Enforced Expression of Roquin Protein in T Cells Exacerbates the Incidence and Severity of Experimental Arthritis*

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Background: Roquin regulates inflammation in autoimmune disease.

Results: Roquin overexpression promoted the induction of collagen-induced arthritis (CIA).

Conclusion: Enforced Roquin expression was produced in mice and used to demonstrate the importance of inflammation and CIA development in experimental arthritis.

Significance: Roquin overexpression may induce inflammation in some autoimmune diseases.

To investigate the role of Roquin, a RING-type ubiquitin ligase family member, we used transgenic mice with enforced Roquin expression in T cells, with collagen-induced arthritis (CIA). Wild-type (WT) and Roquin transgenic (Tg) mice were immunized with bovine type II collagen (CII). Arthritis severity was evaluated by clinical score; histopathologic CIA severity; proinflammatory and anti-inflammatory cytokine levels; anti-CII antibody levels; and populations of Th1, Th2, germinal center B cells, and follicular helper T cells in CIA. T cell proliferation in vitro and cytokine levels were determined to assess the response to CII. Roquin Tg mice developed more severe CIA and joint destruction compared with WT mice. Production of TNF-α, IFN-γ, IL-6, and pathogenic anti-collagen CII-specific IgG and IgG2a antibodies was increased in Roquin Tg mice. In addition, in vitro T cell assays showed increased proliferation and proinflammatory cytokine production in response to CII as a result of enforced Roquin expression in T cells. Furthermore, the Th1/Th2 balance was altered by an increased Th1 and decreased Th2 population. These findings suggest that overexpression of Roquin exacerbates the development of CIA and that enforced expression of Roquin in T cells may promote autoimmune diseases such as CIA.

Rheumatoid arthritis (RA)4 is a severe inflammatory disease with autoimmune features and an intricate genetic element. RA is characterized by synovial hyperplasia, chronic inflammation, and infiltration of lymphocytes; it is driven by diverse cellular and humoral autoimmune responses and results in the destruction of cartilage and bone (1, 2). Autoimmune arthritis is induced in the collagen-induced arthritis (CIA) mouse model via immunization with an emulsion of complete Freund’s adjuvant and type II collagen (CII) (3). Considerable data implicate CII-reactive CD4+ cells as the primary mediators of disease induction and autoantibody production by B cells as a major immune mechanism leading to a localized chronic inflammatory response (4–6). The CIA model has been used extensively to explain various mechanisms related to human RA and to identity potential targets for therapy. Therefore, we used the CIA model to study gene function in autoimmune diseases such as RA.

T cell activation and differentiation are regulated not only by T cell receptor recognition of the antigen-major histocompatibility complex (MHC) but also by interactions between many co-stimulatory molecules (7). Although the interactions among various immune cells are complex, optimal responses are induced by controlled and coordinated expression of T cell co-stimulatory receptors. CD28, the first co-stimulatory molecule to be identified and arguably the most important in naïve T cell activation, binds to B7.1 (CD80) and B7.2 (CD86), which are expressed on the surface of professional antigen-presenting cells. CD28 co-stimulation enhances T cell proliferation, cytokine production, and survival (8–10). Studies using T cells derived from CD28-deficient mice demonstrated its role in T cell activation (11). Consistent with this, CD28 knock-out mice with the DBA/1 background are resistant to CIA (12). Inducible T cell co-stimulator (ICOS), another member of the CD28 family, is not expressed by naïve T helper (Th) cells but is induced

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after T cell activation (13, 14). Recently, several groups have constructed and analyzed ICOS-deficient mice (15–17). T cells derived from ICOS-deficient mice exhibit reduced proliferation and IL-2 production, indicating a role for ICOS in T cell activation (15, 17), and are resistant to CIA (7).

Roquin was recently identified as a RING-type ubiquitin ligase family member. Roquin mRNA is ubiquitously expressed, and Roquin protein has been predicted to be intracellular. Roquin contains an N-terminal RING-1 zinc finger at residues 14–53 based on consensus sequences with the E3 ubiquitin ligase family. The C terminus of the RING finger is a highly conserved novel protein domain known as ROQ, and a zinc finger domain of the CCCH type that conforms precisely to the RNA-binding zinc finger occurs in the center of Roquin. The C terminus of the CCCH domain is a proline-rich region representing potential sites for binding the Src homology 3 (SH3) domains of interacting proteins (19, 20).

Notably, one report identified a novel repressor of ICOS, Roquin, which is the defective gene in sanroque mice (20). san/san mice have a point mutation in Roquin and are highly susceptible to autoimmunity. CD4+ T cells from san/san mice express higher levels of ICOS than those of wild-type (WT) controls. Particularly striking are the accumulation of follicular helper T (Tfh) cells and the increased size of germinal centers. However, in our previous study, Roquin overexpression induced up-regulation of CD28 expression as well as down-regulation of ICOS during T cell activation by anti-CD3/CD28. Secretion of IL-2 and proinflammatory cytokines was increased by enforced expression of Roquin in T cell activation (21). Thus, decreased ICOS expression may repress T cell function and prevent pathogenic immune responses. However, conflicting data on the role of ICOS in allograft survival have been reported, and ICOS blockade can exacerbate or reduce experimental autoimmune encephalomyelitis severity depending upon the treatment approach (15, 22). Moreover, increased CD28 may regulate T cell activation and lead to an autoimmune response.

To our knowledge, there are no reports regarding the function of Roquin in RA in the literature. Therefore, we used Roquin-overexpressing mice to investigate the role of Roquin in the CIA mouse as a model of autoimmune disease. The results indicate that Roquin overexpression promoted the induction of CIA even when reinforced immunization protocols were applied. Immune cell populations, including Th cells, Tfh cells, and germinal center B cells, were unaffected by Roquin overexpression in non-immunized mice. However, Th1/Th2 balance was affected by Roquin overexpression in the CIA model. Therefore, these findings have important implications for our understanding of the role of Roquin in T cells in autoimmune diseases such as RA.

EXPERIMENTAL PROCEDURES

Mice—Roquin transgenic mice were generated as described previously (21). Six generations of Roquin transgenic mice were backcrossed onto the DBA/1 background. All mice used in these experiments were 8–12 weeks old. All animals were raised and kept under specific pathogen-free conditions.
staining with anti-CD4 and anti-CD8 antibodies). Cells were stained with fluorochrome-conjugated anti-mouse Tbet-PerCP Cy5.5, GATA3-AF647, CD44-FITC, CD44-PE, CD44-APC, CD4-PerCP, CXCR5-FITC, PD-1-PE, B220-PerCP, GL-7-FITC, CD95-PE, ICOS-PE, and CD28-PE antibodies and analyzed on a FACScan using CellQuest software (BD Biosciences).

**Lymphocyte Proliferation and Cytokine Analysis—Spleen and draining lymph node cells were cultured at 2 × 10^6 cells/ml for up to 96 h in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mM l-glutamine, 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 mg/ml streptomycin in a humidified 5% CO_2 atmosphere. Cells were stimulated with graded concentrations of CII. Cell proliferation was quantified using a DNA bromodeoxyuridine (BrdU) incorporation assay (Roche Applied Science). The amount of incorporated BrdU is a measure of the rate of DNA synthesis and thus indirectly of cell proliferation. The cell proliferation kit was used according to the manufacturer’s instructions. Supernatants from parallel triplicate cultures were stored at 70 °C until estimation of cytokine content by enzyme-linked immunosorbent assay (ELISA).

**Measurement of Anti-CII Antibodies—Serum was collected on days 20 (diluted 1:5000) and 45 (diluted 1:20,000), and anti-type II collagen antibody levels (total IgG, IgG1, and IgG2a) were measured using ELISA kits purchased from Chondrex.

**Cytokine Assays—Total interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6, IL-4, IL-10, and IL-17 levels in supernatants were determined using a commercially available ELISA kit (R&D Systems) according to the manufacturer’s recommendations. The IL-21 levels were determined using a commercial ELISA kit (Biolegend).

**Statistical Analysis—The results are expressed as the mean ± S.E. of at least three independent experiments. The significance of differences between groups was calculated using a two-tailed Student’s t test. Differences with a p value of less than 0.05 were considered statistically significant.

**RESULTS**

**Generation of Roquin Overexpression on CIA**

FIGURE 1. Generation of Roquin-overexpressing transgenic mice and detection of cell populations in WT and Tg mice. A, Western immunoblot analysis using HA antibody to detect Roquin expression in transgenic mice. B, the populations of ICOS- and CD28-positive cells among CD4^+ T cells from naive DBA/1 and Roquin Tg mice were examined by flow cytometry. Mean fluorescence intensity (MFI) values were measured and compared with those of WT mice. Error bars indicate mean ± S.E. of triplicates. *, p < 0.05. C, representative flow cytometric graphical analysis of Tbet^+CD44^hi^CD4^+^, GATA3^+^CD44^hi^CD4^+^, B220^+^GL-7^+^CD95^+^, and PD-1^+^CXCR5^+^CD4^+^ cells in WT and Roquin transgenic mice. Black bars, wild-type (WT) mice; gray bars, Roquin Tg mice. Data are representative of two independent experiments (n = 4 per group). p values are indicated on the graphs.

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No Effect on the Number of Lymphocytes in the Spleens of Naive Mice—To evaluate the lymphocyte population in naive mice, we analyzed the lymphocyte subsets in the spleen. The percentages of Tbet<sup>+</sup>CD4<sup>+</sup>CD44<sup>hi</sup>CD4<sup>+</sup> (Th1) and GATA3<sup>+</sup>CD4<sup>+</sup>CD44<sup>hi</sup>CD4<sup>+</sup> (Th2) cells were not altered in the Roquin Tg mice compared with the WT mice (p = 0.33 and p = 0.10, respectively). Similarly, the percentages of B220<sup>+</sup>GL-7<sup>+</sup>CD95<sup>+</sup> (germinal center B) and PD-1<sup>hi</sup>CXCR5<sup>+</sup>CD4<sup>+</sup> (Tfh) cells were not altered in the Roquin Tg mice (Fig. 1C). These results demonstrate that T cells and germinal center B cells developed normally in the spleens of the Roquin Tg mice.

**CIA Promotion and Severity in Roquin Tg Mice**—To assess whether T cell-specific Roquin expression affects the development of arthritis, we examined experimental CIA in Roquin Tg and WT mice. The clinical arthritis score was significantly higher in the Roquin Tg mice than in the WT mice (p = 0.033 and p = 0.010, respectively). Similarly, the percentages of B220<sup>+</sup>GL-7<sup>+</sup>CD95<sup>+</sup> (germinal center B) and PD-1<sup>hi</sup>CXCR5<sup>+</sup>CD4<sup>+</sup> (Tfh) cells were not altered in the Roquin Tg mice (Fig. 1C). These results demonstrate that T cells and germinal center B cells developed normally in the spleens of the Roquin Tg mice.

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**Induction of Proinflammatory Cytokine Production and Signaling Modulation in Roquin Tg Mice**—Because enforced Roquin expression in T cells promoted the development of CIA, we examined whether Roquin regulates the secretion of inflammatory cytokines. We isolated serum from mice at 20 and 45 days after immunization and then assessed the levels of various cytokines by ELISAs. In a previous study, CD28-induced IL-2 secretion was increased by Roquin overexpression in vitro (21). To confirm whether IL-2 secretion is increased in vivo, we performed an ELISA on serum collected from CIA mice at 20 and 45 days after immunization. IL-2 secretion was significantly increased in the Roquin Tg mice compared with the WT mice (p < 0.05) (Fig. 3A). At 20 days after immunization, the secretion of TNF-α, IL-6, and IL-17 was significantly elevated in Roquin Tg mice compared with WT mice. However, the levels of IFN-γ, IL-10, and IL-21 were unchanged. At 45 days after immunization, the levels of...
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Alteration of CII-specific IgG Production in Roquin Tg Mice—Because CII-specific IgG levels correlated well with the development of arthritis, we examined CII-specific IgG production in Roquin Tg mice. Serum was isolated from each mouse at 20 and 45 days after immunization. CII-specific total IgG levels measured by ELISA were significantly higher in Roquin Tg mice than in WT mice. At 20 and 45 days after immunization, CII-specific IgG2a, which is associated with Th1 class switching, was higher in Roquin Tg mice, but CII-specific IgG1, which is associated with Th2 class switching, was lower in Roquin Tg mice compared with WT mice (Fig. 5). Thus, enforced expression of Roquin in T cells caused increased production and differential regulation of CII-specific IgG by B cells.

Increased Immune Response in the Spleen and Draining Lymph Nodes—We examined whether Roquin alters the immune response to CII. Spleen and draining lymph node cells were obtained from each group of mice at 20 and 45 days after CIA immunization, and their ability to produce IFN-γ, TNF-α, IL-6, IL-10, and IL-17 or to proliferate in response to CII dose-dependently was investigated. After in vitro antigenic stimulation with CII, proliferation of spleen and draining lymph node cells was higher in Roquin Tg mice than in WT mice at 20 and 45 days after immunization in a dose-dependent manner (Fig. 6A). Moreover, the secretion of IFN-γ, TNF-α, IL-6, and IL-17 was higher in cells from the Roquin Tg mice than in those from WT mice, whereas IL-10 secretion was lower in cells from the Roquin Tg mice than in those from WT mice in a dose-dependent manner (Fig. 6B). These data show that enforced expression of Roquin in T cells increased their proliferation and cytokine production in vitro.

Altered Number of Lymphocytes in the Spleens of CIA Mice after Immunization—To evaluate the CD4+CD28+ or CD4+/ICOS+ T cell populations, we isolated T cells from the spleens of mice with CIA using a CD4+ T cell isolation kit. Expression of ICOS in CD4+ T cells was lower in Roquin Tg mice than in WT mice, but CD28 expression in CD4+ T cells was higher in Roquin Tg mice than in WT mice (Fig. 7A). Also, to evaluate the population of lymphocytes in Roquin Tg mice with CIA after immunization, we analyzed lymphocyte subsets in the spleen. The proportion of Tbet+CD44highCD4+ T (Th1) cells was elevated in Roquin Tg mice compared with WT mice. However, that of GATA3+CD44highCD4+ T (Th2) cells decreased in Roquin Tg mice compared with WT mice (Fig. 7B). Also, the proportion of B220+GL-7+CD95+ (germinal center B) cells was higher in Roquin Tg mice, but that of PD-1highCXCR5+CD4+ (Tfh) cells was unaffected (Fig. 7C). These results indicate that enforced expression of Roquin in the T cells of mice with CIA altered lymphocyte proportions and their expression of co-stimulatory molecules after immunization.

DISCUSSION

Previous studies of Roquin in autoimmunity suggested that its main role was suppression of autoimmune disease via ICOS down-regulation (23). However, our data suggest that enforced expression of Roquin in T cells accelerates induction of CIA.

Germ-line Roquin-deficient mice are perinatally lethal, so tissue-specific ablation of Roquin in T cells, B cells, and hematopoietic cells does not cause autoimmunity (24). For gain of
function via Roquin overexpression, we established Roquin Tg mice. We performed FACS analysis to determine the composition of the immune cell population. The Th1, Th2, germinal center B, and Tfh cell populations were unaffected under normal, non-immunized conditions. Although ICOS expression was down-regulated in the Roquin Tg mice, immune cell development was unaffected. Roquin Tg mice may not exhibit an immune defect because ICOS, another member of the CD28 family, is not expressed by naive T cells (13, 14).

Our data show that enforced expression of Roquin in T cells down-regulated ICOS and up-regulated CD28 expression in arthritis. Also, CD28 up-regulation and ICOS down-regulation were modulated by enforced expression of Roquin in EL-4 cells by anti-CD3/CD28 activation in our previous in vitro study (21). Both CD28 and ICOS are capable of stimulating the development of Th cells (25). However, ICOS is expressed only on T cells following activation, whereas CD28 is constitutively expressed. CD28-stimulated cells, but not ICOS-stimulated T cells, produce IL-2 (26). Thus, CD28 is important in the early stages of T cell activation, whereas ICOS has a major role in its maintenance. Also, ICOS provides a stimulatory co-signal to activated T cells rather than to naive T cells. Ablation of CD28 in mice induces resistance to CIA (12). Targeted deletion of ICOS or ICOS ligand in mice altered the number of Th cells and reduced disease activity in the CIA model of arthritis (7). Our results suggest that Roquin increases the development of arthritis, further suggesting that CD28 up-regulation by Roquin may have a greater effect than ICOS down-regulation in the initiation of T cell activation.

T cells stimulated by TNF-α, IFN-γ, and IL-17 become Th1 and Th17 helper cells, whereas those stimulated by IL-4 and IL-10 develop into Th2 helper cells (27, 28). TNF-α, IFN-γ, and IL-6 are proinflammatory cytokines produced by T cells and are involved in diverse biological processes such as final maturation of B cells into plasma cells and T cell activation. These cytokines seem to be of pivotal importance in arthritis (29–31). Our results show that serum TNF-α, IFN-γ, IL-6, and IL-17 levels were higher in Roquin Tg mice after immunization. In contrast, levels of IL-10, an anti-inflammatory cytokine (32), were lower in the serum of Roquin Tg mice after immunization. Therefore, these data suggest that Roquin Tg mice exhibit exacerbated development of CIA compared with WT mice.

CIA is a well known model of Th1-mediated autoimmune disease (33). This study demonstrated a novel role of Roquin function in the initiation and development of arthritis through its influence on Th1/Th2 differentiation and balance. CD4+ T cells are classified into two major types: Th1 cells are primarily associated with cellular immunity and class switching to IgG2a, whereas Th2 cells are involved mainly in humoral immunity and class switching to IgG1 (34, 35). In the CIA model, immunization with CII generates a spectrum of antibodies with reactivities similar to those in human RA (4). Also, passive transfer of serum from CII-immunized animals or purified anti-CII antibodies can cause arthritis in syngeneic recipients (18, 36, 37).
Therefore, the production of IgG antibodies by Th1/Th2 cells plays a critical role in arthritis. Our data indicate increased IgG2a and decreased IgG1 levels in the serum of the Roquin Tg mice after CII immunization; thus, alterations in the levels of IgG2a and IgG1 may effectively alter the Th1/Th2 balance. Our data demonstrate that Roquin may enhance Th1 development, and enforced expression of Roquin in T cells may result in an elevated Th1-type response after immunization. Th cell differentiation, a crucial step in the immune response, is the result of complex cellular and molecular regulation. Recent years have seen progress in understanding the transcriptional mechanisms of Th lineage commitment and effector cytokine expression. However, how these T cell-intrinsic mechanisms are regulated by environmental stimuli such as co-stimulation by antigen-presenting cells needs further definition. In the future, downstream signals of co-stimulatory receptors will be elucidated and likely found to operate via Roquin-regulated mechanisms. Thus, Roquin may be another "double-edged

FIGURE 6. **Cell proliferation and cytokine production are increased by CII treatment.** A, spleen and draining lymph node cells were harvested on days 20 and 45 and cultured with graded CII concentrations for 96 h. The degree of cell proliferation was evaluated by a BrdU ELISA method after 96 h. Error bars indicate mean ± S.E. of triplicates and are representative of three independent experiments. *, p < 0.05. B, cytokine concentrations in culture supernatant were determined by ELISA after 72 h. Solid symbols, wild-type controls; open symbols, Roquin transgenic mice. Error bars represent the mean ± S.E. of triplicates and are representative of three independent experiments. *, p < 0.05.
swords” for the immune system that can be turned from a requirement of autoreactivity to a regulator of Th1/Th2 differentiation and balance. In conclusion, elevated expression of Roquin in T cells may promote autoimmune diseases such as CIA.

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FIGURE 7. Altered T cell populations in the spleen of collagen-induced arthritis mice after immunization. A, populations of ICOS- and CD28-positive cells in CD4+ T cells from WT and Roquin Tg mice on day 45 were determined by flow cytometry. Mean fluorescence intensity (MFI) values were measured and compared with those of WT mice. Error bars indicate the S.E. of triplicates. *p < 0.05. B, representative flow cytometric plots (left) and graphic analysis (right) of Tbet+CD44highCD4+ and GATA3+CD44highCD4+ in WT and Roquin Tg mice on day 45. C, representative flow cytometric graphic analysis of B220−GL7−PD-1highCXCR5+CD4+ cells in WT and Roquin Tg mice on day 45. Black bars, WT mice; gray bars, Roquin Tg mice. Data are representative of two independent experiments (n = 4 per group). p values are indicated on the graphs.
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