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Sustained T cell Rap1 signaling is protective in the collagen-induced arthritis model of rheumatoid arthritis

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Altered version submitted for publication

Abstract

The small GTPase Rap1 plays a critical role in T lymphocyte trafficking and integrin-dependent adhesion to antigen presenting cells. Genetic inactivation of Rap1 in murine T cells leads to the accumulation of potentially auto-reactive T cells in lymphoid compartments, and Rap1 signaling is blocked in synovial T cells from rheumatoid arthritis (RA) patients. As these data imply that Rap1 inactivation might contribute to T cell-mediated autoimmunity, we examined the consequences of maintaining T cell Rap1 activation in an experimental murine model of rheumatoid arthritis. We find that disease incidence and severity of collagen-induced arthritis (CIA) is dramatically reduced in mice expressing active RapV12, an active mutant of Rap1, in T cells. Protection against pathology in CIA is accompanied by defective TNF-α production in $CD8^+$ T cells, reminiscent of clonal T cell exhaustion observed during chronic viral infection. Additionally, B cell immunoglobulin class switching of auto-antibodies is diminished in RapV12 mice. Defects in qualitative T cell immune responses during CIA are paralleled by inadequate up-regulation of the T cell co-stimulatory molecules ICOS and CD40L. Our results suggest that modulation of T cell Rap1 signaling may be beneficial in attenuating pathologic contributions of T cells to RA and other immune-mediated inflammatory diseases.

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 production, the stimulation of B cells producing autoimmune antibodies, and the promotion of osteoclast differentiation[1]. An active role for the T cell antigen receptor (TCR) in the initiation and perpetuation of disease in RA is suggested by associations between expression of specific MHC HLA-DR1 and DR4 epitope alleles with enhanced disease risk and disease severity in RA patients[2]. 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, ICOS and VLA-4 proteins[1;3]. These T cells are primarily pro-inflammatory Th1 and Th17 cells, and display hyperresponsive cytokine responses to TCR/CD28 stimulation. In established RA, inflammatory cytokines present in the synovial tissue, such as IL-6, IL-12, IL-8 and TNF-α, rather than antigen stimulation, may drive T cell contributions to the perpetuation of inflammation[4;5].

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[6-8]. TCR stimulation results in the activation of guanine nucleotide exchange factors, such as C3G and CalDAG-GEFs which promote accumulation of Rap1 in an active GTP-bound form[9;10]. TCR-dependent Rap1 activation is exquisitely sensitive to costimulatory signals provided by antigen-presenting cells (APCs) such as CD28, which acts through the Rap1 GTPase activating protein RapGAP1 and suppresses Rap1 activation[10;11]. Conversely, CTLA-4 ligation, which opposes CD28 signaling, promotes accumulation of GTP-bound Rap1[12;13]. Once activated, Rap1 regulates several distinct signaling pathways 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 APCs[14]. Additionally, under certain experimental conditions, Rap1 suppresses TCR-dependent ERK activation and IL-2 production, either directly, through blocking Ras-dependent Raf kinase activation[9;11], or indirectly, through diminishing TCR-dependent reactive oxygen species production[6].

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 for Spa-1, a RapGAP expressed in T cells, demon-

strate age-dependent defects in T cell proliferative responses to stimulation by the TCR, mitogens, and recall antigen[15]. 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 immunoglobulin (Ig) class switching[16]. Defective T cell responses in these mice is attributed to both suppression of ERK activation in effector cells, as well as increases in the frequency and functional capacity of CD103-expressing regulatory T cells (Tregs). Conversely, transgenic expression of RapGAP1 in T cells, suppressing Rap1 function, leads to an accumulation of T cells in lymph nodes[17]. These T cells express high levels of CD69 under homeostatic conditions, possibly reflecting an autoreactive phenotype. Alternatively, accumulation of activated T cells in lymph nodes of RapGAP1 transgenic mice may indicate defects in T cell trafficking, a phenotype displayed in Rap1A knockout mice[18;19].
Despite these studies, it is still unclear if Rap1 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 the synovium of RA patients, likely a result of CD28-dependent interactions with synovial APCs[6;7]. Moreover, suppressed Rap1 function in RA synovial T cells was associated with enhanced T cell ROS production and TCR-dependent cytokine responsiveness, indicating that defects in Rap1 signaling may contribute to T cell-dependent pathology in RA[7;8]. To determine if maintenance of T cell Rap1 signaling might protect against autoimmunity, we here examined the effects of transgenic expression of active Rap1 on pathogenesis in the murine collagen-induced model of RA (CIA). We find that transgenic expression of an active mutant of Rap1, RapV12, in murine T cells potently suppresses disease incidence and severity. Protection against disease was not associated with general defects in lymphocyte trafficking or immune responsiveness, but rather specific defects in TNF-α production by $CD8^+$ T cells and ineffective upregulation of the costimulatory proteins ICOS and CD154 on T helper cells, needed for Ig class-switching by autoimmune B cells. Indeed, production of autoantibodies was also reduced in RapV12 transgenic mice. These results suggest that strategies aimed at enhancing T cell Rap1 function may be beneficial in the treatment of human immune-mediated inflammatory diseases.

Materials and methods

Animals

RapV12 transgenic C57BL/6 mice were kindly provided by Dr. D. Cantrell (University of Dundee, Dundee, U.K.)[20]. RapV12 and WT littermate control mice were housed under conventional conditions at the animal facility of the Academic Medical Center (Amsterdam, The Netherlands). Feeding was ad libitum. The animal ethical committee of the Academic Medical Center approved all experiments.

Cell staining and flow cytometry

Single cell suspensions were obtained from spleen and axial lymph nodes (LN) by grinding tissue over 40 μm cell strainers (BD Biosciences). Erythrocytes were removed using erylysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 1 mM EDTA, pH 7.4). Cells were surface stained with the indicated fluorochrome-conjugated antibodies for 30 minutes at 4°C in PBS containing 0.5% BSA. For Foxp3 intracellular stainings cells were stained according to manufacturer's instructions. For assessment of T cell cytokine expression, splenocytes and LN cells were stimulated for 1 hour with PMA (10 ng/ml, Sigma-Aldrich) and ionomycin (1 μM, Sigma-Aldrich) or anti-CD3 (10 μg/ml) and anti-CD28 (4 μg/ml) antibodies (kindly provided by Dr. L. Boon, Bioceros BV, Utrecht). 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 using specific antibodies listed in Supplemental Materials and Methods. For examination of T cell surface molecules following in vitro activation, splenocytes and LN cells were stimulated for 24 hours in the presence of 10 μg/ml anti-CD3 and 4 μg/ml anti-CD28 antibodies. Surface marker and cytokine expression were monitored using FACSCalibur or Canto flow cytometers (BD Biosciences).

In vitro T cell differentiation

For T cell isolation, splenocytes were incubated 30 min at 4°C with a saturating mix-

ture of hybridoma culture supernatants of the following rat anti-mouse antibodies: anti-CD11b (clone M1/70), anti-Ly6G (clone RB6-8C5), anti-CD45R (B220; clone RA3-6B2), anti−MHC class II (clone M5-114) and anti-TER119. After washing, cells were incubated with goat-anti-rat Ig microbeads (Miltenyi) and isolated by MACS. Purified T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies as above for 3 days in 96 well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in the presence of 50 U/ml recombinant murine IL-2 (Invitrogen) supplemented with 3 ng/ml human TGF-β and 20 ng/ml murine IL-6 (both from R&D Systems) when indicated.

Induction and assessment of CIA

Chicken collagen type II (cCII) (Sigma-Aldrich, St. Louis, MO) was dissolved overnight at 4°C with 0.1 M acetic acid at a final concentration of 2 mg/ml and then mixed with 4mg/ml complete Freund's adjuvant (CFA) (Chondrex, Inc., Redmond, WA). 10- to 12- week old mice were injected intradermally on day 0 at the base of the tail with 100 μl of the cCII-CFA emulsion (133 μg cCII and 133 ug CFA in a total volume of 100 μl emulsion). The immunization was repeated on day 21, and animals monitored 3 times weekly through sacrifice at day 60. Methodologies for the scoring of arthritis severity, paw swelling, synovial infiltration, cartilage erosion and radiological damage, as well as determination of serum anti-cCII antibodies, are described in detail in Supplemental Materials and Methods.

Statistical Analysis

Statistical significance was determined using a two tailed Student's t-test. P values < 0.05 were considered statistically significant.

Supplemental Materials and Methods

Assessment of CIA disease scores and paw swelling

Arthritis severity was assessed in a blinded manner, using a semi-quantitative sco-

ring system (0 to 4): 0, normal; 1, redness and/or swelling in one joint; 2, redness and/or swelling in more than one joint; 3, redness and/or swelling in the entire paw; and 4, deformity and/or ankylosis. Hind paw ankle joint swelling was measured using a dial caliper (POCO 2T 0- to 10-mm test gauge; Kroeplin Längenmesstechnik, Schlüchtern, Germany).

Histological analysis

Animals were sacrificed and hind paws fixed in 10% buffered formalin for 48 hours, decalcified in 15% ethylenediaminetetraacetic acid, and embedded in paraffin. Tissue sections (5 μm sagittal serial sections) were stained with haematoxylin and eosin. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severely inflamed joint) based on infiltration by inflammatory cells in the synovium. Cartilage erosions were scored using a semi-quantitative scoring system from 0 (no erosions) to 3 (extended erosions and destruction of bone).

Radiological analysis

Joint destruction of hind paws was analyzed by radiological assessment of x-rays by two observeres blinded to mouse genotype, using a semi-quantitative score (0-4): 0, no damage; 1, minor bone destruction observed in one enlightened spot; 2, moderate changes, two to four spots in one area; 3, severe erosions afflicting the joint; and 4, complete destruction of the joints.

Antibodies for FACS analysis

The following antibodies from eBiosciences were used: anti-CD3 -FITC, -APC, -Alexa 700; anti-CD8 -PE, -FITC, -Alexa 750; anti-CD69-PE; anti-CD44 -FITC; anti-CD62L -APC; anti-CD103 -PE; anti-Foxp3 -PE, -APC; IL-17 –Alexa 488; anti-TNF-α -APC and anti-IFN-γ - APC, -PerCP Cy5.5. Anti-CD4 –FITC, -PE, -PECy7; anti-CD25 –FITC; anti-TNF-α –PE; anti-IL-4 –PE and anti-IL-10 –PE were purchased from BD Biosciences.

Determination of anti-collagen antibodies by ELISA

Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml of chicken collagen type II (cCII) (Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium carbonate buffer (pH 9.7) overnight at 4°C. After blocking for 1 h with 2% milk in phosphate-buffered saline (PBS) at room temperature (RT), sera were added at an initial dilution of 1/100 in 2% milk in PBS and 1/3 serial dilutions, and incubated overnight at 4°C. Plates were subsequently washed and incubated with 1 μg/ml biotinylated rat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, Alabama) of the indicated isotype in 2% milk in PBS for 1 hour at RT. After washing, plates were incubated with streptavidin-conjugated alkaline phosphatase (AP) (Jackson ImmunoResearch, Newmarket, Suffolk, UK) for 1 hour at RT, washed, and developed with p-Nitrophenyl Phosphate (pNPP) substrate (Sigma-Aldrich, St Louis, MO). The reaction was stopped with 2M H2SO4, and the optical density (OD) was measured at 415 nm.

Results and discussion

In RA, a block in synovial T cell Rap1 activation is associated with their pathogenic behavior[6-8]. To determine if 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, driven by the human CD2 promoter, in murine CIA[20]. We chose to examine RapV12 transgenic mice for several specific reasons. RapV12 is expressed in T cells of these mice at levels equivalent to endogenous Rap1A, and Rap1 activity contributed by the transgene product is similar in magnitude to that obtained by PMA/I-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 and RapGAP1 transgenic mice, RapV12 mice have no obvious alterations in T cell homeostasis[12;16;20]. Finally, unlike the active Rap1E63 mutant, RapV12 can still cycle between active and inactive states, albeit at a highly reduced rate[21]. We therefore reasoned that responses of RapV12 mice in CIA might most closely mimic therapeutic interventions aimed at restoring the function of endogenous T cell Rap1.
As phenotypes of genetically modified mice can be strongly influenced by differences in housing conditions, we examined thymocyte development and T cell maturation in RapV12 mice housed in our facilities. Consistent with previous studies[20],

we observed no differences between RapV12 mice and wild-type (WT) littermates in terms of percentages of double negative (DN), double positive (DP), or CD4 and CD8 single positive thymocytes (Supplementary Figure 1A). Additionally, within the DN thymocyte compartment, RapV12 failed to influence CD44 and CD25 expression (Supplementary Figure 1B), as previously described[20]. As thymocyte development and T cell maturation appeared normal in RapV12 mice, we extended our analyses to peripheral T cell compartments. $CD4^+$ and $CD8^+$ T lymphocytes were represented at normal percentages in both spleens and lymph nodes (LN) of RapV12 mice (Figure 1A). RapV12 failed to influence the activation status of splenic and LN T cells under homeostatic conditions, as assessed by CD25 and CD69 staining (Figure 1B). Normal proportions of naive, effector/memory (EM), and central memory (CM) T cells were also observed (Figure 1C). Moreover, we detected no influence of RapV12 on the frequency of $CD4^+FoxP3^+$ Tregs or activated $CD4^+$ $CD103^+$ T cells (Figure 1D). Additionally, RapV12 T cells were capable of producing similar amounts of IL-2, TNF-α, IFN-γ and IL-17 following TCR/CD28 in vitro stimulation (Figure 2A). To rule out the possibility that RapV12 may regulate the induction of peripheral Tregs and/or Th17 cells, we isolated T cells from the spleens of healthy WT and RapV12

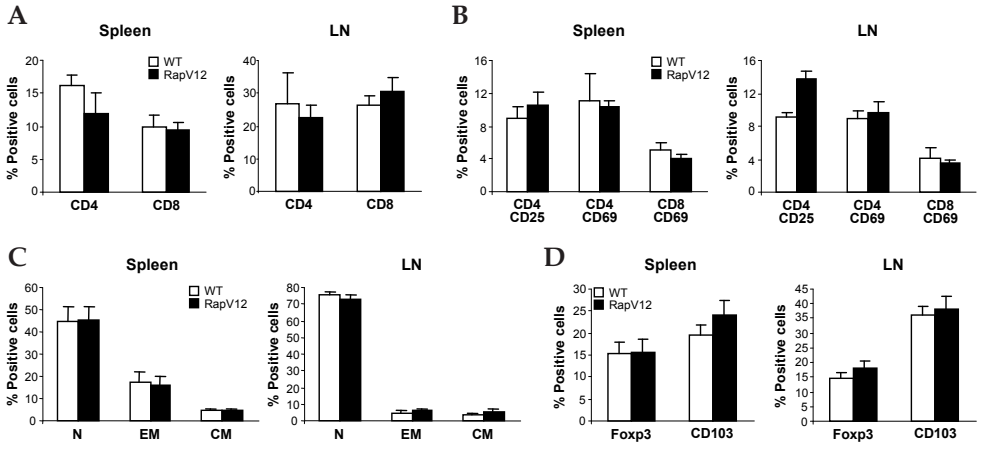


Figure 1. Peripheral T cell homeostasis is normal in RapV12 Tg mice. *(A) Expression levels of $CD4^+$ and $CD8^+$ T cells, (B) CD25 and CD69 cell surface makers on $CD4^+$ and on $CD4^+$ and $CD8^+$ T cells, respectively, and (C) expression levels of naive $CD44^-CD62L^+$ (N), effector memory $CD44^+CD62L^-$ (EM), and central memory $CD44^+CD62L^+$ (CM) $CD4^+$ T cells, present in the spleen and LN of WT or RapV12 Tg mice (n=3). (D) Foxp3 and CD103 expression in $CD4^+$ T cells derived from spleens (Foxp3 n=13; CD103 n=10) and LN (Foxp3 n=10; CD103 n=8) of 8-10 weeks old animals. Values are expressed as mean ± SEM.*

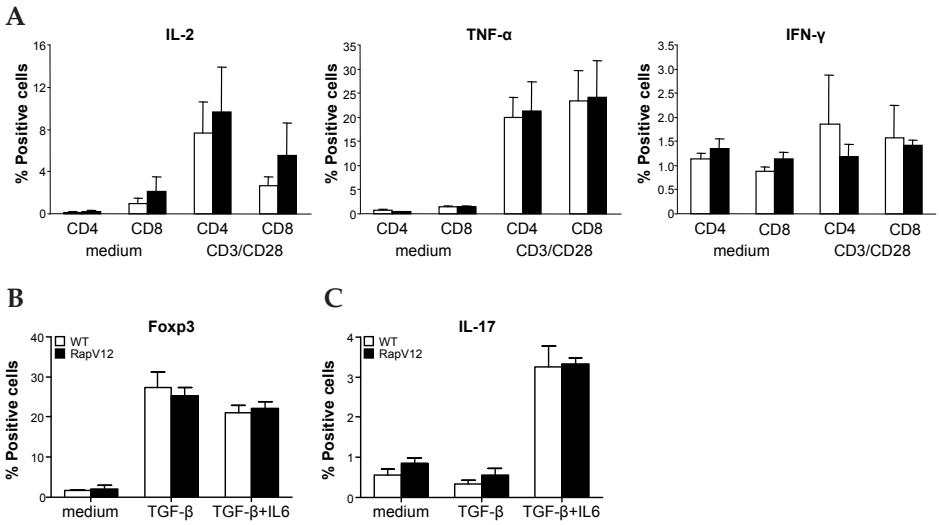


Figure 2. RapV12 Tg mice have normal cytokine production and induction of regulatory and Th17 T cells in vitro. *(A) Splenocytes from WT and RapV12 mice were stimulated for 5h with anti-CD3/CD28 and analyzed for the intracellular expression of IL-2, TNF-α and IFN-γ (n=3). (B,C) T cells were isolated from spleens and stimulated with anti-CD3 and anti-CD28 antibodies for 3 days in the presence of the indicated cytokines. (B) Percentage of Foxp3+ expressing and (C) IL-17 producing T cells induced in the different conditions. Values are expressed as mean ± SEM (n=3).*

mice. In vitro stimulation of these cells with TGF-β induced a robust increase in FoxP3$^+$ T cell numbers, equivalent to that observed in WT T cells (Figure 2B). RapV12 Th17 cells could also be induced in vitro at frequencies equivalent to those observed in WT mice (Figure 2C). Thus, unlike RapGAP1 transgenic, Rap1E63 transgenic and Spa-1 knockout mice, RapV12 mice display no apparent alterations in the T cell compartment. We concluded from these results that potential differences in responses of RapV12 mice in CIA would not be secondary to altered T cell homeostasis, but rather, specific influences of active Rap1 in this disease model.

Following induction of CIA, all WT mice developed clinical signs of disease within two weeks (Figure 3A). Remarkably, only 20% of the RapV12 Tg mice developed arthritis, while the rest remained disease free until the end of the experiment. Throughout the experiment, clinical arthritis scores of RapV12 mice were dramatically lower than those of WT mice ($P < 0.0005$) (Figure 3B), and paw swelling in RapV12 mice was almost completely suppressed compared to WT mice ($P < 0.005$) (Figure 3C). Histologic analysis of murine hind paws by hematoxylin staining re-

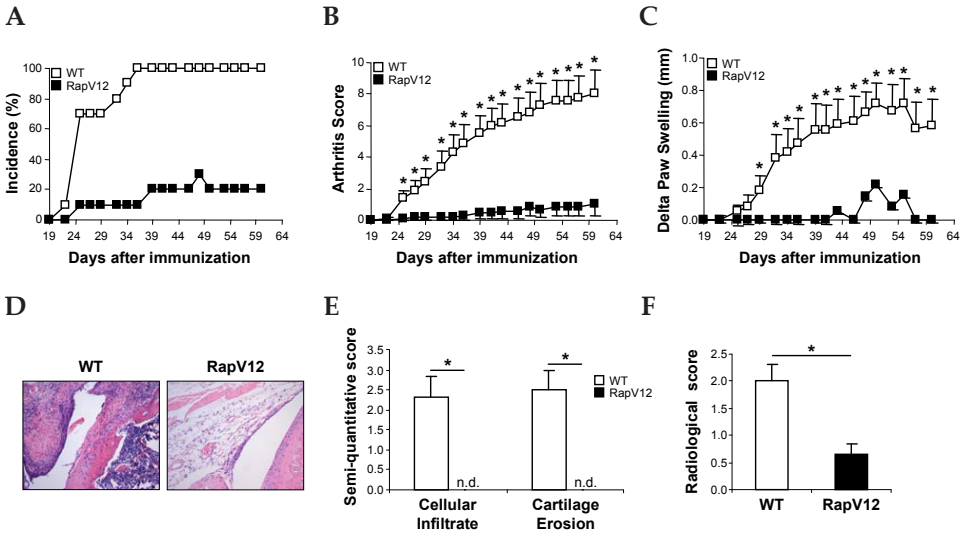


Figure 3. RapV12 Tg mice are protected from arthritis. *WT (open squares) and RapV12 mice (black squares) were immunized with cCII in CFA and boosted on day 21. (A) Hind paw swelling and inflammation of the four limbs were monitored for each mouse during the course of disease and the percentage of affected animals (incidence) as well as their (B) disease scores were plotted at the indicated times. (C) Paw swelling was measured during the course of disease using calipers. Delta hind paw swelling was calculated by subtracting the paw diameter before onset of disease from the measured diameter. (D) Representative pictures of paraffin-embedded sections of hind paws stained with haematoxylin and eosin analyzed for (E) cellular infiltration and cartilage erosion. (F) X-rays from hind paws scored for bone damage. Values are depicted as mean ± SEM (n=10) and are representative of two independent experiments. * P < 0.05.*

vealed that unlike WT mice, RapV12 mice displayed an almost complete absence of joint infiltration by white blood cells (Figure 3D). This was confirmed by semi-quantitative analysis of synovial cell infiltration (Figure 3E) ($P < 0.001$). RapV12 mice also escaped destruction of cartilage (Figures 3E) ($P < 0.001$) and erosive bone damage, as determined by radiology, which was reduced by approximately 75% compared to WT mice (Figure 3F) ($P < 0.001$).

To better characterize how sustained Rap1 activation in T cells protected against disease induction, we sacrificed mice 42 days after primary immunization, prior to the peak of clinical arthritis in WT mice, and examined splenic and LN T cells. RapV12 mice displayed normal numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (Figure 4A) as well as naive (N) ($CD44^-CD62L^+$), effector memory (EM) ($CD44^+CD62L^-$) and central memory (CM) ($CD44^+CD62L^+$) T cells (Figure 4B). We also examined

potential influences of RapV12 on the Treg compartment. However, no significant differences in the numbers of FoxP3$^+$ T cells or CD4$^+$CD103$^-$CD62L$^+$ naive T regs and CD4$^+$CD103$^+$CD62L$^-$ activated/EM Treg subsets were observed (Figure 4C).
We next examined the quality of T cell responses in WT and RapV12 mice during disease onset. Splenocytes and LN cells were harvested from mice at day 42 and re-stimulated in vitro with PMA/I. Production of Th1 and Th17 cytokines involved in the pathology of CIA was measured by intracellular staining and FACS analysis. No differences in the number of CD4$^+$ and CD8$^+$ T cells producing IFN-γ were observed between RapV12 and WT mice, either in the spleen or LN (Figure 5A). Remarkably, hardly any CD8$^+$ T cells producing TNF-α were detected in RapV12 LN compared to WT mice ($P < 0.05$) (Figure 5B). A similar trend toward reduced TNF-α production was also observed in RapV12 LN CD4$^+$ T cells, but did not reach statistical significance. In contrast, similar numbers of TNF-α-producing T cells were detected in WT and RapV12 splenocytes. Depressed TNF-α production by LN CD8$^+$ T cells from arthritic RapV12 mice appeared to be dependent on in vivo inflammatory con-

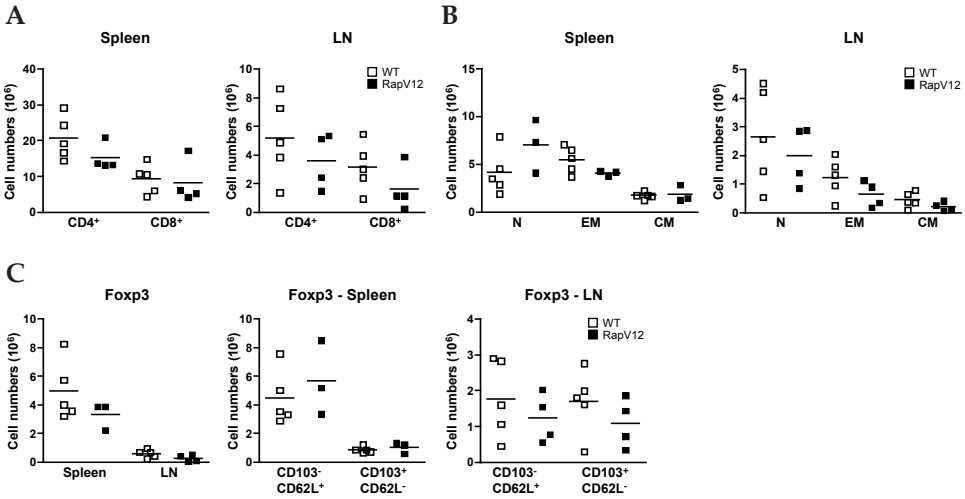


Figure 4. RapV12 Tg mice have normal numbers of effector and regulatory T cells. *Spleens and LN were collected from mice 42 days after the induction of arthritis. (A) Absolute numbers of CD3+CD4+ and CD3+CD8+ T cell subsets and (B) absolute numbers of naive CD44-CD62L+ (N), effector memory CD44+CD62L- (EM) and central memory CD44+CD62L+ (CM) CD4+ T cells, present in the spleen and LN of WT or RapV12 Tg mice. (C) Absolute numbers of regulatory Foxp3+CD4+ T cells and of Foxp3+CD103-CD62L+ and Foxp3+CD103+CD62L- T cells. Values are depicted as mean ± SEM (WT n=5, RapV12 Tg n=4).*

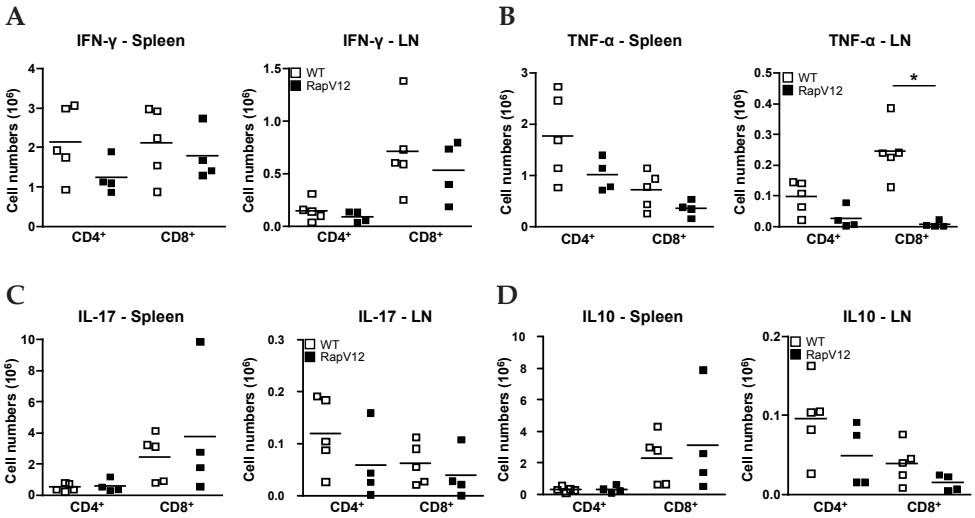


Figure 5. RapV12 Tg mice have defective TNF-α production. *(A) Intracellular expression of IFN-γ, (B) TNF-α, (C) IL-17 and (D) IL-10 cytokines in $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells upon 5h PMA/I stimulation (WT n=5, RapV12 Tg n=4). Values are depicted as mean ± SEM. * P < 0.002.*

ditions, as no such defect was observed in T cells isolated from the LN of healthy mice (data not shown). Defects in RapV12 LN T cell TNF-α production were highly selective, as in addition to IFN-γ, no differences were observed between WT and RapV12 mice in regards to IL-17 (Figure 5C) or IL-10 production (Figure 5D). Thus, qualitative analysis of arthritic WT and RapV12 mice T cell responses indicates that the protective effects of RapV12 are not a result of generalized T cell anergy or unresponsiveness, but rather a selective defect in T cell TNF-α production.

As 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 collagen type II (c-CII) production in arthritic WT and RapV12 mice. We found a significant reduction in the serum levels of anti-cCII IgG2a and IgG2b in RapV12 mice relative to WT mice (relative IgG2a: WT 100% ± 6.4, RapV12 60.5% ± 8.5; $P < 0.005$; relative IgG2b: WT 100% ± 6.7, RapV12 65.8% ± 9.7; $P < 0.05$) (Figure 6A). In Rap1E63 transgenic mice and Spa-1 knockout mice, defects in Ig class switching are associated with increased Treg function and accumulation of anergic $CD44^{high}$ $CD4^+$ T cells, respectively[15;16]. However, we did not observe these altered T cell phenotypes in arthritic RapV12 mice (Figures 4B and 4C).

Recent studies have demonstrated essential roles for the T cell costimulatory proteins ICOS and CD40L expressed by follicular Th cells in effective B cell activation and Ig class switching[22]. ICOS, a CD28-related protein which is upregulated on activated T cells and provides costimulatory signals distinct from CD28, is required for effective T cell-dependent immune responses[23]. ICOS may contribute to Ig class switching directly, or indirectly by promoting CD40L expression on T cells[24]. CD40L binding to B cell CD40 in turn induces proliferation, Ig production, isotype switching and upregulation of B cell costimulatory molecules, important for T cell activation. Blockade of ICOS signaling in murine CIA or knock down of ICOS renders mice resistant to disease, with decreased anti-CII antibodies and decreased T cell cytokine production[25;26]. Notably, disruption of ICOS signaling in CIA results in defective follicular

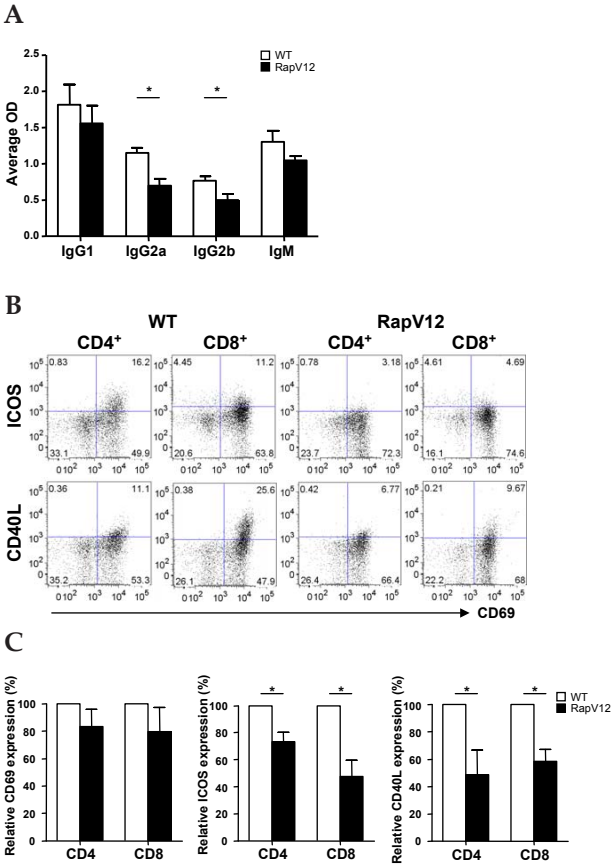


Figure 6. RapV12 Tg mice have defective anti-collagen antibody production. *(A) Serum from WT (white bars) or RapV12 Tg mice (black bars) was collected at day 60 and the levels of specific anti-collagen IgG detected (n=10). Represented values (mean ± SEM) were obtained within linear regions of the serum dilution curve. *P < 0.05. (B, C) T cells were isolated from spleens and stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours, followed by analysis of the expression levels of CD69, ICOS and CD40L surface molecules Values are expressed as mean ± SEM (n=5).*

Th function[27]. Interference with CD40L signaling in CIA also blocks development of disease and production of anti-CII antibodies[28]. To examine if RapV12 expression may influence activation-dependent expression of proteins required for functional interactions with B cells, we isolated splenocytes and LN cells from non-arthritic WT and RapV12 mice. Cells were stimulated for 24 hours in the presence of activating TCR/CD28 antibodies, and T cells analyzed for expression of CD69, ICOS, and CD40L. In line with previous studies, RapV12 had no influence on Ras-dependent expression of CD69 (Figure 6B and 6C)20. In contrast, TCR/CD28-dependent upregulation of both ICOS and CD40L (Figure 6B and 6C) was specifically suppressed in both $CD4^+$ and $CD8^+$ RapV12 T cells.

ICOS and CD40L signaling are required for induction of pathology in murine models of arthritis, and are also thought to contribute to T cell pathogenic behavior in RA. RA synovial T cells have increased ICOS expression compared to disease controls[29]. Several studies have shown the ability of ICOS signaling to enhance CD8 effector T cell responses. Importantly, in vivo studies have shown that ICOS delivers important signals for TNF-α production[30]. In this context, decreased TNF-α production in RapV12 Tg $CD8^+$ 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[31]. Although 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 synovial fluid and the synovium are sufficient to induce T cell CD40L expression[3;4]. T cell CD40L expression might not only promote auto-antibody production in RA, but also promote antigen-independent, cell contact-dependent activation of macrophages and stromal cells[32-34].

Decreased activation-dependent expression of ICOS and CD40L on RapV12 T cells offers a possible explanation for the protective effects of T cell Rap1 activation in CIA. However, the magnitude of disease resistance in RapV12 mice suggests that other mechanisms may be at play. Previous studies have predicted that Rap-dependent effects on T cell trafficking and anergy induction might influence T cell-dependent responses in vivo. However, although Rap1 regulates T cell trafficking in vivo[19], we observed no overt changes in effector T cell or Treg numbers in the spleen or LN of RapV12 mice. Our data are also in discordance with other models suggesting that Rap1 promotes T cell anergy. Elevated Rap1 activity has been observed in anergized murine and human T cells[9;35]. Additionally, Spa-1 knockout mice display an age-dependent accumulation of $CD44^{high}$ T cells which are unresponsive to antigen and mitogen stimulation[15]. However, in RapV12 T cells, where Rap1 activity approximates

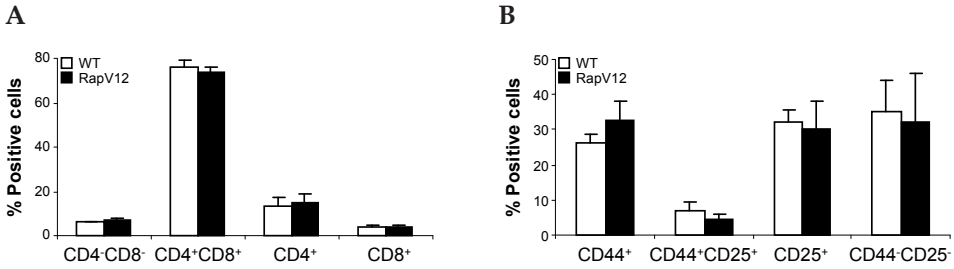
that of pharmacological activation of endogenous Rap1, no defects are observed in antigen-dependent IL-2 production or T cell proliferation[20]. In vitro, we found that RapV12 and WT T cells also produce similar amounts of IL-2, TNF-α, IFN-γ, and IL-17 following TCR/CD28 stimulation. In vivo, RapV12 T cell defects are limited to TNF-α production. Together, these data argue that T cell anergy is not the underlying mechanism in regard to protection against CIA. A more likely possibility might be that we are detecting the deletion or exhaustion of specific auto-antigen –specific T cell clonal populations. Elegant studies in vitro have previously illustrated that sustained Rap1 activation can promote Fas-dependent apoptosis of antigen-specific T cells[36]. Interestingly, the specific loss of TNF-α production by $CD8^+$ cells is also observed during chronic viral infection. LCMV infection of mice results in the clonal exhaustion of antigen-specific $CD8^+$ T cell clones. Here, this process is accompanied by a hierarchal loss of cytokine responses, in which TNF-α defects precede loss of IFN-γ production[37;38]. A similar phenomenon has been observed in human $CD8^+$ T cells following HIV infection[39].

Here we show that maintenance of T cell Rap1 activation decreases arthritis incidence and severity in the CIA model, and provide the first evidence that T cell Rap1 function is important in the regulation of inflammatory disease. Strategies disrupting CD28 co-stimulation, such as CTLA4-Ig therapy, have proven clinical efficacy in the treatment of RA[40]. The pathogenic behavior of synovial T cells is associated with a block in Rap1 activation, which is dependent upon synovial cell stimulation of CD28 and can be disrupted by CTLA4-Ig[6;7]. We propose that the development of specific therapies that prevent Rap1 inactivation in RA T cells might be of clinical benefit for RA and other auto-immune diseases driven by improper activation of T cells.

Acknowledgments

We would like to thank Dr. D. Cantrell (University of Dundee, UK) for providing the RapV12 Tg mice, and Dr. R. van Lier and Dr. H. Schuitemaker (our institute) for critical reading of the manuscript. This work was supported in part by a grant from the Dutch Arthritis Association to KAR.

Supplementary figure



Supplementary figure 1. Thymocyte development is normal in RapV12 Tg mice. *Thymus from WT (white bars) or RapV12 Tg mice (black bars) were collected and analyzed for cell surface expression of (A) CD4 and CD8 and (B) CD25 and CD44 cell surface markers. Values are expressed as mean ± SEM (n=3).*

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