Targeting intracellular signaling pathways at the interface of T lymphocyte and innate immunity in immune-mediated inflammatory diseases
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Sustained T cell Rap1 signaling is protective in the collagen-induced arthritis model of rheumatoid arthritis

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Abstract

**Objective:** Defective activation of T cell receptor–proximal signaling proteins, such as the small GTPase Rap1, is thought to contribute to the pathologic behavior of rheumatoid arthritis (RA) synovial T cells. This study was undertaken to determine whether maintaining Rap1 signaling in murine T cells modifies disease onset or severity in collagen-induced arthritis (CIA).

**Methods:** CIA experiments were conducted using wild-type and RapV12-transgenic mice, which express an active mutant of Rap1 in the T cell compartment. Mice were assessed using macroscopic, microscopic, and radiologic measures, and serum levels of anticollagen antibodies were measured by enzyme-linked immunosorbent assay. Phenotypic and functional characterization of wild-type and RapV12-transgenic T cells under homeostatic conditions and during disease onset was performed by flow cytometry.

**Results:** Disease incidence and severity, synovial infiltration, joint destruction, and anticollagen antibody production were significantly reduced in RapV12-transgenic mice. Although the numbers and percentages of CD3+, CD4+, and CD8+ (naive, effector, and memory) T cells, Treg cells, and Th17 cells were equivalent in wild-type and RapV12-transgenic mice, a significant decrease in the percentage of tumor necrosis factor secreting CD8+ T cells was observed in RapV12-transgenic mice during CIA. RapV12-transgenic T cells also inefficiently expressed inducible costimulator and CD40L costimulatory proteins involved in B cell immunoglobulin class switching.

**Conclusion:** Our findings indicate that maintenance of T cell Rap1 signaling in murine T cells reduces disease incidence and severity in CIA, which are associated with specific defects in T cell effector function. Therefore, the restoration of Rap1 function in RA synovial T cells may have therapeutic benefit in RA.
Introduction

T cells contribute to synovitis and joint destruction in rheumatoid arthritis (RA), a prototype immune-mediated inflammatory disease, through the pleiotropic activation of macrophages and synovial stromal cells via cell–cell contacts and interleukin-17 (IL-17) production, the stimulation of B cells producing autoimmune antibodies, and the promotion of osteoclast differentiation. An active role for the T cell receptor (TCR) in the initiation and perpetuation of disease in RA is suggested by associations between expression of specific major histocompatibility complex HLA–DR1 and DR4 epitope alleles with enhanced disease risk and disease severity in RA patients. T cells derived from RA synovial tissue or synovial fluid (SF) display characteristics suggestive of recent TCR stimulation, including surface expression of CD45RO, CD69, CD154, HLA–DR, inducible costimulator (ICOS), and very late activation antigen 4 proteins. These T cells are primarily proinflammatory Th1 and Th17 cells and display hyperresponsive cytokine responses to TCR/CD28 stimulation. In established RA, inflammatory cytokines that are present in the synovial tissue, such as IL-6, IL-12, IL-8, and tumor necrosis factor α (TNFα), rather than antigen stimulation, may drive T cell contributions to the perpetuation of inflammation.

Although the molecular mechanisms underlying altered T cell function in RA are unknown, recent studies have indicated that inactivation of the small GTPase Rap1 may contribute to the pathogenic behavior of T cells in the synovium. TCR stimulation results in the activation of guanine nucleotide exchange factors, which promote accumulation of Rap1 in an active GTP-bound form. TCR-dependent Rap1 activation is exquisitely sensitive to costimulatory signals provided by antigen-presenting cells such as CD28, which acts through the Rap1 GTPase activating protein RapGAP and suppresses Rap1 activation. Conversely, CTLA-4 ligation, which opposes CD28 signaling, promotes accumulation of GTP-bound Rap1. Once activated, Rap1 regulates several distinct signaling pathways that are predicted to contribute to the quality of T cell immune responses in vivo. Activation of Rap1 by TCR ligation, chemokines, and adhesion molecules promotes remodeling of the cytoskeleton and integrin activation, needed for T cell trafficking and adhesion to antigen-presenting cells. Additionally, under certain experimental conditions, Rap1 suppresses TCR-dependent ERK activation and IL-2 production, either directly, through blocking Raf kinase activation, or indirectly, through diminishing TCR-dependent reactive oxygen species (ROS) production.

Genetic manipulation of Rap1 signaling pathways in vivo has provided further evidence that the activation status of Rap1 in T cells can have qualitative effects on immune responses. Mice deficient in Spa-1, a RapGAP expressed in T cells, demonstrate age-dependent defects in T cell proliferative responses to stimulation by the TCR, mitogens, and recall antigen. Transgenic mice expressing an active Rap1E63 mutant in T cells exhibit defects in both primary and secondary T cell proliferative responses.
as well as defects in B cell Ig class switching. Defective T cell responses in these mice are attributed to both the suppression of ERK activation in effector cells and increases in the frequency and functional capacity of CD103-expressing Treg cells. Conversely, transgenic expression of RapGAP1 in T cells, suppressing Rap1 function, leads to an accumulation of T cells in lymph nodes (LNs). These T cells express high levels of CD69 under homeostatic conditions, possibly reflecting an autoreactive phenotype. The accumulation of activated LN T cells of RapGAP1-transgenic mice may also indicate defects in T cell trafficking, a phenotype displayed in Rap1A-knockout mice. Despite previous investigations, it is still unclear if Rap1-mediated T cell functions are relevant to immune responses in human disease. We have previously demonstrated that Rap1 activation is suppressed in T cells obtained from RA SF, likely a result of CD28-dependent interactions with synovial antigen-presenting cells. Suppressed Rap1 function in RA synovial T cells is associated with enhanced T cell ROS production and TCR-dependent cytokine responsiveness, indicating that defects in Rap1 signaling may contribute to the T cell–dependent pathologic process in RA. To determine whether maintenance of T cell Rap1 signaling might protect against autoimmunity, we examined the effects of transgenic expression of active Rap1 on pathogenesis in murine collagen-induced arthritis (CIA). We show that transgenic expression of an active mutant of Rap1, RapV12, in murine T cells potently suppresses disease incidence and severity in CIA, indicating that enhancing T cell Rap1 function may be beneficial in the treatment of human immune-mediated inflammatory diseases.

Methods

Animals
RapV12-transgenic C57BL/6J mice were kindly provided by Dr. D. A. Cantrell (University of Dundee, Dundee, UK) and were maintained as heterozygous transgenic by breeding with wild-type C57BL/6J mice (Charles River). RapV12-transgenic mice were backcrossed onto the wild-type C57BL/6J background for at least 10 generations prior to their use in these experiments, and wild-type littermates obtained in these breedings were used as experimental controls. RapV12-transgenic and wild-type littermate control mice were housed under conventional conditions at the animal facility of the Academic Medical Center (Amsterdam, The Netherlands). Mice were fed ad libitum. The animal ethics committee of the Academic Medical Center approved all experiments.

Induction and assessment of CIA
Mice (10–12 weeks of age) were injected intradermally on day 0 at the base of the tail with 100 µl of an emulsion of chicken type II collagen (CII; Sigma-Aldrich) and Freund’s complete adjuvant (CFA; Chondrex) (133 µg of chicken CII and 133 µg of CFA in a total emulsion volume of 100 µl). The immunization was repeated on day 21, and animals
were monitored 3 times per week until they were killed on day 60. Arthritis severity was assessed in a blinded manner, using a semiquantitative scoring system (with scores ranging from 0 to 4 for each paw), as previously described. Hind paw ankle joint swelling was measured using a dial caliper (Kroeplin Längenmesstechnik). After animal sacrifice, the hind paws were fixed, decalcified, and embedded in paraffin, and sagittal serial sections were stained with hematoxylin and eosin (H&E). Graded scales of 0–3 were used to score inflammation and cartilage erosions, and a scale of 0–4 was used for radiologic assessment of bone destruction. Anticollagen antibody production in mouse serum was measured by adding serial dilutions of mouse serum to 96-well plates coated with 5 µg/ml of chicken CII, followed by sequential incubation with biotinylated rat anti-mouse Ig (Southern Biotechnology Associates) of the indicated isotype and streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch). Plates were developed with p-nitrophenyl phosphate substrate (Sigma-Aldrich), and the optical density at 415 nm was measured.

Cell staining and flow cytometry
Single-cell suspensions were obtained from spleen and axillary LNs, and erythrocytes were removed. Cells were stained with the indicated fluorochrome-conjugated antibodies for surface markers and intracellular cytokines. The antibodies fluorescein isothiocyanate (FITC)–conjugated anti-CD3, allophycocyanin (APC)–conjugated anti-CD3, Alexa 700–conjugated anti-CD3; phycoerythrin (PE)–conjugated anti-CD8, FITC-conjugated anti-CD8, Alexa 750–conjugated anti-CD8, PE-conjugated anti-CD69, FITC-conjugated anti-CD44, APC-conjugated anti-CD62L, PE-conjugated anti-CD103, PE-conjugated anti-FoxP3, APC-conjugated anti-FoxP3, Alexa 488–conjugated IL-17, APC-conjugated anti-TNFα, and APC-conjugated and PerCP-Cy5.5–conjugated interferon-γ (IFNγ) were obtained from eBioscience. FITC-conjugated anti-CD4, PE-conjugated anti-CD4, PE-Cy7–conjugated anti-CD4, FITC-conjugated anti-CD25, PE-conjugated anti-TNFα, APC-conjugated anti–IL-2, PE-conjugated anti–IL-4, and PE-conjugated anti–IL-10 were from BD Biosciences.

FoxP3 intracellular stainings were performed according to the recommendations of the manufacturer. For assessment of T cell cytokine expression, cells were stimulated for 1 hour with phorbol myristate acetate (PMA; 10 ng/ml) (Sigma-Aldrich) and ionomycin (1 µM; Sigma-Aldrich) or plate-bound anti-CD3 antibodies (5 µg/ml) and anti-CD28 antibodies (5 µg/ml) (kindly provided by Dr. L. Boon, Bioceros BV, Utrecht, The Netherlands). Brefeldin A (10 µg/ml; Sigma-Aldrich) was added for the final 4 hours of stimulation, and cells were harvested and stained with CD4 and CD8 antibodies. Cells were then fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and labeled for intracellular cytokines. Activation-dependent expression of T cell surface markers was assessed by stimulating cells for 24 hours with 10 µg/ml of anti-CD3 and 4 µg/ml anti-CD28 antibodies.
of anti-CD28 antibodies. Surface marker and cytokine expression were monitored using FACSCalibur or Canto flow cytometers (BD Biosciences).

In vitro Treg and Th17 differentiation

Purified splenic CD3+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies, as described above, for 3 days in the presence of 50 units/ml of recombinant murine IL-2 (Invitrogen) supplemented with 3 ng/ml of human transforming growth factor β (TGFβ) and 20 ng/ml of murine IL-6 (both from R&D Systems) where indicated.

Statistical Analysis

Statistical significance was determined using Student’s 2-tailed t-test. P values less than 0.05 were considered significant.

Results

Effects of T cell Rap1 activation on disease incidence and severity in murine CIA

In RA, a block in synovial T cell Rap1 activation is associated with the pathogenic behavior of these cells4,7,8. To determine whether maintenance of T cell Rap1 signaling might limit inflammation and joint destruction in an experimental model of RA, we examined the influence of T cell–specific expression of active RapV12, which is driven by the human CD2 promoter, in murine CIA19. We chose to examine RapV12-transgenic mice specifically for several reasons. RapV12 is expressed in the T cells of these mice at levels equivalent to endogenous Rap1, and Rap1 activity contributed by the transgene product is similar in magnitude to that obtained by phorbol ester/ionomycin–stimulated endogenous Rap1A. Although RapV12 T cells have enhanced integrin function, no defects are observed in TCR-dependent ERK activation or proliferative responses, and unlike Rap1E63-transgenic and RapGAP1-transgenic mice, RapV12-transgenic mice have no obvious alterations in T cell homeostasis12,16,19. Finally, unlike the active Rap1E63 mutant, RapV12 can still cycle between active and inactive states, albeit at a highly reduced rate21. We therefore reasoned that the responses of RapV12-transgenic mice with CIA might most closely mimic therapeutic interventions aimed at restoring the function of endogenous T cell Rap1.

Following the induction of CIA, 21 days after primary immunization, all wild-type littermate mice developed clinical signs of disease within 2 weeks (Figure 1A). Notably, only 20% of the RapV12-transgenic mice developed arthritis, while the remaining RapV12-transgenic mice remained disease free until the end of the experiment. Throughout the experiment, clinical arthritis scores in RapV12-transgenic mice were dramatically lower
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than those in wild-type mice ($P < 0.0005$) (Figure 1B), and paw swelling was almost completely suppressed in RapV12-transgenic mice in contrast to wild-type mice ($P < 0.005$) (Figure 1B). Histologic analysis of murine hind paws by H&E staining revealed that, unlike wild-type mice, RapV12-transgenic mice displayed an almost complete absence of joint infiltration by white blood cells (Figure 1C). This was confirmed by semiquantitative analysis of synovial cell infiltration ($P < 0.001$ versus wild-type mice) (Figure 1C). RapV12-transgenic mice showed no destruction of cartilage ($P < 0.001$ versus wild-type mice) (Figure 1C). Erosive bone damage, as determined by radiology, was reduced by $\sim 75\%$ in RapV12-transgenic mice compared with wild-type mice ($P < 0.001$).

Effects of T cell Rap1 activation on T cell homeostasis and cytokine production in healthy mice

To determine whether the protective effect of RapV12 expression was secondary to influences on T cell development or homeostasis, we phenotypically characterized T
Figure 2. Phenotypic characterization of T cells in wild-type (WT) and RapV12-transgenic mice under homeostatic conditions. (A–C), Percentages of CD4+ and CD8+ T cells (A), CD4+CD25+, CD4+CD69+, and CD8+CD69+ T cells (B), and naive (N) CD44−CD62L+, effector memory (EM) CD44+CD62L−, and central memory (CM) CD44+CD62L+ CD4+ T cells (C), present in the spleen and lymph nodes (LNs) of wild-type and RapV12-transgenic mice (n = 3 per group). (D), FoxP3 and CD103 expression in CD4+ T cells derived from the spleens (n = 13 for FoxP3; n = 10 for CD103) and LNs (n = 10 for FoxP3; n = 8 CD103) of wild-type and RapV12-transgenic mice. Bars show the mean and SEM. No significant differences were observed between groups.

Analysis of peripheral T cell compartments revealed that CD4+ and CD8+ T lymphocytes were represented at normal percentages in both the spleens and LNs of RapV12-transgenic mice (Figure 2A). RapV12 did not influence the activation status of splenic and LN T cells under homeostatic conditions, as assessed by CD25 and CD69 staining (Figure 2B). Normal proportions of naive (CD44−CD62L+), effector memory (CD44+CD62L−), and central memory (CD44+CD62L+) T cells were also observed (Figure 2C), and we detected no influence of RapV12 on the frequency of CD4+FoxP3+ Treg cells or activated CD4+CD103+ T cells (Figure 2D).

Since these data indicated that RapV12 expression had no influence on T cell maturation or homeostasis, we next examined the ability of T cells to respond to antigenic and inflammatory stimuli. RapV12 expression had no effect on the production of IL-2, TNFα, or IFNγ following TCR/CD28 in vitro stimulation of T cells isolated from the spleens (Figure 3A) or LNs (data not shown) of healthy mice. Also, RapV12 failed to influence the generation of FoxP3+ Treg cells or Th17 cells. In vitro stimulation of splenic T cells
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Figure 3. Cytokine and differentiation responses of T cells from healthy, untreated wild-type (WT) mice (open bars) and RapV12-transgenic mice (solid bars). (A), Intracellular expression of interleukin-2 (IL-2), tumor necrosis factor α (TNFα), and interferon-γ (IFNγ) in splenocytes from healthy wild-type and RapV12-transgenic mice that were stimulated for 5 hours with anti-CD3 and anti-CD28 antibodies and analyzed by flow cytometry. (B and C) Percentages of FoxP3-expressing (B) and IL-17–producing (C) T cells induced following isolation of splenocytes and stimulation with anti-CD3 and anti-CD28 antibodies for 3 days in the presence of IL-2 and the indicated cytokines, as measured by flow cytometry. Bars show the mean and SEM (n = 3 mice per group). No significant differences were detected between groups. TGFβ = transforming growth factor β.

from RapV12-transgenic mice with CD3/CD28 antibodies in combination with IL-2 and TGFβ induced a robust increase in FoxP3+ T cell numbers, equivalent to that observed in wild-type T cells (Figure 3B). RapV12-transgenic T cells could also be converted to Th17 cells in vitro at frequencies equivalent to those observed in wild-type mice (Figure 3C). The frequencies of Th17 cells induced by TGFβ and IL-2 in both RapV12-transgenic and wild-type mice were comparable with those previously observed under these experimental conditions, where total T cells, rather than fractionated naive CD4+CD25–FoxP3– T cells, were used as input. Thus, unlike RapGAP1-transgenic, Rap1E63-transgenic, and Spa-1–knockout mice, RapV12-transgenic mice display no apparent alterations in the T cell compartment.

Characterization of the quality of T cell immune responses in wild-type and RapV12-transgenic mice during CIA.

Collectively, our analyses suggested that the protective effect of T cell Rap1 activation in CIA was not secondary to the suppression of TCR-proximal signaling or altered T cell homeostasis, but rather, could be attributed to specific influences occurring during
the induction of inflammation in vivo. To address this, we repeated CIA experiments with wild-type and RapV12-transgenic mice, examining the splenic and LN T cells of mice killed on day 42, prior to the peak of clinical arthritis in wild-type mice. RapV12-transgenic mice displayed normal numbers of CD3+CD4+ and CD3+CD8+ T cell subsets (A) and absolute numbers of naive CD44−CD62L+, effector memory CD44+CD62L−, and central memory CD44+CD62L+ CD4+ T cells (B) present in the spleen and LNs of wild-type and RapV12-transgenic mice. (C), Absolute numbers of regulatory FoxP3+CD4+ T cells in the spleen and LNs (left panel), and FoxP3+CD103−CD62L+ and FoxP3+CD103−CD62L− CD4+ T cells in the spleen (middle panel) and LNs (right panel) of wild-type and RapV12-transgenic mice. Horizontal lines show the mean; boxes represent individual mice (n = 3–5 mice per group). See Figure 2 for definitions.

We next examined the quality of T cell responses in wild-type and RapV12-transgenic mice during disease onset. Splenocytes and LN cells harvested from mice on day 42 after primary immunization were restimulated in vitro with PMA/ionomycin, and the production of Th1 and Th17 cytokines involved in CIA was measured by intracellular staining and fluorescence-activated cell sorting analysis. No differences in the numbers of CD4+ and CD8+ T cells producing IFNγ were observed between RapV12-transgenic and wild-type mice, either in the spleen or LN (Figure 5A). Notably, hardly any CD8+ T cells producing TNFα were detected in RapV12-transgenic LNs compared with
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Figure 5. Cytokine responses of T cells in wild-type and RapV12-transgenic mice during the onset of arthritis. Spleens and LNs of wild-type mice (n = 5) and RapV12-transgenic mice (n = 4) were collected 42 days after primary immunization, and intracellular cytokine production was assessed by flow cytometry. Intracellular expression of (A), IFNγ, (B), TNFα, (C), IL-17, and (D), IL-10 in CD3+CD4+ and CD3+CD8+ T cells following 5 hours of stimulation with phorbol myristate acetate/ionomycin is shown. Horizontal lines show the mean; boxes represent individual mice. P = P < 0.002. See Figure 3 for definitions.

Assessment of autoantibody production and T cell costimulatory protein expression in wild-type and RapV12-transgenic mice

Since previous studies have demonstrated that chronic T cell Rap1 activation can suppress B cell antibody production and Ig class switching\(^{15,16}\), we examined anti–chicken CII antibody production in arthritic wild-type and RapV12-transgenic mice. We found a significant reduction in the serum levels of anti–chicken CII IgG2a and IgG2b in RapV12-
Figure 6. Anticollagen antibody production and T cell costimulatory expression in wild-type (WT) and RapV12-transgenic mice. (A), Serum from wild-type or RapV12-transgenic mice was collected on day 60, and the levels of specific anticollagen IgG were detected by enzyme-linked immunosorbent assay (n = 10 mice per group). Values were obtained within linear regions of the serum dilution curve. Bars show the mean and SEM. \( P = P < 0.05 \). (B and C), T cells were isolated from the lymph nodes of healthy untreated wild-type and RapV12-transgenic mice and stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours, followed by analysis of the expression levels of CD69, inducible costimulator (ICOS), and CD40L surface molecules by flow cytometry. Dot plots from a representative experiment are shown in (B). Bars in (C) show the mean and SEM (n = 3 mice per group). \( P = P < 0.05 \).
transgenic mice relative to wild-type mice. (Relative mean ± SEM serum levels of IgG2a were 100 ± 6.4% in wild-type mice and 60.5 ± 8.5% in RapV12-transgenic mice [P < 0.005], and relative serum levels of IgG2b were 100 ± 6.7% in wild-type mice and 65.8 ± 9.7% in RapV12-transgenic mice [P < 0.05]) (Figure 6A). In Rap1E63-transgenic mice and Spa-1–knockout mice, defects in Ig class switching are associated with increased Treg frequency and accumulation of anergic CD44^high^CD4^+^ T cells, respectively. However, we did not observe these altered T cell phenotypes in arthritic RapV12-transgenic mice (Figures 4B and C).

Recent studies have demonstrated an essential role for follicular T helper cell expression of the costimulatory proteins ICOS and CD40L in promoting effective B cell activation and Ig class switching. To examine if RapV12 expression may influence activation-dependent expression of proteins required for functional interactions with B cells, we isolated LN cells from nonarthritis wild-type and RapV12-transgenic mice. Cells were stimulated for 24 hours in the presence of activating TCR/CD28 antibodies, and T cells were analyzed for expression of CD69, ICOS, and CD40L. Consistent with the results of previous studies, RapV12 had no influence on Ras-dependent expression of CD69 (Figures 6B and C). In contrast, TCR/CD28-dependent up-regulation of both ICOS and CD40L (Figures 6B and C) was specifically suppressed in both CD4^+^ and CD8^+^ RapV12-transgenic T cells.

Discussion

Defects in the expression or function of TCR-proximal signaling proteins, such as CD3ζ, the adaptor protein LAT, and Rap1, either intrinsic or secondary to chronic inflammatory stimulation, are thought to contribute to the pathogenic nature of synovial T cells in RA. Decreased expression of CD3ζ, the TCR component responsible for initiating antigen-dependent T cell intracellular signaling pathways, has been documented in RA SF and synovial T cells, correlating with decreased T cell proliferative responses and IL-2 production ex vivo. Successful therapeutic treatment in RA is associated with the release of synovial CD3ζ^{dim} T cells into peripheral blood. ROS-dependent misfolding of LAT in RA synovial T cells also leads to defective IL-2 production due to an inability of LAT to properly localize to the cellular membrane and participate in TCR signaling cascades. However, the consequences of decreased CD3ζ or LAT function with regard to the pathologic process in RA are not understood. Importantly, recent studies have demonstrated that RA SF T cell hyporesponsiveness is secondary to ex vivo apoptosis, and that the TCR signaling capacity of freshly isolated cells is intact, if not enhanced.

In this study, we demonstrated that sustained T cell Rap1 signaling, rescuing defective Rap1 function observed in RA SF T cells, prevents disease in CIA. Sustained T cell Rap1 activity may prevent induction of arthritis by one of several mechanisms. Previous
studies have predicted that Rap-dependent effects on T cell trafficking and anergy induction might influence T cell–dependent responses in vivo. However, we observed no overt changes in effector T cell or Treg numbers in the spleen or LNs of RapV12-transgenic mice, either under homeostatic conditions or during CIA. Our data also fail to support models suggesting that Rap1 activation may promote T cell anergy in vivo. Elevated Rap1 activity has been observed in anergized murine and human T cells, and Spa-1-knockout mice display an age-dependent accumulation of CD44-high T cells, which are unresponsive to antigen and mitogen stimulation. However, in RapV12-transgenic T cells, where Rap1 activity approximates that of pharmacologic activation of endogenous Rap1, no defects are observed in antigen-dependent IL-2 production or T cell proliferation. We found that in vitro, RapV12-transgenic and wild-type T cells from healthy mice produce similar amounts of IL-2, TNFα, and IFNγ following TCR/CD28 stimulation, while in vivo, RapV12-transgenic T cell defects are limited to TNFα production. Taken together, these data indicate that induction of T cell anergy by RapV12 is not the underlying mechanism of protection against CIA. Our study identifies a novel mechanism by which Rap1 activity regulates T cell function: suppression of activation-dependent expression of costimulatory proteins, namely, ICOS and CD40L. Failure to efficiently up-regulate these 2 costimulatory proteins could contribute to the observed defects in both B cell help and T cell TNFα production. ICOS may contribute to Ig class switching directly, or indirectly by promoting CD40L expression on T cells. CD40L binding to B cell CD40 in turn induces proliferation, Ig production, isotype switching, and up-regulation of B cell costimulatory molecules, which are important for T cell activation. Blockade of ICOS signaling in murine CIA or knockdown of ICOS renders mice resistant to disease, decreasing T cell cytokine production and follicular Th function, and consequently preventing accumulation of anti-CII antibodies. Interference with CD40L signaling in CIA also blocks development of disease and production of anti-CII antibodies. ICOS and CD40L signaling are also thought to contribute to T cell pathogenic behavior in RA. RA synovial T cells have increased ICOS expression compared with disease controls. Importantly, in vivo studies have shown that ICOS delivers important signals for TNFα production. In this context, decreased TNFα production in RapV12-transgenic CD8+ T cells, and to a lesser extent in CD4+ T cells, could be a consequence of decreased ICOS expression. Similarly, CD40L is also frequently expressed on RA synovial T cells. Intriguingly, we observed that while RapV12 modestly suppresses T cell CD40L and ICOS expression, as well as anti-CII antibody production in vivo, T cell RapV12 protection against CIA is almost complete. One potential explanation for this is that sustained T cell Rap1 activation partially suppresses multiple, independent T cell functions in CIA that are required to surpass an inflammatory threshold needed to induce disease. Alternatively, we may be underestimating the functional impact of sustained T cell Rap1 activation in specific autoreactive T cell populations needed to establish disease. Consistent with
this notion, elegant studies in vitro have previously demonstrated that sustained Rap1 activation can promote Fas-dependent apoptosis of antigen-specific T cells\(^{37}\).

The most striking T cell–intrinsic phenotype that we observed in RapV12-transgenic mice during CIA was the specific loss of CD8+, and to a lesser extent CD4+, TNF\(\alpha\)–producing LN T cells. It is therefore possible that TNF\(\alpha\) is serving as a surrogate marker for an autoreactive T cell population that is deleted or inactivated in RapV12-transgenic mice. Alternatively, the specific loss of TNF\(\alpha\) production by CD8+ cells we observed in CIA has also been noted during chronic viral infection in mice. Lymphocytic choriomeningitis virus infection results in the clonal exhaustion of antigen-specific CD8+ T cell clones, a process accompanied by the hierarchical loss of cytokine responses, in which TNF\(\alpha\) defects precede the loss of IFN\(\gamma\) production\(^{38,39}\). These possibilities can be tested in the future, pending the generation of reagents for monitoring chicken CII–specific T cells in C57BL/6 mice.

Our findings clearly demonstrate that reversing the block in Rap1 signaling that is observed in RA synovial tissue T cells protects against CIA, and provide the first direct evidence that Rap1 signaling critically regulates the quality of T cell responses in a disease setting. However, further analyses are required to determine whether the therapeutic benefit of T cell Rap1 activation observed in CIA is achieved by cellular mechanisms which can translate into similar protective effects in RA. For instance, it is as yet unclear if Rap1 is also inactivated in wild-type murine synovial T cells during CIA; current biochemical techniques used to assess Rap1 activation status require affinity precipitation from isolated cell populations, and no specific biomarker for Rap1 activation has been identified.

In CIA, it is likely that RapV12 is already influencing T cell activation or survival at sites distant from the joint, since we were unable to detect any inflammatory infiltration of the synovium in RapV12-transgenic mice. It will therefore be of interest to develop models in which T cell Rap1 can be selectively activated following the induction of arthritis. However, our observations suggest that some of the consequences of sustained T cell Rap1 activation will be relevant to inflammatory T cell behavior in RA. For instance, while CD40L expression on RA synovial T cells has historically been interpreted as an indication of recent antigen-dependent stimulation, it is now recognized that inflammatory cytokines present in RA SF and the synovium are sufficient to induce T cell CD40L expression\(^{3,5}\). T cell CD40L expression might not only stimulate autoantibody production in RA, but also promote antigen-independent activation of macrophages and synovial stromal cells\(^{40-42}\), a process which might be abrogated by restoring synovial T cell Rap1 activity.

Finally, since Rap1 inactivation in RA synovial T cells is dependent upon synovial cell stimulation of CD28, it will be of interest to determine whether rescue of T cell Rap1 function underlies at least in part the mechanism of action of abatacept treatment in RA\(^{7,8}\). Since disruption of CD28 costimulation has proven clinical efficacy in the
treatment of RA, identification of other T cell costimulatory proteins that regulate Rap1 activity may aid in the development of therapeutic strategies to suppress or augment T cell responses in immune-mediated inflammatory diseases\textsuperscript{43}.

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Reference List


