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The role of Roquin overexpression in the modulation of signaling during *in vitro* and *ex vivo* T-cell activation

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ABSTRACT

The T-cell receptor (TCR) engages with an antigen and initiates a signaling cascade that leads to the activation of transcription factors. Roquin, a protein encoded by the RC3H1 gene and characterized as an immune regulator, was recently identified as a novel RING-type ubiquitin ligase family member, but the mechanisms by which Roquin regulates T-cell responses are unclear. We used the EL-4 murine lymphoma cell line to elucidate the role of Roquin *in vitro*. Roquin-overexpressing EL-4 cells became hyperresponsive after anti-CD3/CD28 stimulation *in vitro* and were a major source of the cytokines IL-2 and TNF-α. Upon activation, these cells showed particularly enhanced production of IL-2 and TNF-α. To clarify the important role played by Roquin in T-cell responses *ex vivo*, we generated T-cell-specific Roquin transgenic (Tg) mice. Roquin-Tg $CD4^+$ T-cells showed enhanced production of IL-2 and TNF-α in response to TCR stimulation with anti-CD28 co-stimulation. Further studies are necessary to investigate the role of Roquin in the regulation of primary T-cell activation, survival, and differentiation.

1. Introduction

The immune response consists of complex mechanisms that function to protect the host from disease, destroy pathogens and attack tumor cells. Disorders of the immune system can result in autoimmune diseases, inflammatory diseases, and cancer [1]. During adaptive immune responses, $CD4^+$ T-cells are activated by MHC molecules that present antigen peptides. A co-stimulatory signal initiated by members of CD28 family is also indispensable for activating $CD4^+$ T-cells. This co-stimulatory signal is essential for maintaining the full activation status of antigen-stimulated T-cells by sustaining their cellular proliferation and differentiation and preventing the onset of anergy and induction of apoptosis [2]. During the immune response, the binding of the TCR to its cognate antigen initiates a signaling cascade that leads to the activation of three main transcription factors: AP-1, NF-κB, and NFAT, all of which are critically involved in cytokine production. These transcription factors regulate the expression of early cytokines, especially interleukin-2 (IL-2), which mediates the T-cell expansion phase [3]. IL-2 was first identified as a growth factor for T-cells [4,5]. T-cell proliferation induced by concanavalin A or anti-CD3 stimulation is substantially reduced in IL-2-deficient mice [6]. Therefore, the proper secretion of IL-2 is important for maintaining a well-balanced immune response.

Roquin, a protein encoded by the RC3H1 gene, was recently identified as a novel RING-type ubiquitin ligase family member. Roquin mRNA transcripts are ubiquitous and, based on consensus sequences with the E3 ubiquitin ligase family, are predicted to encode an intracellular protein with an amino-terminal RING-1 zinc finger at residues 14–53. In a previous study, Roquin was shown to regulate an inducible T-cell co-stimulator (ICOS) that is part of the CD28 superfamily. ICOS is capable of providing co-stimulation in T-cell responses in place of CD28 [7].

This study focused on the functional role of Roquin in the T-lymphocyte-mediated immune response and provides important clues about the role that Roquin plays in T-cell activation. We found that AKT and JNK phosphorylation are required for the modulation of Roquin upon TCR engagement. Our results also demonstrate that signaling pathways other than those involved in inflammation are influenced by Roquin. We provide experimental evidence that Roquin modulates the secretion of various cytokines. Our study suggests that Roquin plays a significant role in modulating early T-cell activation, including the expression of IL-2 induced by TCR/CD28 engagement.

2. Materials and methods

2.1. Cell culture and transfection

EL-4 cells, which are a murine T lymphocytic cell line, were cultured in RPMI 1640 medium (Gibco BRL, NY, USA) supplemented with 10% heat inactivated fetal calf serum (Gibco BRL, NY, USA),

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50 U/ml penicillin, and 50 mg/ml streptomycin. EL-4 cells were transfected with an Amaxa Nucleofector device (Amaxa Biosystems, Basel, Switzerland). The full-length mouse Roquin cDNA, which contains an EcoRI restriction enzyme site, was cloned into the pEGFP-N1 vector and driven by the CMV promoter. The vector was prepared for cell transfection by digesting with ApaLI cells (2×10^6) were transfected with 4 μg of plasmid DNA in solution L using program C-009. To establish stable EL-4 cell lines, the cells were initially selected using medium containing 1 mg/ml geneticin and were subsequently maintained in medium containing 0.3 mg/ml geneticin (Invitrogen, CA, USA). EL-4 cells were seeded into 24-well plates and were either left untreated or were stimulated with 1 μg/ml anti-CD3, 4 μg/ml soluble anti-CD28 (BD Biosciences, CA, USA) and 4 μg/ml anti-ICOS (eBioscience, CA, USA) monoclonal antibodies. To inhibit AKT and JNK signaling, we used 20 μM LY294002 and 10 μM JNK inhibitor I (Calbiochem, Darmstadt, Germany). After EL-4 cells were treated with LY294002 for 1 h or JNK inhibitor I for 30 min, these cells were stimulated with anti-CD3/CD28 for 24 h, and the cell supernatants were harvested. An ELISA was used to determine the concentration of IL-2 in cell culture supernatants.

2.2. RNA extraction and RT-PCR

Total RNA was extracted from EL-4 cells using TRIzol® Reagent (Invitrogen, CA, USA). The extracted RNA was then treated with DNase I for 30 min to remove genomic DNA. The DNase I enzyme was then inactivated by incubating samples at 65 °C. cDNA synthesis was performed using an RT-PCR kit (Takara, Kyoto, Japan) with 1 μg of RNA. The reverse transcription reaction was performed using an oligo (dT) primer with moloney murine leukemia virus (MMLV) reverse transcriptase. The amount of first strand reaction added to the PCR was titrated for each set of primers. The primer sequences for GAPDH are as follows: forward 5′-AATGCATCCTG CACCACCAA-3′ reverse 5′-GTAGCCATATTCATTGTCATA-3′. The cycling profile parameters were as follows: 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s for a total of 25 cycles.

2.3. Quantitative real-time PCR

Quantitative real-time PCR was performed on an Applied Biosystems PRISM 7300 detection system. The expression levels of ICOS and CD28 were determined using TaqMan probes and GAPDH as an endogenous control. TaqMan Universal PCR Master Mix, the ICOS primer/probe set (Part No. Mm00497600_m1), the CD28 primer/probe set (Part No. Mm00483137_m1), and the GAPDH primer/probe set (Part No. Mm99999915_g1) were purchased directly from Applied Biosystems. Relative quantification was performed using the comparative cycle threshold method as described in the *User Bulletin* edit provided by Applied Biosystems.

2.4. Western blots

EL-4 cell extracts (2×10^6 cells) were prepared using lysis buffer (Cell Signaling, MA, USA). The protein concentration of the clarified supernatants was determined using a Bradford protein assay (Bio-Rad, CA, USA) with BSA as a standard. After blocking the membranes with 5% non-fat dry milk in Tris buffered saline (25 mM Tris HCl [pH 7.4], 150 mM NaCl) containing 0.05% Tween 20, the blots were exposed to mAb at room temperature, rinsed in Tris buffered saline–Tween 20, and exposed to horseradish peroxidase–conjugated secondary antibodies (Santa Cruz, CA, USA) for 2 h at room temperature. The blots were probed for β-actin as the loading control. Immunoreactivity was determined using an enhanced chemiluminescence detection system (GE Healthcare, Munich, Germany). The anti-Roquin antibody was purchased from Novus biologicals, and the anti-HA antibody was purchased from Roche. Antibodies against phospho-AKT (Ser473), AKT, phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK (Thr183/Tyr185), and JNK were obtained from Cell Signaling Technology (MA, USA).

2.5. Generation and screening of transgenic mice

The murine Roquin gene was amplified from mouse spleen RNA using primers flanking the ORF. The T lymphocyte-specific expression cassette included the human CD2 promoter, a multiple cloning site, a bovine growth hormone polyadenylation signal (BGH pA), the locus control region (LCR) and the transgene tagged with HA. The plasmid DNA used for microinjection was purified using a plasmid midi-prep kit (Qiagen, CA, USA) and was prepared by digesting the recombinant vector DNA with Bgl II and Not I. Roquin-transgenic (Tg) mice were generated using a standard procedure [8]. All mice were raised and housed under specific pathogen-free conditions. Genomic DNA was extracted from biopsied offspring tails, and the presence of the Roquin transgene was determined by PCR. The primers used were forward 5′-ACTCCACCAGTCTCACTTCA-3′ and reverse 5′-CATCACGTTTGGTGACCTTG-3′. Amplification was carried out with a thermal cycler (Takara, Kyoto, Japan) using the following amplification protocol: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for a total of 30 cycles.

2.6. $CD4^+$ T-cell purification and activation

Single cell suspensions were prepared from the lymph nodes and spleens of wild-type and Roquin Tg mice. Then, the cell suspensions were 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. $CD4^+$ T-cells were positively selected using anti-CD4 antibody-coupled magnetic cell sorting microbeads (Miltenyi, CA, USA). $CD4^+$ T-cells were isolated to more than 95% purity as analyzed by flow cytometric staining with anti-CD4 and anti-CD8 antibodies. Purified $CD4^+$ T-cells (5×10^5) were then seeded into 24-well tissue culture plates precoated with 1 μg/ml anti-CD3 and 4 μg/ml soluble anti-CD28 monoclonal antibodies.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The total levels of IL-2, TNF-α, and IFN-γ in cell culture supernatants were determined using a commercially available ELISA kit (R&D Systems, MN, USA), according to the recommendations of the manufacturer.

2.8. Statistical analysis

The results are expressed as means ± SEM from at least three independent experiments. Groups were compared by ANOVA. Statistical significance was set at $P < 0.05$.

3. Results

3.1. A Roquin overexpressing EL-4 cell line regulates T-cell activation

To confirm the role of Roquin in T-lymphoma cells *in vitro*, we generated a stable EL-4 cell line that overexpressed the Roquin protein. Compared with control EL-4 cells transfected with mock vector, EL-4 cells transfected with the Roquin expression vector exhibited high levels of Roquin mRNA and protein (Fig. 1A and B).

Based on the RT-PCR analysis, ICOS expression in Roquin overexpressing EL-4 cells was significantly decreased compared with control EL-4 cells (Fig. 1C; $^{**}P < 0.001$), confirming the results found in a previous study [7]. In contrast, the level of CD28 mRNA in Roquin

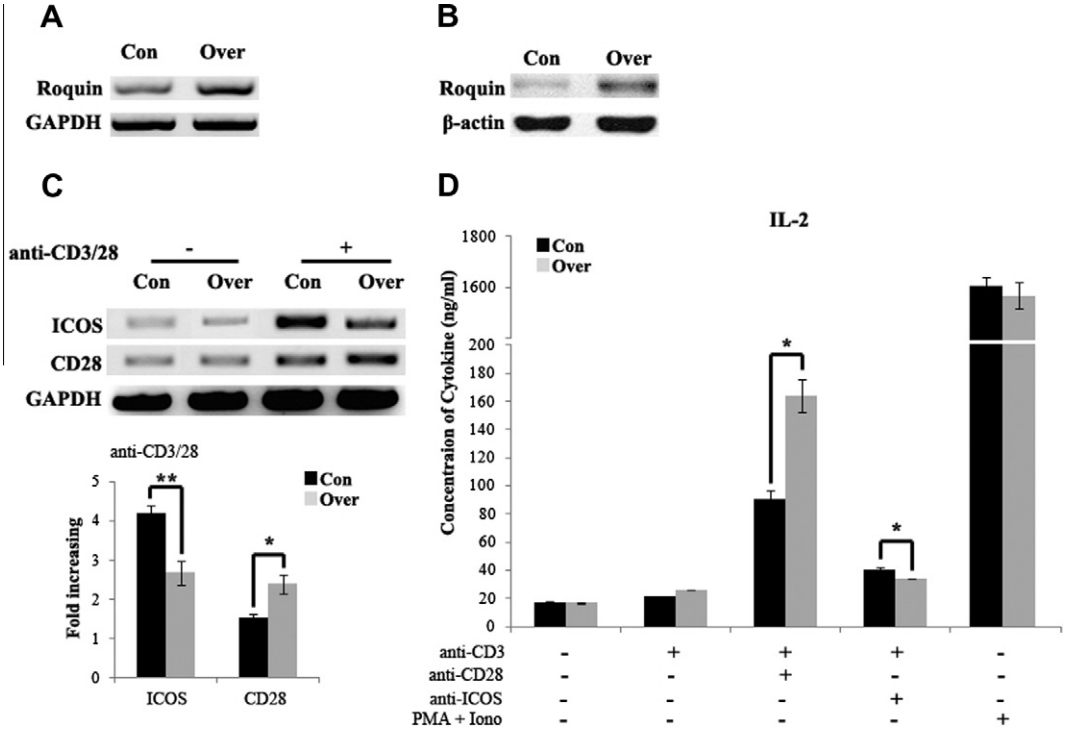

Fig. 1. The establishment of a stable Roquin-overexpressing cell line to elucidate the role of Roquin in T-cell activation. (A) Roquin expression was quantitatively determined with reverse-transcription PCR. Roquin appears as a 459 bp DNA fragment in the sample from the stable Roquin-overexpressing cell line. (B) The Roquin protein was detected in Roquin-overexpressing cells by Western blot using an antibody against Roquin. (C) ICOS and CD28 expression were detected by reverse-transcription PCR. Roquin-overexpressing EL-4 cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (4 μg/ml) for 24 h to activate T-cells. (–), no treatment; (+), treatment with anti-CD3/CD28. The results shown are representative of at least three independent experiments. The data were graphed using ImageJ software. The value represents the mean ± SD of three independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$. (D) Quantification of IL-2 in the culture supernatants from non-transfected and Roquin-overexpressing EL-4 cells. Cells were stimulated for 24 h with plate-bound anti-CD3 and soluble anti-CD28 or anti-ICOS Abs or PMA (5 ng/ml) and ionomycin (Iono; 100 nM). Con, non-transfected EL-4 cells; Over, Roquin-overexpressing cells. Data are representative of three independent experiments. $^{*}P < 0.01$.

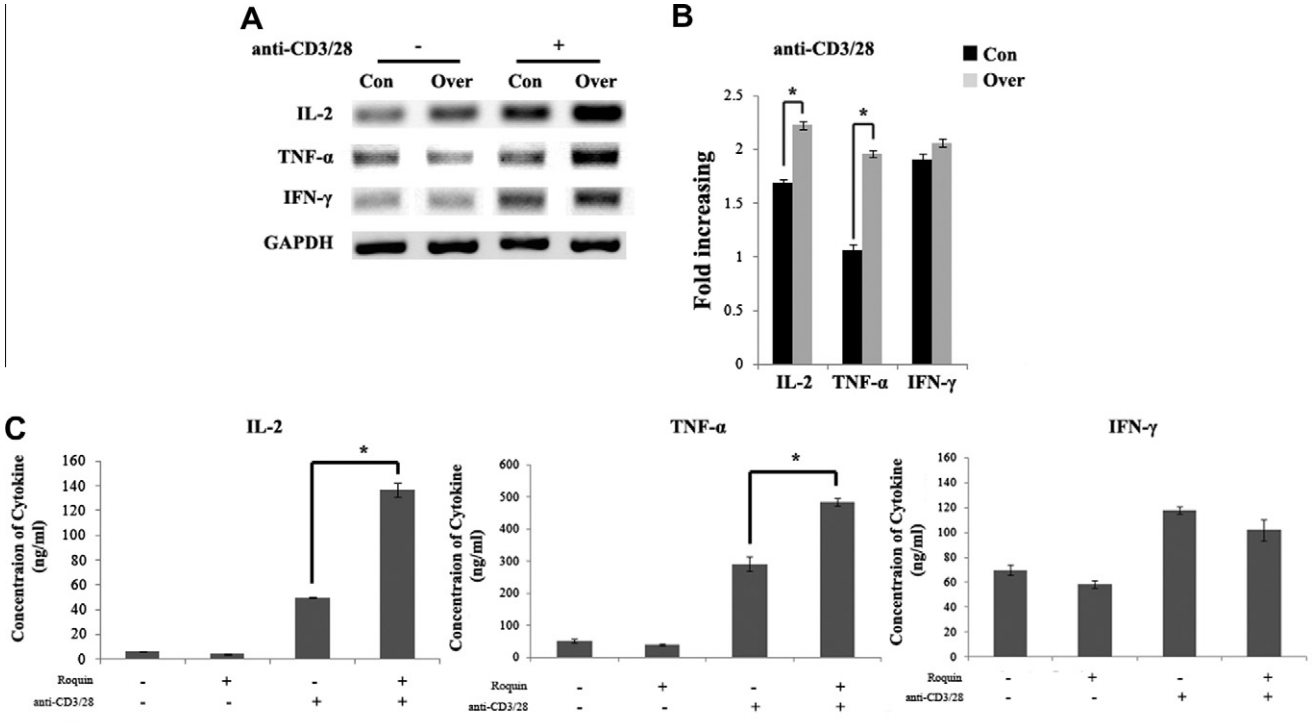

Fig. 2. The transcriptional activity and secretion of various cytokines after T-cell activation via CD3/CD28 *in vitro*. (A) Cytokine expression was detected by reverse-transcription PCR. Roquin-overexpressing EL-4 cells were treated with anti-CD3 (1 μg/ml) and anti-CD28 (4 μg/ml) for 24 h to activate T-cells. (–), no treatment; (+), treatment with anti-CD3/CD28; Con, non-transfected EL-4 cells; Over, Roquin-overexpressing cell line. The right panel shows the data from the left panel graphed using ImageJ software. The value shown represents the mean ± SD of three independent experiments. $^{*}P < 0.001$. (B) Various cytokines were detected by ELISA in anti-CD3/CD28-stimulated EL-4 cells. Each value represents the mean ± SD of three independent experiments. $^{*}P < 0.001$.

overexpressing EL-4 cells was significantly increased in comparison to control cells, which was unexpected (Fig. 1C; $^{*}P < 0.01$). To investigate the regulation of CD28 and ICOS by Roquin during T-cell activation, Roquin overexpressing EL-4 cells were treated with anti-CD3/CD28, anti-CD3/ICOS, or PMA/ionomycin, and IL-2 secretion was measured. IL-2 secretion by Roquin overexpressing EL-4 cells decreased significantly after stimulation with anti-CD3/ICOS. In contrast, IL-2 secretion by Roquin overexpressing EL-4 cells increased significantly after stimulation with anti-CD3/CD28. However, treatment with PMA/ionomycin, which acts through a TCR/CD28-independent pathway, did not affect IL-2 secretion in Roquin overexpressing cells (Fig. 1D; $^{*}P < 0.01$). These results demonstrate that Roquin overexpression affects T-cell activation in a TCR/CD28-dependent manner by upregulating CD28 expression.

3.2. Roquin overexpression regulates cytokine expression and secretion induced by CD3/CD28 engagement

The expression and secretion of various cytokines by Roquin overexpressing EL-4 cells were analyzed. We used RT-PCR to determine the transcription levels of various cytokines in Roquin overexpressing EL-4 cells after stimulation with anti-CD3/CD28. The IL-2 and TNF-α mRNA levels increased significantly after stimulation with anti-CD3/CD28, but the IFN-γ levels did not (Fig. 2A and B; $^{*}P < 0.001$). To determine the effect of Roquin on pro-inflammatory cytokine secretion *in vitro*, we used an ELISA to measure the cytokine levels in the supernatants from EL-4 cells stimulated with anti-CD3/CD28 for 24 h. The levels of the pro-inflammatory cytokines IL-2 and TNF-α in the cell culture supernatants were increased significantly in Roquin overexpressing EL-4 cells compared to control cells (Fig. 2B; $^{*}P < 0.001$). These data show that Roquin overexpression affects cytokine secretion.

3.3. Roquin may modulate T-cell activation via the AKT and JNK signaling pathways

To identify any modulatory effects that Roquin has on signal transduction, we activated cells with anti-CD3/CD28 for 24 h and observed the phosphorylation of the signaling kinases AKT, JNK, and ERK, all of which are involved in signal transduction after T-cell activation [9]. Prior to activation, the levels of phospho-AKT, phospho-JNK, and phospho-Erk in Roquin overexpressing and control

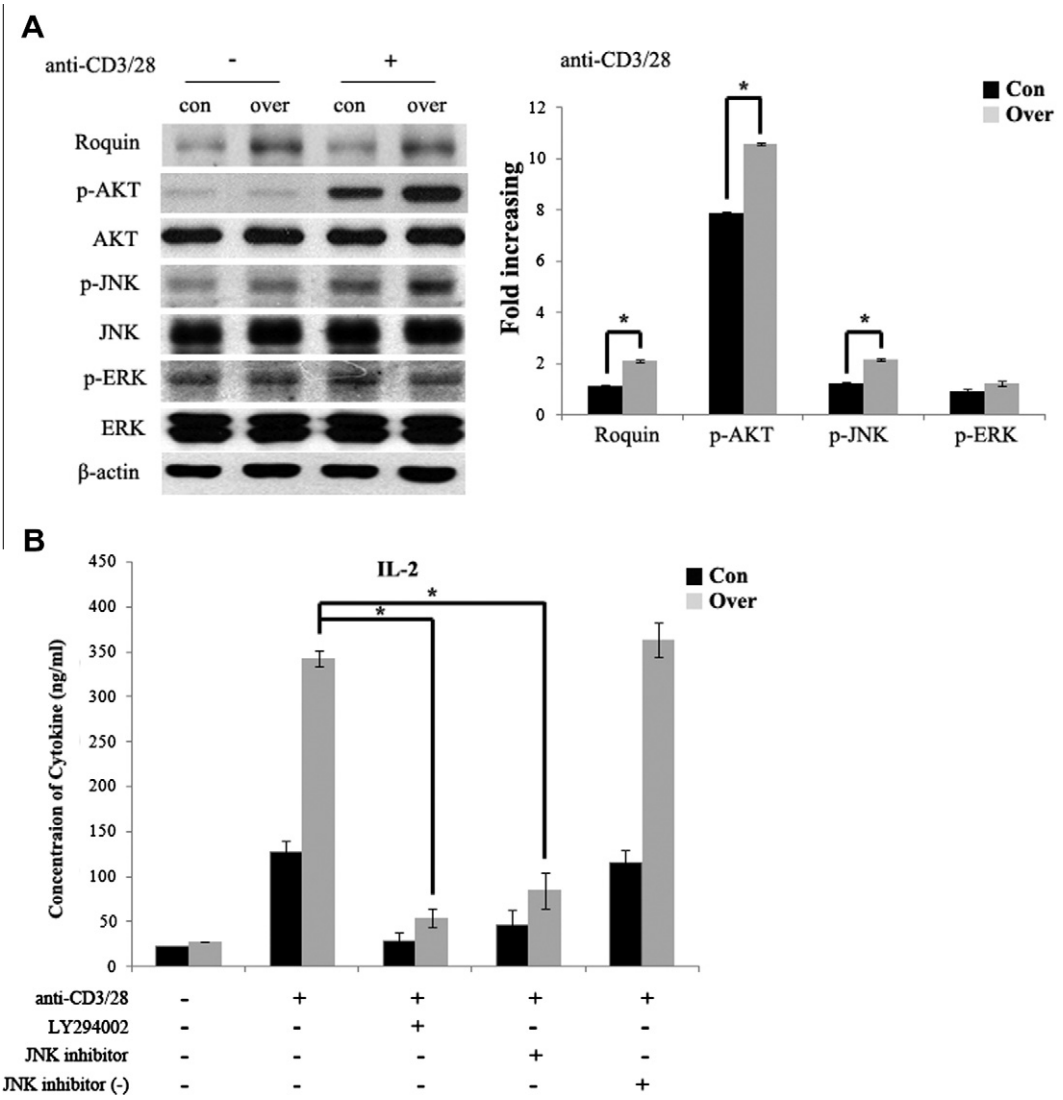

Fig. 3. The modulation of T-cell activation signaling by Roquin after CD3/CD28 stimulation *in vitro*. (A) The modulation of Roquin as examined by Western blot. Lanes 1 and 3, non-transfected EL-4 cells; lanes 2 and 4, EL-4 cells overexpressing Roquin; lanes 1 and 2, unstimulated cells; lanes 3 and 4, cells stimulated with anti-CD3/CD28. The right panel shows data from the left panel that was graphed using ImageJ software. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.001$. (B) The secretion of IL-2 from cells activated with anti-CD3/CD28 and subsequently treated with AKT and JNK inhibitors was determined by ELISA. Con, non-transfected EL-4 cells; Over, Roquin-overexpressing cell line. The data shown are the mean values of at least three individual experiments. $^{*}P < 0.001$.

EL-4 cells did not differ. After anti-CD3/CD28 treatment, the levels of phospho-AKT and phospho-JNK were significantly increased in Roquin overexpressing EL-4 cells compared to control cells (Fig. 3A; $^{*}P < 0.001$), while the phospho-ERK level did not change. Next, we treated Roquin overexpressing EL-4 cells with AKT and JNK inhibitors. After treating the control and Roquin overexpressing EL-4 cells with these inhibitors, we measured IL-2 production by ELISA. Roquin overexpressing cells treated with AKT and JNK inhibitors produced significantly lower levels of IL-2 than control cells treated with AKT and JNK inhibitors (Fig. 3B; $^{*}P < 0.001$). These results suggest that Roquin overexpression may modulate T-cell activation via the AKT and JNK signal transduction pathways.

3.4. The expression of T-cell co-stimulatory molecules and cytokine secretion in activated T-cells is regulated by Roquin

To examine the effects of Roquin overexpression on T-cells *in vivo*, we used a plasmid vector containing an HA-tagged Roquin transgene under the control of the T-cell-specific hCD2 promoter to generate transgenic (Tg) mice that specifically overexpress Roquin in T-cells (Fig. 4A). Roquin-Tg mice were identified by PCR screening of total genomic DNA extracted from the biopsied tails of founder candidates. Two mice showing insertion of the transgene into their genomic DNA were selected (Fig. 4B). The founder mice were mated with C57BL/6 mice, and all of the pups produced offspring carrying the transgene. We confirmed western blot by anti-HA antibody in spleen, sorted T-cells, non-T-cells from Roquin-Tg mice. As a results, spleen and sorted T-cells were detected HA positive, but not non-T-cells (Fig. 4C).

Real-time PCR was performed to examine the regulation of CD28 and ICOS expression by Roquin-Tg mice $CD4^{+}$ T-cells. With activation by anti-CD3/CD28 treatment, ICOS mRNA expression decreased and CD28 expression increased significantly in $CD4^{+}$ T-cells from Roquin-Tg mice compared with $CD4^{+}$ T-cells of WT mice (Fig. 4D; $^{*}P < 0.001$).

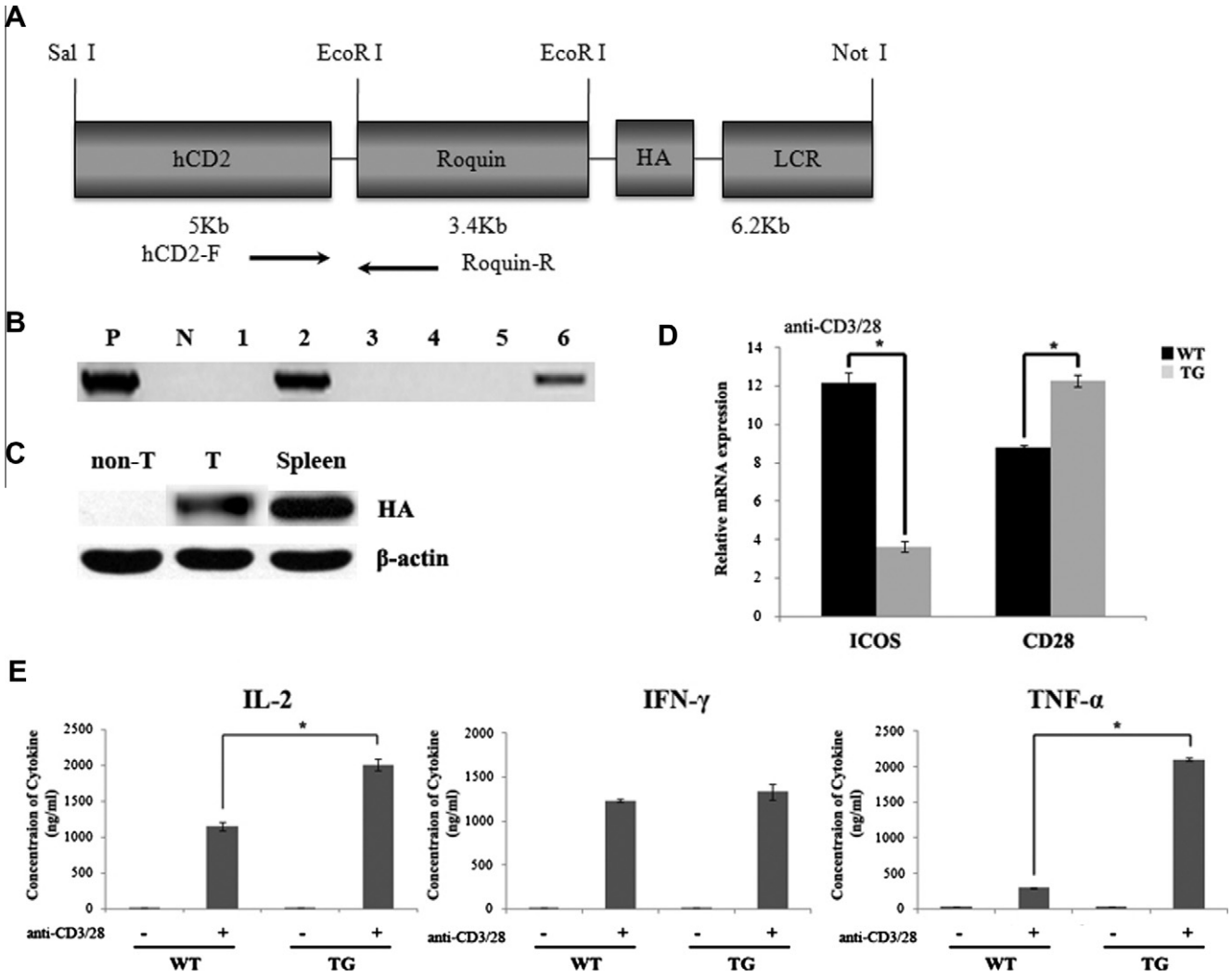

Fig. 4. The modulation of T-cell activation-induced CD28 expression and cytokine secretion by Roquin overexpression *ex vivo*. (A) The transgenic construct used to achieve T-cell-specific expression of the Roquin gene. The primers used for the tail biopsy PCR are indicated by arrows. (B) Identification of transgenic mice harboring the recombinant hCD2-Roquin gene. Two mice were determined to be positive by PCR and used as founders. P, positive control; N, negative control. (C) T-cell-specific expression of Roquin was determined by probing a Western blot with an anti-HA antibody. Spleen tissue, $CD4^{+}$ T-cells and non-T-cells were isolated from Roquin Tg mice. $CD4^{+}$ T-cells were prepared using a $CD4^{+}$ T-cell isolation kit and the remaining cells were classified as non-T-cells. β-actin was used as a loading control. (D) The expression levels of ICOS and CD28 in $CD4^{+}$ T-cells from naïve C57BL/6 mice and Roquin transgenic mice after stimulation with anti-CD3/CD28 for 24 h were determined by real-time PCR. The expression levels of ICOS and CD28 were normalized to GAPDH. WT, wild-type mice (black bar); TG, Roquin transgenic mice (gray bar). Error bars indicate the SD of triplicates. $^{*}P < 0.001$. (E) $CD4^{+}$ T-cells from WT and Tg mice were cultured for 72 h in the presence of anti-CD3/CD28, and the culture supernatant was analyzed for cytokine content using antibody arrays. (–), no treatment; (+), treatment with anti-CD3/CD28; WT, wild-type mice; TG, Roquin transgenic mice. Data represent the mean ± SD of three independent experiments. * $P < 0.001$.

To investigate the effects that Roquin has on $CD4^+$ T-cell cytokine secretion, we measured the levels of pro-inflammatory cytokines using ELISA. $CD4^+$ T-cells from Roquin-Tg spleens expressed high levels of Roquin mRNA compared with cells from WT mice, indicating transgene activity (data not shown). IL-2 was also highly expressed in $CD4^+$ T-cells from Roquin-Tg mice compared with cells from WT mice. In addition, stimulating $CD4^+$ T-cells from Roquin-Tg mice with anti-CD3/CD28 increased the level of the pro-inflammatory cytokine TNF-α in comparison to control cells (Fig. 4E; $^*P < 0.001$). The secretion of IFN-γ did not change upon T-cell activation. These results indicate that Roquin overexpression regulates the secretion of pro-inflammatory cytokines from $CD4^+$ T-cells activated by stimulation with anti-CD3/CD28.

4. Discussion

In the present study, IL-2 production and T-cell activation were enhanced by Roquin overexpression. The importance of IL-2 as a key cytokine for T-cell activation, proliferation, and the immune response has extensive experimental support [10]. The binding of IL-2 to its receptor was initially demonstrated to be critical for inducing T-cell proliferation *in vitro* [11], and IL-2 functions as an auto- and paracrine growth factor during the first 48–72 h of T-cell activation. The abnormal control of IL-2 production is known to affect many immune cell functions in addition to those directly related to regulatory T-cells [12]. Therefore, it is likely that Roquin participates in the regulation of IL-2 secretion and T-cell activation in the immune response or in autoimmune diseases.

To determine the magnitude of the cytokine response caused by Roquin overexpression during T-cell activation, we measured the cytokine levels in supernatants from $CD4^+$ T-cell and EL-4 cultures. As a pro-inflammatory cytokine, TNF-α plays an important role in the immune response to infections and cancer and has been shown to function in the regulation of inflammation [13]. Our study demonstrates that Roquin overexpression increases TNF-α production.

The TCR and CD28 stimulate independent signaling pathways that can be distinguished based on their biophysical and biochemical characteristics. Nevertheless, the TCR and CD28 signaling pathways share many downstream signal transducers [14], thus making it difficult to differentiate the effects of the two independent pathways. Analysis of the CD28-dependent signaling pathways in primary $CD4^+$ T-cells in a TCR-independent manner revealed that the PI3K and NF-κB pathways were the most relevant biochemical targets of CD28 [15–17]. The TCR/CD3 and CD28 have unique roles in the activation of the MEK1/2- and PI3K/AKT-dependent pathways [18]. Our results show that phospho-AKT and phospho-JNK are induced in Roquin overexpressing EL-4 cells and that phospho-ERK is not regulated by Roquin. We plan to further study the role of Roquin overexpression in the modulation of the TCR and CD28 signaling pathways.

ICOS was identified by the generation of monoclonal antibodies against activated human T-cells [19]. The establishment of ICOS knockout mice exhibiting defective B-cell responses, altered immunoglobulin class switching, and the absence of germinal centers in secondary lymphoid follicles has increased interest in ICOS [20,21]. T-cells activated through TCR engagement upregulate ICOS, encouraging T-cell proliferation and IL-4 and IL-10 production [21]. The role of ICOS in T-cell development and maintenance was initially reported in sanroque mice, which possess a single recessive mutation in the Roquin gene [22]. Sanroque mice develop severe autoimmune disease and form spontaneous germinal centers enriched in mature T-cells. A previous study showed that Roquin induced the degradation of ICOS mRNA in T-cells through its repressor activity [7]. In general, naïve or primary T-cell responses are more dependent on CD28 than secondary responses. ICOS expression is restricted to activated and memory T-cells. Therefore, we used naïve $CD4^+$ T-cells to ensure that our results accurately reflected the effects that Roquin overexpression has on CD28 signaling. In the future, we will study how Roquin overexpression affects activated or memory T-cells generated by immunization.

Thus, we propose to examine the mechanisms by which Roquin regulates ICOS and IL-2. A previous study has shown that anti-CD3/CD28 stimulation has a greater effect on T-cell activation and IL-2 secretion than anti-CD3/ICOS stimulation. Based on our results, increased IL-2 production may positively regulate the immune response rather than degrade ICOS, as IL-2 is secreted prior to ICOS degradation. This hypothesis will be further explored *in vivo* to help clarify the role of Roquin in the immune response and autoimmune mechanisms.

In summary, we generated Tg mice expressing a Roquin transgene driven by a T-cell-specific promoter. Roquin overexpression regulated the activation and proliferation of $CD4^+$ T-cells and modulated the secretion of pro- and anti-inflammatory cytokines. Furthermore, based on our *in vitro* results, Roquin regulated the transcription and secretion of cytokines as well as the phosphorylation of AKT and JNK. The present report describes the role of Roquin in T-cell activation and proliferation and its possible involvement in immune diseases.

Acknowledgments

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References

[1] L.M. Coussens, Z. Werb, Inflammatory cells and cancer: think different!, J Exp. Med. 193 (2001) F23–F26.
[2] Y.T. Teng, Protective and destructive immunity in the periodontium: Part 2–T-cell-mediated immunity in the periodontium, J. Dent. Res. 85 (2006) 209–219.
[3] H. Schorle, T. Holtschke, T. Hunig, A. Schimpl, I. Horak, Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting, Nature 352 (1991) 621–624.
[4] T. Taniguchi, Y. Minami, The IL-2/IL-2 receptor system: a current overview, Cell 73 (1993) 5–8.
[5] K.A. Smith, T-cell growth factor, Immunol. Rev. 51 (1980) 337–357.
[6] D.M. Willerford, J. Chen, J.A. Ferry, L. Davidson, A. Ma, F.W. Alt, Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment, Immunity 3 (1995) 521–530.
[7] D. Yu, A.H. Tan, X. Hu, V. Athanasopoulos, N. Simpson, D.G. Silva, A. Hutloff, K.M. Giles, P.J. Leedman, K.P. Lam, C.C. Goodnow, C.G. Vinuesa, Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA, Nature 450 (2007) 299–303.
[8] C.F.A.L.E. Hogan B, Microinjection of DNA into pronuclei. (1986).
[9] M.L. Kemp, L. Wille, C.L. Lewis, L.B. Nicholson, D.A. Lauffenburger, Quantitative network signal combinations downstream of TCR activation can predict IL-2 production response, J. Immunol. 178 (2007) 4984–4992.
[10] M.A. Burchill, J. Yang, K.B. Vang, M.A. Farrar, Interleukin-2 receptor signaling in regulatory T cell development and homeostasis, Immunol. Lett. 114 (2007) 1–8.
[11] M. Hatakeyama, M. Tsudo, S. Minamoto, T. Kono, T. Doi, T. Miyata, M. Miyasaka, T. Taniguchi, Interleukin-2 receptor beta chain gene: generation of three receptor forms by cloned human alpha and beta chain cDNA's, Science 244 (1989) 551–556.
[12] J.C. Crispin, G.C. Tsokos, Transcriptional regulation of IL-2 in health and autoimmunity, Autoimmun. Rev. 8 (2009) 190–195.
[13] A.K. Abbas, K.M. Murphy, A. Sher, Functional diversity of helper T lymphocytes, Nature 383 (1996) 787–793.
[14] O. Acuto, F. Michel, CD28-mediated co-stimulation: a quantitative support for TCR signalling, Nat. Rev. Immunol. 3 (2003) 939–951.

[15] L. Tuosto, A. Costanzo, F. Guido, B. Marinari, S. Vossio, F. Moretti, M. Levrero, E. Piccolella, Mitogen-activated kinase kinase kinase 1 regulates T cell receptor- and CD28-mediated signaling events which lead to NF-kappaB activation, Eur. J. Immunol. 30 (2000) 2445–2454.

[16] B. Marinari, A. Costanzo, A. Viola, F. Michel, G. Mangino, O. Acuto, M. Levrero, E. Piccolella, L. Tuosto, Vav cooperates with CD28 to induce NF-kappaB activation via a pathway involving Rac-1 and mitogen-activated kinase kinase 1, Eur. J. Immunol. 32 (2002) 447–456.

[17] E. Piccolella, F. Spadaro, C. Ramoni, B. Marinari, A. Costanzo, M. Levrero, L. Thomson, R.T. Abraham, L. Tuosto, Vav-1 and the IKK alpha subunit of I kappa B kinase functionally associate to induce NF-kappa B activation in response to CD28 engagement, J. Immunol. 170 (2003) 2895–2903.

[18] L.J. Appleman, A.A. van Puijenbroek, K.M. Shu, L.M. Nadler, V.A. Boussiotis, CD28 costimulation mediates down-regulation of p27kip1 and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells, J. Immunol. 168 (2002) 2729–2736.

[19] A. Hutloff, A.M. Dittrich, K.C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, R.A. Kroczek, ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28, Nature 397 (1999) 263–266.

[20] C. Dong, A.E. Juedes, U.A. Temann, S. Shresta, J.P. Allison, N.H. Ruddle, R.A. Flavell, ICOS co-stimulatory receptor is essential for T-cell activation and function, Nature 409 (2001) 97–101.

[21] C. Dong, U.A. Temann, R.A. Flavell, Cutting edge: critical role of inducible costimulator in germinal center reactions, J. Immunol. 166 (2001) 3659–3662.

[22] C.G. Vinuesa, M.C. Cook, C. Angelucci, V. Athanasopoulos, L. Rui, K.M. Hill, D. Yu, H. Domaschenz, B. Whittle, T. Lambe, I.S. Roberts, R.R. Copley, J.I. Bell, R.J. Cornall, C.C. Goodnow, A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity, Nature 435 (2005) 452–458.