Biochemical and Biophysical Research Communications 452 (2014) 822–827

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Over-expression of Roquin aggravates T cell mediated hepatitis in transgenic mice using T cell specific promoter

Young Rae Ji [a,1], Hei Jung Kim [a,1], Dong Hun Yu [a], Ki Beom Bae [a], Seo Jin Park [a], Si Jun Park [a], Woo Young Jang [a], Min-Cheol Kang [a], Jain Jeong [a], Yong Hun Sung [a], Minjee Choi [a], Taejun Park [b], Taesun Park [c], Jong Won Yun [b], Hyun-Shik Lee [a], Sanggyu Lee [a], Myoung Ok Kim [d], Zae Young Ryoo [a,*]

[a] School of Life Science, KNU Creative BioResearch Group (BK21 plus project), Kyungpook National University, Buk-ku, Daegu, Republic of Korea
[b] Department of Biotechnology, Daegu University, Kyungsan, Kyungbuk, Republic of Korea
[c] Department of Food and Nutrition, Brain Korea 21 PLUS Project, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul, Republic of Korea
[d] Departments of Animal Science, Kyungpook National University, Sangju, Republic of Korea

ARTICLE INFO

Article history:
Received 22 August 2014
Available online 6 September 2014

Keywords:
Roquin
Transgenic mice
Hepatitis
T cell activation
Concanavalin A

ABSTRACT

Chronic hepatitis is a major cause of liver cancer, so earlier treatment of hepatitis might be reducing liver cancer incidence. Hepatitis can be induced in mice by treatment with Concanavalin A (Con A); the resulting liver injury causes significant $CD4^+$ T cell activation and infiltration. In these T cells, Roquin, a ring-type E3 ubiquitin ligase, is activated. To investigate the role of Roquin, we examined Con A-induced liver injury and T cell infiltration in transgenic (Tg) mice overexpressing Roquin specifically in T cells. In Roquin Tg mice, Con A treatment caused greater increases in both the levels of liver injury enzymes and liver tissue apoptosis, as revealed by TUNEL and H&E staining, than wild type (WT) mice. Further, Roquin Tg mice respond to Con A treatment with greater increases in the T cell population, particularly Th17 cells, though Treg cell counts are lower. Roquin overexpression also enhances increases in pro-inflammatory cytokines, including IFN-γ, TNF-α and IL-6, upon liver injury. Furthermore, Roquin regulates the immune response and apoptosis in Con A induced hepatitis via STATs, Bax and Bcl2. These findings suggest that over-expression of Roquin exacerbates T-cell mediated hepatitis.

1. Introduction

Liver diseases, including viral and autoimmune hepatitis, are a common health problem. Advances in the understanding of liver disease pathogenesis have improved the management and treatment of hepatitis, the main cause of liver cancer [1].

A common murine model of T cell-mediated liver injury employs treatment with Concanavalin A (Con A), a member of the plant lectin family [2]. Con A, which stimulates proliferation of $CD4^+$ T cells, is widely used in investigations of immune regulation [3,4]. Con A-induced liver injury is partly driven by up-regulation of cytokines, including TNF-α, IFN-γ, and IL-6 [5,6]. Recent studies of Con A-induced hepatitis have reported that neutrophils infiltrate the liver [7,8] and regulate $CD4^+$ T cell recruitment and production of TNF-α, IFN-γ, and IL-4 [3,9]. *In vitro* experiments have shown that Kupffer cells also contribute to the production of various cytokines by lymph nodes, including TNF-α, IFN-γ, and IL-4, in response to Con A treatment [10]. Since TNF-α, IFN-γ, and IL-4 are known to play a key role in liver injury in hepatitis [11,12], Con A-induced hepatitis in mice is considered a suitable model for the study of certain mechanisms that give rise to hepatitis in patients, including the involvement of immune system processes.

Autoimmune processes are modulated by Roquin, a RING-type E3 ubiquitin ligase family member localized in cytosolic stress

Abbreviations: Con A, concanavalin A; Tg, transgenic; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling; H&E, hematoxylin and eosin; WT, wild type; Th17, interleukin-17 secreting CD4 T helper cells; Treg, regulatory T helper cells; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor-alpha; IL, interleukin; Bax, Bcl2 associated X; Bcl-2, B cell lymphoma 2; ICOS, inducible T cell co-stimulator; Tfh, follicular T helper cells; CIA, collagen induced arthritis; FACS, fluorescence activated cell sorter; ELISA, enzyme linked immunoassay.

* Corresponding author. Address: School of Life Sciences and Biotechnology, Kyungpook National University, 1370 Sankyuk-dong, Buk-ku, Daegu, Republic of Korea. Fax: +82 53 943 6925.
E-mail address: jaewoong64@hanmail.net (Z.Y. Ryoo).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.09.001
0006-291X/

granules in T cells. Roquin inhibits the expression of the inducible T cell co-stimulator (ICOS) protein, a member of the CD28 superfamily, by binding sequences in the 3′-UTR of the corresponding mRNA and preventing translation [13,14]. Inhibition of ICOS expression prevents differentiation of follicular B helper T cells (Tfh cells), and, thus, attenuates autoimmune reaction and the activation of B cells. ICOS is also capable of co-stimulating T cell responses in place of CD28 [13,14]. In a previous study, transgenic overexpression of Roquin aggravated the severity of collagen-induced arthritis (CIA), a model of rheumatoid arthritis (RA). Roquin activates the CD28 signaling pathway and its component proteins, including Akt, JNK, and NF-κB, and upregulates production of several pro-inflammatory cytokines, such as TNF-α, IL-2, IL-6, and IFN-γ [15].

To investigate the effects of Roquin on the development of hepatitis, in particular on T cell subpopulations, secretion of inflammatory cytokines, and liver injury, we used Con A to induce hepatitis in transgenic mice that constitutively expressed Roquin specifically in T cells.

2. Materials and methods

2.1. Mice

Roquin-transgenic (Roquin Tg) mice were generated using a standard procedure [16]. In brief, the cDNA of the gene encoding Roquin, *Rc3h1*, was amplified using RNA from mouse spleen. For T lymphocyte-specific expression, we placed the *Rc3h1* cDNA under the control of the human *CD2* promoter [17]. All mice were raised and housed under special, pathogen-free conditions in a temperature-controlled room at 22 °C. Genomic DNA was extracted from tail biopsies of offspring, and the presence or absence of the *CD2* promoter driven *Rc3h1* transgene was determined by PCR. The primers used were 5′-ACTCCACCAGTC TCACTTCA-3′ (forward) and 5′-CATCACGTTTGGTGACCTTG-3′ (reverse). Amplification was carried out with a thermal cycler (Takara, Kyoto, Japan) using the following protocol: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, for a total of 30 cycles.

2.2. Concanavalin A injections

Roquin Tg mice on a C57BL/6 mice background were used along with corresponding age-matched WT mice. Mice were injected intravenously (i.v.) with 10 mg/kg of Concanavalin A (Con A) (Sigma, St. Louis, MO, USA), or with phosphate buffered saline (PBS) as control, at around 7–10 weeks of age. All experiments were performed according to the guidelines of the Kyungpook National University Intramural Animal Use and Care Committee.

2.3. Assessment of hepatotoxicity

After Con A treatment, the mice were killed; blood samples were taken, and then liver samples were collected. The collected blood samples were centrifuged at 10,000×*g* for 10 min at 4 °C, and the separated sera were stored at −80 °C until analysis. Plasma samples were used in biochemical analysis of alanine transaminase (ALT) and aspartate transaminase (AST) levels. Measurements were performed by an auto-analyzer.

2.4. Histological analysis

After PBS or Con A treatment, the mice were killed, and each liver was collected. Livers were fixed with 4% paraformaldehyde for 2 days, dehydrated in a graded series of ethanol and xylene, and embedded in paraffin. Thin sections (5 μm) were stained with hematoxylin and eosin (H&E). To detect apoptosis, an *in situ* cell apoptosis detection kit (Trevigen, Gaithersburg, MD, U.S.A.) was used according to the manufacturer's protocol.

2.5. Western blot

Liver and spleen lysates were prepared using lysis buffer, consisting of 20 mM Tris–HCl (pH 8.0), 200 mM NaCl, 0.25% Triton X-100, 10 μg/mL aprotinin, 1 g/mL pepstatin, 10 μg/mL leupeptin, and 2 M phenylmethylsulfonyl fluoride, and then cleared by centrifugation. The protein concentrations of the resulting clarified lysate supernatants were determined using a Bradford protein assay (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard. Proteins in whole organ lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), run in tris–glycine buffer, and transferred to nitrocellulose membranes. The membranes were incubated in primary antibody overnight at 4 °C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Levels of proteins of interest were determined using an enhanced chemiluminescence detection system (GE Healthcare). Primary antibodies against phospho-STAT-1, phospho-STAT-3, phospho-STAT-6, Bax, and Bcl-2 were obtained from Cell Signaling Technology.

2.6. Flow cytometric analysis

Single cell suspensions were prepared from the livers and spleens, which was eliminated blood using heart perfusion methods, and then treated with red blood cell lysing solution (0.15 M NH_4Cl and 0.1 mM Na_2EDTA) for 5 min at 4 °C to eliminate erythrocytes. To isolate mononuclear cells, single cell suspensions were mixed with Percoll (Sigma) and the mixtures were centrifuged. $CD4^+$ T cells were positively isolated to more than 95% purity, using anti-CD4 monoclonal antibody-coupled magnetic cell-sorting micro beads (Miltenyi, San Diego, CA, U.S.A.). Cells were stained with fluoro-conjugated anti-mouse CD4, CD69, CD25, IL-17, FoxP3, CD44, NK1.1, and CXCR5 antibodies and analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA, U.S.A.), using Cell Quest software (BD Biosciences).

2.7. Cytokine assay

Total levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10) (R&D Systems, Minneapolis, MN, U.S.A.), and interleukin-13 (IL-13) (Invitrogen, Carlsbad, CA, USA) in supernatants were determined using a commercially available ELISA kit according to the manufacturer's recommendations.

2.8. Statistical analysis

The results are expressed as the mean and standard error (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, for each of various groups. Differences with a *p*-value of less than 0.05 were considered statistically significant.

3. Results

3.1. Roquin is induced in the liver and spleen by Con A treatment

To determine the effect of Con A treatment on expression of Roquin, we conducted western blots of protein samples from livers and spleens harvested from mice 24 h after Con A or PBS injection. Expression of Roquin was greater in organs from Con A-injected

mice than from PBS-injected mice (Fig. 1A). These data indicated that Roquin was induced in the liver and spleen of mice with Con A-induced hepatitis. Con A-induced hepatitis has been investigated for its accuracy as a model for T cell-mediated liver injury [12]. In a previous study, we generated a transgenic mouse that over-expressed Roquin specifically in T cells, under the control of the human *CD2* promoter [17]. As we are interested in the effects of T cell-specific overexpression of Roquin on T cell-mediated hepatitis, we also compared expression of Roquin in livers and spleens from Roquin Tg mice, versus from WT mice, and observed more hepatic and splenic Roquin expression in transgenic mice (Fig. 1B).

3.2. Overexpression of Roquin in T cells exacerbates Con A-induced hepatitis

To examine the effect of Roquin overexpression in T cells on Con A-induced liver injury, we examined the extent of liver injury, by H&E staining, at 24 h after injection with Con A or with PBS as control. Livers from PBS treated Roquin Tg and WT mice were equally healthy, with no significant differences. In contrast, more necrotic tissue was observed in liver sections from Con A treated Roquin Tg mice than from Con A treated WT mice. Similarly, a higher degree of infiltration by mononuclear cells, such as T cells, was seen in livers of Roquin Tg mice than in those of WT mice (Fig. 1C). We also assessed expression of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are normally upregulated upon liver injury, and both were expressed at higher levels in Roquin Tg mice than in WT mice, at 6 and 24 h after Con A injection. Incidentally, expression of both AST and ALT was greater at 6 h than at 24 h (Fig. 2A).

3.3. Roquin overexpression enhances Con A-induced inflammatory cytokine secretion

Con A treatment induces expression of various inflammatory cytokines in the liver [12]. To examine whether Roquin overexpression altered production of cytokines in the liver, we assayed

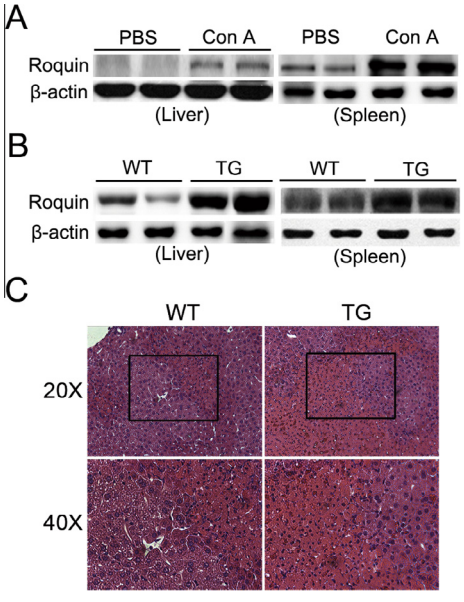

Fig. 1. Liver injury was increased with Roquin after the administration of Con A. (A) Roquin expression was detected by Western blot analysis in liver and spleen of wild type (WT) mice at 24 h after PBS or Con A injection. (B) Roquin expression was detected by Western blot analysis in liver and spleen of WT and Roquin transgenic (TG) mice at 24 h after Con A injection. (C) Histological changes were showed in the liver from WT and Roquin Tg mice at 24 h after Con A injection by H&E staining.

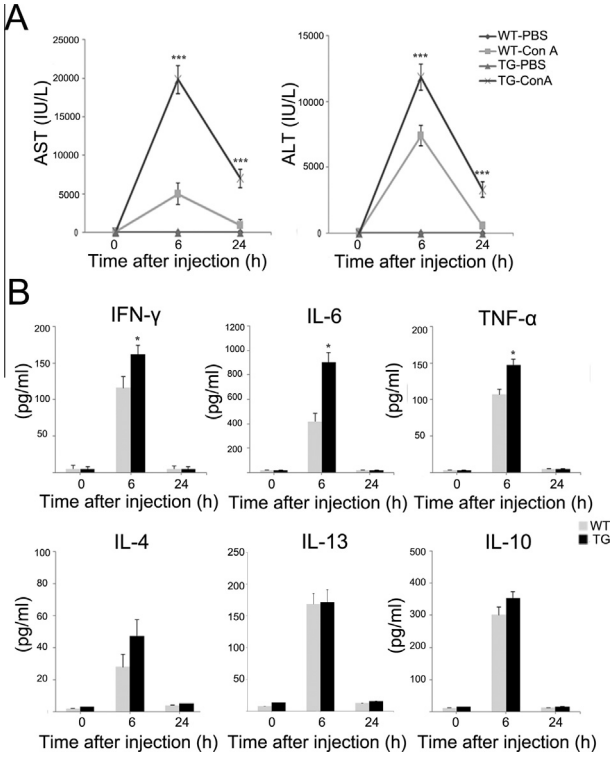

Fig. 2. Roquin up-regulates secretion of inflammatory cytokine in Con A injected mice. (A) Serum of WT mice and Roquin Tg mice after PBS and Con A injection were measured to AST and ALT levels in each time, 0, 6 and 24 h. (B) Secretion of IFN-γ, TNF-α, IL-6, IL-4, IL-13, and IL-10 was detected by ELISA at 0, 6 and 24 h after Con A injection from serum of WT and Roquin Tg mice. *Black bars*, WT mice; *gray bars*, Roquin Tg mice. *Error bars* indicated mean ± S.E. of all immunized mice of genotype ($n = 6$). $^{*}p < 0.05$; $^{***}p < 0.001$.

cytokine secretion over time by ELISA. Secretion of IFN-γ, IL-6, and TNF-α was significantly greater in liver from Roquin Tg mice, compared with liver from WT mice, at 6 h after Con A injection, but secretion of IL-4, IL-13, and IL-10 did not differ (Fig. 2B). As the increases in expression were specific to pro-inflammatory cytokines, these data suggested that Roquin overexpression activated inflammatory responses in the liver.

3.4. Roquin regulates Con A-induced activation of hepatic lymphocytes

To find out which subsets of cells exhibited enhanced infiltration of the liver in Roquin Tg mice, we next evaluated the effect of Roquin overexpression on Con A-induced alterations to $CD4^+$ and $CD8^+$ T cell populations, to iNKT cell and to $B220^+$ B cell populations, in the liver. The number of $CD4^+$ T cells was significantly larger in Roquin Tg mice than in WT mice, but there was no difference in the number of iNKT cells or $CD8^+$ T cells or $B220^+$ B cells (Fig. 3A). Therefore, the mononuclear cells that contributed to the more extensive infiltration seen in Fig. 1C might be $CD4^+$ T cells. Flow cytometry, using labeled antibodies against $CD69^+$ and $CD25^+$, markers of T cell activation, revealed greater numbers of activated $CD4^+$ cells (expressing $CD69^+$ or $CD25^+$) in Roquin Tg mice than in WT mice (Fig. 3B and C). The distribution of other T cell types was also assessed by flow cytometry, using antibodies against IL-17 and Foxp3, markers for helper and regulatory T cells, respectively. After Con A injection, the number of IL-17^+ T cells in the liver and spleen was higher in Roquin Tg mice than in WT mice (Fig. 3D), while the number of $FoxP3^+$ cells was lower (Fig. 3E). This result indicated that overexpression of Roquin increases T cell activation and regulates differentiation of pro-inflammatory Th17 and

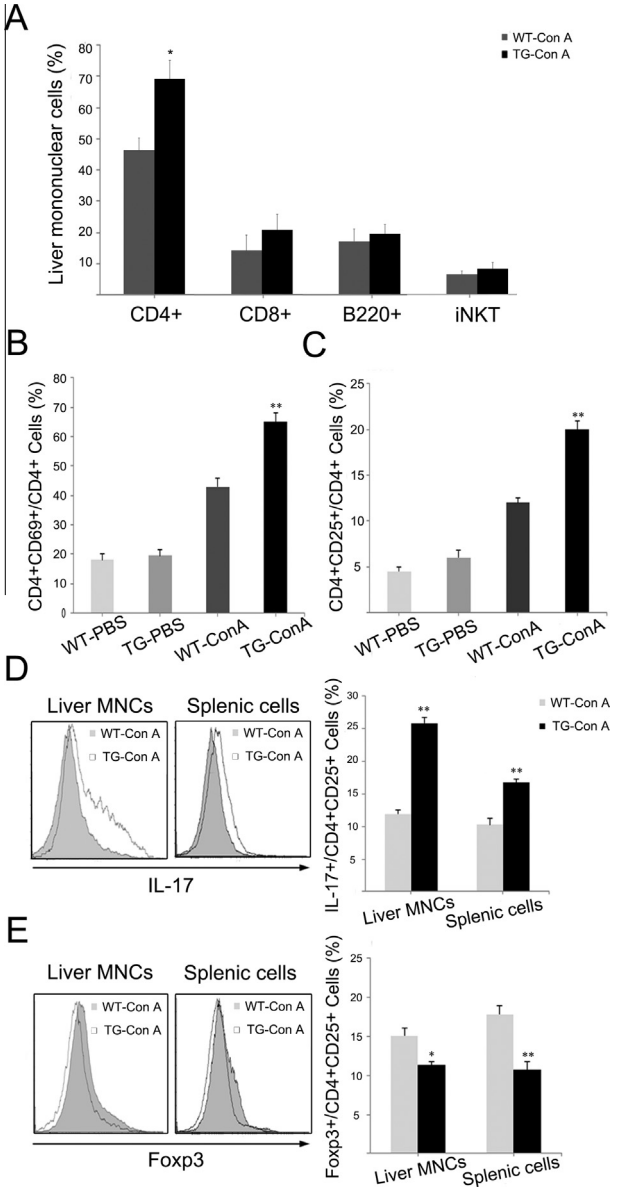

Fig. 3. Roquin regulates activation of hepatic and splenic lymphocytes after Con A injection. (A) Analysis of lymphocyte population, including $CD4^+$, $CD8^+$, $B220^+$ B cell, and iNKT cells is confirmed in liver from WT mice and Roquin Tg mice at 24hrs after Con A injection. (B) $CD69^+$ population is detected to activation of T cell by FACS analysis in WT and Roquin Tg mice at 24 h after PBS and Con A injection. (C) $CD25^+$ population is detected to activation of T cell by FACS analysis in WT mice and Roquin Tg mice at 24 h after PBS and Con A injection. (D) IL-17^+, $CD4^+$ and $CD25^+$ population is detected to Th17 cells from liver and spleen in WT and Roquin Tg mice at 24 h after Con A injection. Representative flow cytometric plots (left) and graphic analysis (right). (E) FoxP3^+, $CD4^+$ and $CD25^+$ population is detected to Treg cells from liver and spleen in WT and Roquin Tg mice at 24 h after Con A injection. *Error bars* indicate mean ± S.E. of all immunized mice of each genotype. Liver MNCs: liver mononuclear cells. $^*p < 0.05$, $^{**}p < 0.01$.

anti-inflammatory Treg cells, consistent with the promotion of inflammation in the liver.

3.5. Roquin activates pro-inflammatory and pro-apoptotic signaling

To examine whether the scale of liver injury correlated with the extent of apoptosis, we performed TUNEL assays. Con A injection induced more apoptosis in Roquin Tg mice than in WT mice, in both necrotic and non-necrotic areas (Fig. 4A). In combination with the

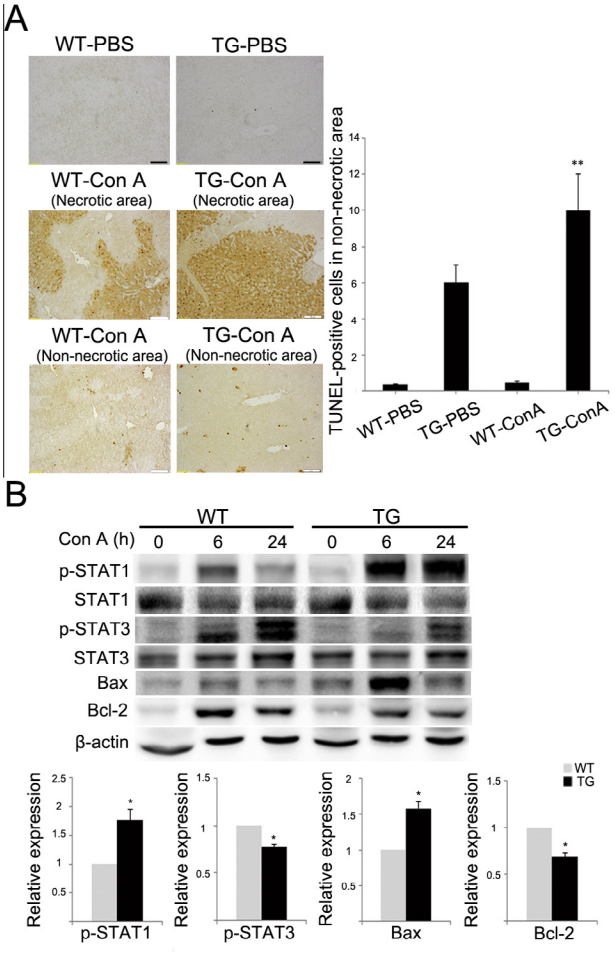

Fig. 4. Hepatic cellular signaling and apoptotic factor is regulated by Roquin overexpression in mice. (A) Apoptotic cells were showed in the liver from WT and Roquin Tg mice at 24 h after Con A injection by TUNEL. Representative Immunohistochemistry (left) and graphic analysis for cell counts per 0.2 mm^2 (right). (B) Hepatic cellular signals, which are Stat1 and Stat3, were confirmed phosphorylation by Western blot from liver lysates at 0, 6, and 24 h in WT mice and Roquin Tg after Con A treatment. Also, apoptosis related factor, which are Bax and Bcl2, were detected by Western blot from liver lysates at 0, 6, and 24 h in WT mice and Roquin Tg after Con A treatment. Representative Western blots (upper) and graphic analysis for results at 6 h (bottom). Scale bars: A, white 50 μm and black 100 μm. *Error bars* indicated mean ± S.E. of all immunized mice of genotype ($n = 6$). $^*p < 0.05$; $^{**}p < 0.01$.

H&E staining results, these findings indicated that Roquin exacerbated liver injury in Con A-induced hepatitis via apoptosis. To examine the signaling pathways underlying the augmented inflammatory response in Roquin transgenic mice, we assessed the phosphorylation status of STAT-family proteins, which regulate cell proliferation, the immune response, and oncogenesis [18,19]. Under pro-inflammatory conditions, the phosphorylation of STAT1 is increased, but that of STAT3 is decreased [20]. Following Con A injection, more STAT1, but less STAT3, was phosphorylated in Roquin Tg mice than in WT mice (Fig. 4B). To evaluate the signaling mechanism responsible for enhanced apoptosis in Roquin Tg mice, we measured the expression levels of Bax, a pro-apoptotic marker, and Bcl-2, an anti-apoptotic marker, in Con A-induced hepatitis. After Con A injection, expression of Bax was increased in Roquin Tg mice, while that of Bcl-2 was decreased, relative to WT mice (Fig. 4B). These results demonstrated that, in the context of Con A-induced hepatitis, Roquin stimulated the immune response and the occurrence of apoptosis via regulation of STATs, Bax, and Bcl2.

4. Discussion

Our data, using Roquin-overexpressing transgenic mice, suggest that Con A-induced hepatitis is promoted by the activation and differentiation of specific T cell populations, which are regulated, at least in part, by Roquin. In a previous study, we established Roquin transgenic mice, in which the *Rc3h1* cDNA followed the T cell-specific human *CD2* promoter, and confirmed overexpression of Roquin mRNA and protein [17]. Here, we showed that Roquin is expressed in liver and spleen after Con A injection, and that induction of hepatitis by Con A is enhanced in Roquin transgenic (Roquin Tg) mice relative to wild type (WT) mice. Roquin has previously been demonstrated to regulate the development of autoimmune diseases such as collagen-induced arthritis [15]; we have now expanded the group of conditions in which Roquin is known to play a role to include the acute inflammation that leads to T cell-mediated liver injury.

Roquin expression was detected in liver and spleen at 24 h after Con A injection, but not at 6 h (data not shown). Because secretion of chemokines that drive immune cell infiltration occurs on a similar time scale [21], this result suggests that Roquin might be expressed in infiltrating immune cells, such as T cells, rather than in hepatocytes. Thus, the mice from this study, which overexpress Roquin specifically within T cells, are likely to be useful for understanding T cell-mediated hepatitis.

Our flow cytometry data revealed that Roquin aggravates liver injury by influencing the differentiation pathways of T cell populations. The numbers of $CD4^+$ T cells that were also $CD25^+$ or $CD69^+$ were increased in Roquin Tg mice relative to WT mice. Within the population of $CD4^+$ $CD25^+$ T cells, the number of $IL\text{-}17^+$ cells was increased, but the number expressing FoxP3 was decreased, suggesting that Roquin overexpression alters the ratio of Th17 to Treg cells. Given that the balance between the populations of Th17 and Treg cells is known to mediate T cell activation [22–24], Roquin overexpression might provoke T cell-activation by affecting the regulation of Th17 and Treg cell differentiation. In our previous study, Roquin was shown to regulate the balance of Th1 and Th2 cells in an arthritis model and to enhance secretion of IL-17. These data are consistent with increases in the Th17 population upon acute liver injury observed in this study. These results indicate that overexpression of Roquin exacerbates Con A-induced hepatitis by influencing Th17 and Treg differentiation.

In a previous study, Roquin has been found to inhibit the translation of inducible T cell co-stimulator (ICOS) [13]. In contrast, Roquin overexpression in a collagen-induced arthritis model enhanced activation of CD28 and increased phosphorylation of Akt, JNK and IκB, which are downstream of CD28 [15]. CD28 is important in the early stages of T cell activation, whereas ICOS plays a major role in its maintenance. Also, ICOS provides a co-stimulatory signal that affects the differentiation of Tfh cells [25]. We found that the populations of Tfh cells and activated B cells did not differ between WT and Roquin Tg mice that were both injected with Con A to trigger T cell-mediated hepatitis (data not shown). In previous study, iNKT cells, which have a critical role in hepatitis, highly expressed ICOS and regulated activation of iNKT cells by ICOS [26]. Our results showed that no difference was iNKT cell activation between WT and Roquin Tg mice via counting of $CD44^+NK1.1^+$ population and measuring IL-13. Thus, the small effect of ICOS, compared to that of CD28, on the development of acute hepatitis, such as in the Con A-induced hepatitis model, is not surprising.

Analysis of cellular signaling and apoptosis-related pathways, by western blot, revealed alterations in the phosphorylation of STAT family proteins, and in the expression of proteins regulating apoptosis. In a previous study of T cell-mediated hepatitis, the phosphorylation of STAT1 and STAT3 was linked to hepatocyte apoptosis [20,27]. We found that phosphorylation of STAT1 was increased, but that of STAT3 was decreased, in livers from Con A-treated Roquin Tg mice, when compared to phosphorylation in livers from Con A-treated WT mice. In addition, the levels of the apoptotic factors Bax and Bcl2 were modified by Roquin overexpression, and necrosis and apoptosis were more severe in Roquin Tg mice than in WT mice. These results indicate that overexpression of Roquin in T cells in the liver activates cellular signaling that promotes hepatocyte apoptosis and a stronger inflammatory response.

In conclusion, Roquin expression in T cells plays a critical role in T cell-mediated hepatitis. We have demonstrated that Roquin overexpression enhances Con A-induced liver injury, hepatocyte cell death, and inflammation, by altering the regulation of T cell activation and differentiation. Therefore, while the mechanism by which Roquin regulates hepatitis deserves further study, targeting the modulation of Th17 and Treg cell differentiation by Roquin could offer a novel approach to the treatment of T cell-mediated liver damage.

Acknowledgments

This work was supported by the Next-Generation BioGreen21 Program (No. PJ 009573), the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2008-0062618).

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