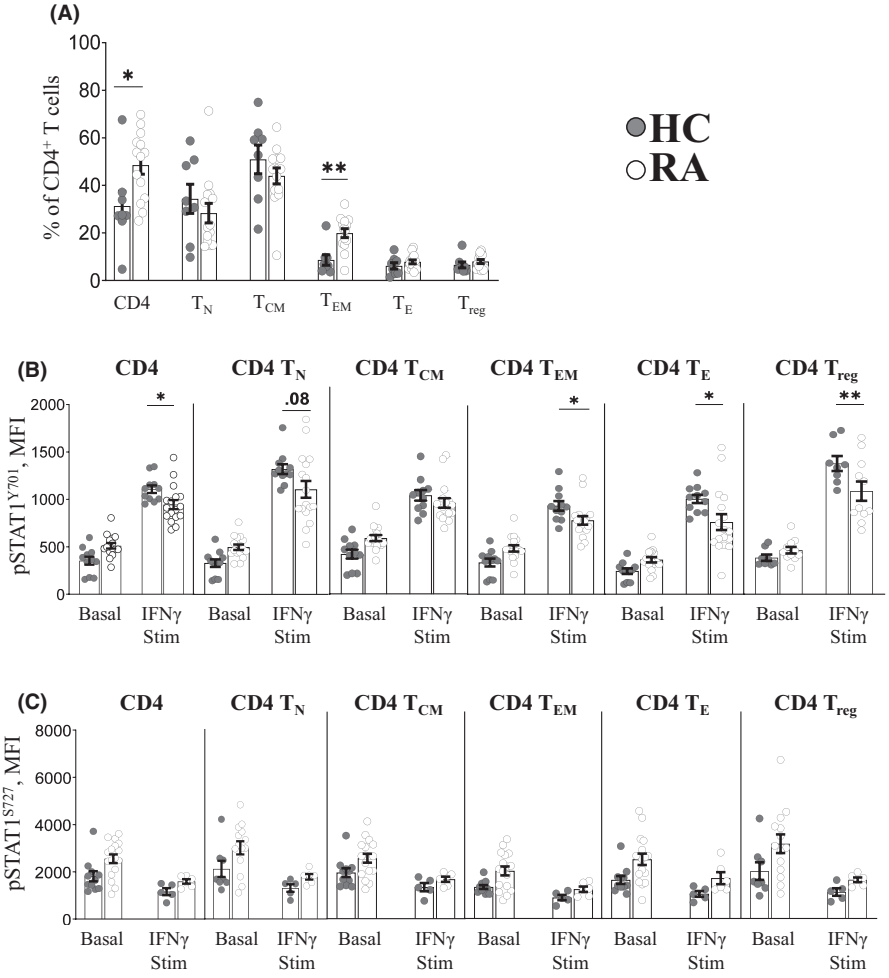

Figure 1. $CD4^+$ T-cell populations and interferon-γ (IFN-γ)–induced phosphorylation of STAT1 in patients with rheumatoid arthritis (RA) compared with healthy controls (HC). **A**, Proportion of total $CD4^+$ T cells and $CD4^+$ T-cell subpopulations in HC and patients with RA. **B**, Levels (mean fluorescence intensity [MFI]) of $pSTAT1^{Y701}$ in total $CD4^+$ T cells and $CD4^+$ T-cell subpopulations from HC and patients with RA at basal conditions or after stimulation with IFN-γ for 15 minutes. **C**, Levels (MFI) of $pSTAT1^{S727}$ in total $CD4^+$ T cells and $CD4^+$ T-cell subpopulations in HC and patients with RA at basal conditions or after stimulation with IFN-γ for 15 minutes. Peripheral blood mononuclear cells from HC (solid circles) and patients with RA (open circles) were characterized for the proportion of $CD4^+$ T cells and $CD4^+$ T-cell subpopulations by flowcytometry. Data shown are mean ± SEM, each dot representing an independent individual donor. Unpaired Student's *t* tests (**A** and **C**) and one-way analysis of variance with uncorrected Fisher's least significant difference tests (**B**) were performed. $^*P < 0.05$; $^{**}P < 0.01$. T_{CM}, central memory T cells ($CD45RA^-$, $CCR7^+$); T_E, effector T cells ($CD45RA^+$, $CCR7^-$); T_{EM}, effector memory T cells ($CD45RA^-$, $CCR7^-$); T_N, naïve T cells ($CD45RA^+$, $CCR7^+$); T_{reg}, regulatory T cells ($CD25^+$, $CD127^{lo}$).

stimulation (Figure 1C). We did not detect active PI3K in RA or HC CD4$^+$ T cells (Supplementary Figure 6), the kinase upstream of STAT1 Ser^{727} phosphorylation (14). As a control, we did find that T-cell receptor (TCR) stimulation (αCD3) induced activation of PI3K (data not shown).

Engaging the TCR complex enhances IFN-γ–induced STAT1Y701 phosphorylation in RA CD4+ T cells. We previously showed in mice that TCR engagement enhances IFN-γ–induced activation of STAT1^{Y701} in CD4$^+$ T cells (15). TCR stimulation has been shown to affect JAK-/STAT-dependent cell signaling in response to other cytokines (16). We therefore investigated whether engaging the TCR would enhance the reduced IFN-γ–induced activation of STAT1 in RA CD4$^+$ T cells. Costimulating CD4$^+$ T cells with αCD3 significantly enhanced IFN-γ–induced STAT1^{Y701}, but not STAT1^{S727}, activation in CD4$^+$ T cells from patients with RA (Figure 2A and Supplementary Figure 7, respectively). This effect was not observed in HC. The enhancing effect of αCD3 costimulation on IFN-γ–induced activation of STAT1 in patients with RA was not limited to any particular CD4$^+$ T-cell subset. Notably, costimulation with αCD3 raised the levels of pSTAT1^{Y701} in RA T cells to the levels seen in HC T cells (Figure 2B).

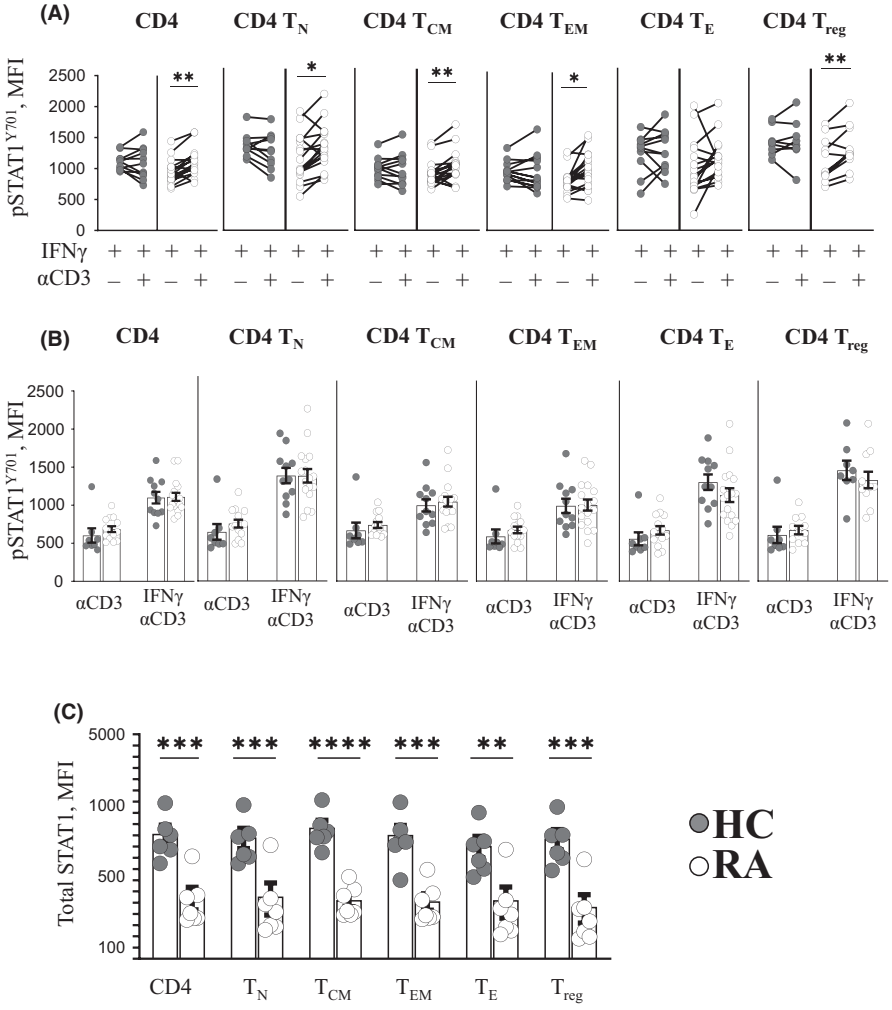

Figure 2. Costimulation of interferon-γ (IFN-γ) + αCD3 induced phosphorylation of STAT1 and total STAT1 levels in patients with rheumatoid arthritis (RA) compared with healthy controls (HC). **A**, Levels (mean fluorescence intensity [MFI]) of pSTAT1^{Y701} in total CD4$^+$ T cells and CD4$^+$ T-cell subpopulations from HC and patients with RA following stimulation with IFN-γ or IFN-γ + αCD3 for 15 minutes. **B**, Levels (MFI) of pSTAT1^{Y701} in CD4$^+$ T cells and CD4$^+$ T-cell subpopulations from HC and patients with RA following stimulation with αCD3 or IFN-γ + αCD3 for 15 minutes. **C**, Levels (MFI) of total STAT1 (unphosphorylated + phosphorylated) in total CD4$^+$ T cells and CD4$^+$ T-cell subpopulations in HC and patients with RA at basal conditions, without any stimulation(s). Peripheral blood mononuclear cells from HC (solid circles) and patients with RA (open circles) were characterized for the proportion of CD4$^+$ T cells and CD4$^+$ T-cell subpopulations by flowcytometry. Data shown are mean ± SEM, each dot representing an independent individual donor. Paired Student's *t* tests (**A**), one-way analysis of variance with uncorrected Fisher's least significant difference tests (**B**), or unpaired Student's *t* tests (**C**) were performed. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. T_{CM}, central memory T cells (CD45RA$^-$, CCR7$^+$); T_E, effector T cells (CD45RA$^+$, CCR7$^-$); T_{EM} = effector memory T cells (CD45RA$^-$, CCR7$^-$); $T_{N,}$ naïve T cells (CD45RA$^+$, CCR7$^+$); T_{reg}, regulatory T cells (CD25$^+$ CD127^{lo}).

IFN-γ–induced activation of STAT1 is enhanced in CD8+ TCM and TEM cells from patients with RA. Although the proportions of total peripheral $CD8^+$ T cells in patients with RA and HC were similar, there was expansion of the T_{EM} and T_E populations and a contraction of the T_N population in patients with RA (Figure 3A). This pattern is consistent with a previous report showing expansion of T_E and T_{EM} $CD8^+$ T cells in patients with RA (17). In contrast to $CD4^+$ T cells (Figure 1B), we found that IFN-γ–induced $pSTAT1^{Y701}$ levels in $CD8^+$ T_{CM} and T_{EM} cells were elevated in patients with RA compared with HC (Figure 3B). Costimulation with αCD3 had no effect on IFN-γ–induced $pSTAT1^{Y701}$ levels in $CD8^+$ T cells from patients with RA or HC (Figure 3C and Supplementary Figure 8).

CD4+ and CD8+ T cells from patients with RA have lower levels of total STAT1. Zimmerman et al (18) showed that monocytes from patients with mutations in the autoimmune regulator (*AIRE*) gene have reduced levels of IFN-γ–induced $pSTAT^{Y701}$ and reduced levels of total STAT1 (phosphorylated + unphosphorylated). Therefore, we investigated whether differences

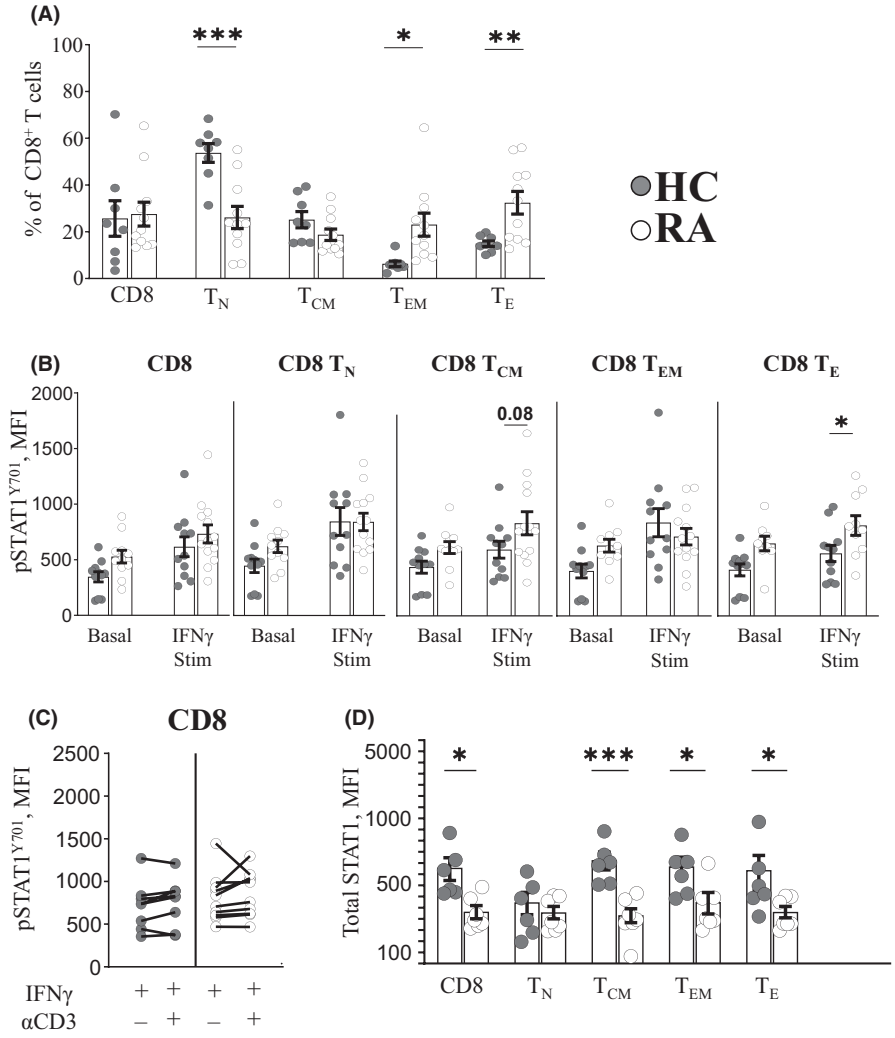

Figure 3. $CD8^+$ T-cell populations and interferon-γ (IFN-γ)–induced phosphorylation of STAT1 and total STAT1 levels in patients with rheumatoid arthritis (RA) compared with health controls (HC). **A**, Proportion of total $CD8^+$ T cells and $CD8^+$ T-cell subpopulations in HC and patients with RA. **B**, Levels (mean fluorescence intensity [MFI]) of $pSTAT1^{Y701}$ in total $CD8^+$ T cells and $CD8^+$ T-cell subpopulations in HC and patients with RA at basal conditions or after stimulation with IFN-γ for 15 minutes. **C**, Levels (MFI) of $pSTAT1^{Y701}$ in total $CD8^+$ T cells in HC and patients with RA following stimulation with IFN-γ or IFN-γ + αCD3 for 15 minutes. **D**, Levels (MFI) of total STAT1 (unphosphorylated + phosphorylated) in total $CD8^+$ T cells or $CD8^+$ T-cell subpopulations in HC and patients with RA at basal conditions, without any stimulation(s). Peripheral blood mononuclear cells from HC (solid circles) and patients with RA (open circles) were characterized for the proportion of $CD4^+$ T cells and $CD4^+$ T-cell subpopulations by flowcytometry. Data shown are mean ± SEM, each dot representing an independent individual donor. Unpaired Student's *t* tests (**A** and **D**), one-way analysis of variance with uncorrected Fisher's least significant difference tests (**B**), or paired Students *t* tests (**C**) were performed. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. T_{CM}, central memory T cells ($CD45RA^-$, $CCR7^+$); T_E, effector T cells ($CD45RA^+$, $CCR7^-$); T_{EM}, effector memory T cells ($CD45RA^-$, $CCR7^-$); T_N, naïve T cells ($CD45RA^+$, $CCR7^+$).

in the levels of total STAT1 might explain to some extent the differences in STAT1 activation seen in RA and HC T-cell subsets. Surprisingly, we found significantly lower levels of total STAT1 in all but one T-cell population from patients with RA ($CD8^+$ T_N cells being the exception) compared with HC (Figures 2C and 3D). Normalizing $pSTAT1^{Y701}$ to STAT1, we find that, proportionately, IFN-γ induced greater activation of STAT1 in RA $CD4^+$ T cells than in HC $CD4^+$ T cells (Supplementary Figure 9).

DISCUSSION

In this study, we show that T_{CM} and T_{EM} and T_E $CD4^+$ T cells and Treg cells from individuals with RA, compared with HC, have significantly dampened responsiveness to IFN-γ stimulation in the context of activation of STAT1. In contrast, IFN-γ–induced activation of STAT1 in RA $CD8^+$ T cells was similar to that in HC $CD8^+$ T cells; the exception was in T_E cells, in which IFN-γ–induced activation of STAT1 was greater in RA $CD8^+$ T cells than in HC $CD8^+$ T cells. The reduced IFN-γ responsiveness in $CD4^+$ T cells observed in this study is not consonant with our previous finding that *IFNGR* expression is increased in RA T cells (9). This might be due to the fact *IFNGR* expression in the previous study was measured in whole blood and not selectively in T cells. It is also possible that *IFNGR* expression is elevated in patients with RA as part of a compensatory response to overcome diminished sensitivity to IFN-γ. IFN-γ did not activate STAT3 and STAT5 in RA and HC T cells (data not shown). From these results, we infer that lower IFN-γ–induced activation of STAT1 in patients with RA is not compensated for by activation of other STAT proteins. The diminished IFN-γ responsiveness in RA $CD4^+$ T cells was restored to levels equivalent to that in HC $CD4^+$ T cells when the TCR was coengaged. Although the mechanism(s) by which TCR engagement increases IFN-γ–induced activation of $pSTAT1^{Y701}$ is currently unknown, we predict that it may be through inactivation of glycogen synthase kinase-3 (15,19). A limitation of this study is the sample size and demographic difference between the RA and control groups, so larger studies will be needed to validate these findings.

Unexpectedly, we discovered that T-STAT1 levels were diminished in both CD4 and CD8 T cells from individuals with RA compared with HC. However, T-STAT1 level is insufficient to explain the differences in IFN-γ–induced activation of STAT1 seen between RA and HC $CD4^+$ and $CD8^+$ T-cell subpopulations. This is supported by the finding that in patients with RA, costimulation with αCD3 restored IFN-γ–induced activation of STAT1 to the levels seen in HC. Low basal levels of T-STAT1 observed in RA T cells might be due to decreased transcription and/or enhanced proteasome-mediated degradation of the STAT1 protein, perhaps mediated by ERK activation (20). In fact, elevated ERK activity is a feature in RA CD4 and CD8 T cells (10).

Our data suggest that in RA CD4 T cells, down modulation of T-STAT1 may be one mechanism for dampening the IFN-γ response. Alternatively, given that IFN-γ function is pleotropic, RA might be exacerbated by dampening the protective effects of IFN-γ. Longitudinal studies examining IFN-γ response in patients with different levels of RA disease activity will be needed to address these questions. Our current study did not address underlying mechanisms of our findings, a goal for future investigation. In this study, all in vitro stimulations were performed by using PBMCs, followed by an analysis of gated populations. This allowed us to interrogate rare cell populations that might otherwise be lost during cytometry/sorting and avoid any stimulation induced by antibodies required for their sorting. We recognize that monocytes within the PBMC mixture can have bystander effect on T cells, but we think this is unlikely to contribute significantly to our findings. Stimulations were only for 15 minutes, a duration likely too short for a paracrine effect to impact STAT phosphorylation in T cells.

In summary, we report several novel findings. First, several $CD4^+$ T-cell populations from patients with RA were less responsive to IFN-γ stimulation than similar populations in HC. Second, coactivation of the TCR complex enhances IFN-γ–induced STAT1 activation in RA $CD4^+$ T cells but not HC $CD4^+$ T cells. Third, most peripheral blood $CD4^+$ and $CD8^+$ T-cell subsets from patients with RA have lower T-STAT1 content than their counterparts from HC. Overall, these findings, if validated, may lead to actionable biomarkers to stratify risk of RA or identify patients with RA with the highest likelihood of response to JAK/STAT inhibitors.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Raman had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sharma, Pope, Bridges, Raman.

Acquisition and design. Sharma, Pope, Santiago, Boland, Bridges, Raman.

Analysis and interpretation of data. Sharma, Sun, Reynolds, Szalai, Bridges, Raman.

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