Acetylation of GATA-3 affects T-cell survival and homing to secondary lymphoid organs

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Acetylation of a transcription factor has recently been shown to play a significant role in gene regulation. Here we show that GATA-3 is acetylated in T cells and that a mutation introduced into amino acids 305–307 (KRR-GATA3) creates local hypoacetylation in GATA-3. Remarkably, KRR-GATA3 possesses the most potent suppressive effect when compared with other mutants that are disrupted in putative acetylation targets. Expressing this mutant in peripheral T cells results in defective T-cell homing to systemic lymphnodes, and prolonged T-cell survival after activation. These findings have significant implications in that the acetylation state of GATA-3 affects its physiological function in the immune system and, more importantly, provides evidence for the novel role of GATA-3 in T-cell survival and homing to secondary lymphoid organs.

Keywords: acetylation/AICD/GATA-3/T-cell homing

Introduction

Acetylation of histones is a characteristic feature of transcriptionally active chromatin. Acetylation of lysine residues in the N-terminus tails of histones facilitates gene activation, presumably by reducing histone tail affinity for DNA and thereby promoting the binding of transcription factors to nucleosomal DNA (Brownell and Allis, 1996; Grunstein, 1997; Struhl, 1998). A number of proteins that possess such histone acetyltransferase (HAT) activity include GCN5, P/CAF, CBP/p300, TAF_{II}250, SRC-1, ACTR and Tip60 (Bannister and Kouzarides, 1996; Brownell *et al.*, 1996; Kuo *et al.*, 1996; Mizzen *et al.*, 1996; Ogryzko *et al.*, 1996; Yang *et al.*, 1996; Chen *et al.*, 1997; Yamamoto and Horikoshi, 1997). Interestingly, recent studies demonstrate that these acetyltransferases have substrates other than nucleosomal histones. CBP/p300 were shown to acetylate tumor suppressor protein p53 (Gu and Roeder, 1997), the erythroid Kruppel-like factor (EKLf) (Zhang and Bieker, 1998), the basal transcription factors, TFIIE, TFIIF (Imhof *et al.*, 1997) and recently reported GATA-1 (Boyes *et al.*, 1998; Hung *et al.*, 1999). p53 is also acetylated by P/CAF at a lysine residue different from that which is acetylated by p300

(Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). The discovery that HATs acetylate substrates other than histones has generated increased interest in the role of acetylation in regulation of gene expression. However, the physiological significance of acetylating non-histone substrates remains an open question.

GATA-3 is the third member of the GATA family of proteins whose expression is abundant in a number of distinct sites during development, including the kidney, central and peripheral nervous system, and T-cell compartment (George *et al.*, 1994). Mice deficient in GATA-3 exhibit multiple physiological abnormalities during development and die on embryonic day 12 (Pandolfi *et al.*, 1995). Along with T-cell ontogeny, GATA-3 is detected in a thymic rudiment from embryonic day 12.5 and is expressed throughout mature peripheral T cells (Oosterwegel *et al.*, 1992; Hattori *et al.*, 1996). Implication for the significance of GATA-3 in T-cell development and function has been reported by identification of binding sites for GATA-3 in the TCR- α , - β and - δ enhancers (Leiden, 1993), the CD8 α promoter/enhancer (Landry *et al.*, 1993), the CD4 enhancer (Wurster *et al.*, 1994), the interferon- γ promoter (Penix *et al.*, 1993) and in the IL-5 promoter (Siegel *et al.*, 1995; Yamagata *et al.*, 1995, 1997; Lee *et al.*, 1998; Zhang *et al.*, 1998). Indeed, a subsequent null mutation analysis with RAG2^{-/-} mice reconstituted with GATA-3^{-/-} ES demonstrated that GATA-3^{-/-} T-cell development is blocked at the earliest double-negative (CD4⁻/CD8⁻) stage, affirming its essential role in early T-cell development (Ting *et al.*, 1996). Most recently, a pivotal role of GATA-3 in CD4⁺ T helper 2 (Th2) differentiation has been demonstrated (Zhang *et al.*, 1997; Zheng and Flavell, 1997; Ouyang *et al.*, 1998, 2000). However, direct assessment of the roles of GATA-3 *in vivo* has been precluded by the early differentiation block of GATA-3^{-/-} T cells, and thus the role of GATA-3, especially at the physiological level, still remains to be investigated.

The KRR mutant of GATA-3 (KRR-GATA3) was originally discovered to be a dominant-negative form of GATA-3 (Smith *et al.*, 1995). In this mutant, the lysine-arginine-arginine (KRR) residues at amino acids 305–307 in GATA-3 are substituted with alanines (KRR→AAA). The validity of using this molecule in probing the role of GATA-3 has been substantiated by recent studies in the Th2 immune response (Ouyang *et al.*, 1998; Zhang *et al.*, 1999). However, to date, neither the molecular event provoked by this mutation nor the mechanism of the dominant-negative effect has been uncovered. In this report, we have shown that the KRR mutation renders the hypoacetylated form of GATA-3, not only by disrupting its own lysine, but also by inhibiting acetylation of neighboring lysine residues. Expressing KRR-GATA3 in peripheral T cells disclosed

an unexpected role of GATA-3 in T-cell homing and survival. Our results provide evidence that acetylation of a transcription factor has bona fide physiological significance through affecting expression of the relevant endogenous genes. These findings have important implications for current views regarding the molecular mechanisms by which acetylated transcription factors affect biological outcome *in vivo*. In addition, our study revealed a non-Th2-related role for GATA-3, which is, as yet, unexplored due to the embryonic lethality and thymic developmental arrest in aforementioned null mutation models.

Results

KRR is the most critical acetylation motif in GATA-3

Recent studies have reported the acetylation of GATA-1 near the second zinc finger domain upon interaction with CBP/p300 (Blobel *et al.*, 1998; Boyes *et al.*, 1998; Hung *et al.*, 1999). Among the seven acetylated lysines in GATA-1, six lysines and neighboring amino acids are well conserved in GATA-3, strongly suggesting GATA-3 is a target of acetyltransferase activities (Figure 1A). Since the final functional output of a transcription factor is to regulate gene transcription, the significance of acetylation may as well be investigated in terms of its transcriptional relevance. To compare the functional contribution of the six acetylation-target motifs we prepared five mutants, in addition to the KRR mutant, whose acetylation-target-lysines and adjacent arginines are substituted with alanines (Figure 1A), and examined their effect on a GATA-reporter plasmid. These mutations did not affect their DNA-binding (data not shown). While each mutant showed somewhat lower transactivation compared with wild-type GATA-3, the M2 mutant (mut-KRR) showed the lowest transactivation of all (Figure 1B). We next investigated how these mutants affect transactivation by wild-type GATA-3. While mutants M1 and M3 had minimal suppressive effect, and mutants M4 and M6 had no additive effect on transactivation by wild-type GATA-3, M2 (mut-KRR) strongly suppressed it (Figure 1C). These results indicate that the lysine-arginine-arginine at position 305–307 is the most transcriptionally relevant acetylation motif among the six potential acetylation sites. The KRR mutant (M2) completely loses transcriptional synergism with p300, indicating that the transcriptional enhancement conferred by p300 interaction is completely shut off by this mutation (Figure 1D). To provide evidence that GATA-3 is acetylated physiologically, we performed an *in vivo* acetylation assay. The [¹⁴C]acetate labeling of mouse splenocytes, stimulated or unstimulated with ConA, followed by immunoprecipitation with antiserum against GATA-3 disclosed the *in vivo* acetylation of GATA-3 (Figure 1E). Intriguingly, the ConA stimulation had no effect on acetylation of GATA-3. These results indicate that GATA-3 is actually acetylated *in vivo* and that the KRR motif has a crucial role in GATA-3 as a transcriptional regulator.

KRR mutation reduces acetylation and transactivation of GATA-3

To investigate directly whether the KRR motif is acetylated by p300 and how this mutation affects acetylation of other lysine residues, we performed an *in vitro* acetylation assay using glutathione *S*-transferase (GST) fusion proteins as substrates. As shown in Figure 2B, GST fusion protein containing wild-type GATA-3 (amino acids 289–357) was strongly labeled by [¹⁴C]acetyl-CoA (Figure 2B, Wild). To evaluate acetylation for Lys305 (K in KRR), we made a mutant with single amino acid substitution at K305 (mut K305A). This mutation caused ~25% reduction in acetylation compared with the wild type (Wild versus K305A). Conversely, the fusion protein harboring both M1 and M3 mutations (M13) showed 75% reduction in acetylation. These results indicate that K305 is actually acetylated by p300, and that K293 and K347 are also the targets of acetylation by p300. Surprisingly, acetylation of the fusion protein with KRR mutation (M2) was drastically reduced, up to 75%, compared with the wild type. These results indicate that while K305A mutation simply disrupts acetylation at K305, KRR mutation not only disrupts acetylation at K305 but also drastically reduces acetylation of the neighboring lysines to create local hypoacetylation in GATA-3. To see if acetylation status correlates with transactivation, we performed a reporter assay with GATA-3 mutants harboring these mutations. As shown in Figure 2C, the mutants that showed a decrease in acetylation concomitantly showed a decrease in transactivation. Thus, the acetylation state correlates with the transactivation ability of GATA-3. We conclude that the potent negative form rendered by the KRR mutation is due to acetylation cancellation at K305 and acetylation inhibition in the neighboring lysines.

Expressing KRR-GATA3 in late T-cell specific fashion by the Lck distal promoter

To date, the physiological significance of transcription factor acetylation is unresolved. To explore the *in vivo* effect of the GATA-3 hypo-acetylated form, we expressed KRR-GATA3 in late T-cell-specific fashion by the Lck distal-promoter cassette. Four lines of founder mice were obtained and northern blot analysis of the transgene was shown for two of the four lines (Figure 3B). Both lines showed strong expression of the 3.2 kb transgenic mRNA in spleen (Figure 3B, lanes 1 and 3), while only the 4.0 kb endogenous GATA-3 mRNA was observed in thymus (lanes 5–8). This is consistent with the expected expression pattern driven by the Lck distal-promoter cassette (Wildin *et al.*, 1995; Hashimoto *et al.*, 1996). The protein expression was verified by an electrophoretic mobility shift assay (EMSA). Splenocytes from both lines of mice expressed relatively high amount of GATA-binding protein compared with the wild-type splenocytes (Figure 3C, lanes 1–3). These shifted bands were demonstrated to be GATA-3 by a supershift experiment using anti-GATA-3 specific antiserum (lanes 4–6). Binding affinity for the EMSA probe was shown to be equal between wild-type GATA-3 and KRR-GATA3 (Figure 3D). A previous study showed that KRR-GATA3 inhibits development of asthma by suppressing the production of Th2 cytokines (Zhang *et al.*, 1999). In line with this, we also confirmed diminished production of

Fig. 1. KRR is the most transcriptionally relevant acetylation motif in GATA-3. **(A)** Conserved acetylation-target motifs in GATA-1 and GATA-3. Six of the seven acetylated lysines in GATA-1 are conserved in GATA-3 (boxed). These motifs were disrupted by alanine substitutions to generate mutants M1 to M6. M2 corresponds to the KRR mutation. **(B)** The effects of the alanine mutations on GATA-mediated transactivation. Jurkat cells were transfected with the GATA reporter plasmid (6xGATA-tk-Luci) and the expression vector (pSSR α) containing wild-type GATA-3 or each GATA-3 mutant M1 to M6 (pSSR α -M1 to M6). Cells were harvested 48 h after transfection and luciferase activity was determined. **(C)** The effect of mutants on transactivation by wild-type GATA-3. Jurkat cells were transfected with 6xGATA-tk-Luci and wild-type GATA-3 along with each GATA-3 mutant. The equal amounts (3 μ g) of wild-type GATA-3 plasmid and the mutant plasmids were transfected to see the dominant-negative effect. **(D)** KRR-GATA3 loses its ability to cooperate with p300. Jurkat cells were transfected with 6xGATA-tk-Luci in the presence or absence of wild-type or KRR GATA3, along with the increasing amount of p300 expression plasmid pCMV β -p300 (0, 2, 4 and 8 μ g for each assay). **(E)** *In vivo* acetylation of GATA-3 in T cells. Mouse splenocytes, stimulated or unstimulated with ConA, were pulse-labeled with [¹⁴C]acetate for 3 h, washed with PBS and subjected to mechanical disruption by sonicator. The soluble cell extract was immunoprecipitated with antiserum against GATA-3 (G3N5) or pre-immune serum, respectively, and resolved by SDS-PAGE.

IL-4 and -5 (Figure 3E), which validates the *in vivo* function of KRR-GATA3 in our mice. The following experiments were mostly performed for one line and confirmed by duplication in another independent line.

Altered T-cell population and distribution in KRR mice
Analysis of wild-type and KRR thymocytes showed almost indistinguishable change in cell number and

Fig. 2. KRR mutation abrogates acetylation and transactivation. (A) Schematic representation of the GST fusion proteins prepared. 'x' indicates the site of acetylation motif disrupted, as in Figure 1A. M13 has both M1 and M3 mutations. In K305A, only K of KRR motif was substituted with A. M2 corresponds to KRR mutation. M123 has all M1+M2+M3 mutations, which is expected to have no target lysine to incorporate ¹⁴C-labeled CH₃CO⁺ residue. (B) *In vitro* acetylation of KRR motif and its effect on adjacent lysines. An equal amount (2.5 μg) of the purified GST fusion proteins were incubated with 100 ng of purified FLAG-p300(1194–1812) and [¹⁴C]acetyl-CoA for 1 h at 30°C, and the reaction mixtures were separated by SDS-PAGE, fixed and stained with Coomassie Brilliant Blue (CBB). The stained gel was dried and ¹⁴C incorporation was visualized using a BAS2000 image analyzer. The amount of incorporated [¹⁴C]acetate for each fusion protein was plotted on a bar chart. (C) Transactivation by GATA-3-mutants harboring mutations in (A). Jurkat cells were transfected with 6xGATA-tk-Luc and 3 μg of each GATA-3-mutant plasmid. Data represents the relative fold induction of each mutant compared with wild-type GATA-3.

CD4:CD8 ratio (Figure 4A), indicating that the differentiation process is not disturbed in KRR mice. In contrast, there was apparent enlargement of spleen in KRR mice. Splenic cell counts showed an increase in total cell number (Table I), and flow cytometric analysis revealed increased T cell:B-cell ratios in KRR mice spleen (0.61 in wild type versus 1.04 in KRR; Figure 4A). The calculated alteration in cell number was an absolute increase in CD3⁺ cells with relatively a small change in B220⁺ cells (Figure 4B). Of the CD3⁺ cells, increase in CD4⁺ cells (2- to 3-fold) was more prominent than in CD8⁺ cells (1.5-fold) (Figure 4C). Analysis with a set of antibodies against different TCR Vβ

Fig. 3. Generation of the KRR-GATA3 mice driven by the Lck distal-promoter cassette. (A) Schematic diagram of the injected transgene fragment. (B) Specific expression of KRR-GATA3 in mature peripheral T cells. Poly A⁺ RNA (10 μg) from thymus and spleen of KRR and litter-mate control mice was hybridized with mouse GATA-3 probe and autoradiographed. Results of the two independent lines of transgenic mice were shown. (C) Detection of KRR-GATA3 protein in the nuclear extract of KRR mice splenocytes. Nuclear extracts from control or two independent lines of KRR mice were incubated with isotope-labeled DNA fragment from TCRα enhancer containing consensus GATA sequence, and subjected to EMSA. Nuclear extracts were also incubated with anti-GATA-3 antiserum (G3N5). The DNA-protein complexes and the supershifted complexes are indicated by arrows. (D) Binding of wild-type and KRR GATA3 to the EMSA probe. COS cells were transfected with either pSSRα-GATA3 or pSSRα-KRR-GATA3, and the nuclear extract from each transfectant was subjected to EMSA as in (C). The amount of protein was equalized with anti-GATA-3 blot shown below. (E) Diminished Th2 cytokine production in KRR T cells. Spleen cells were stimulated with anti-CD3 (10 μg/ml) for times indicated, and the production of IL-4 and IL-5 in the culture supernatant was measured using ELISA (Amersham).

subclasses demonstrated that the T-cell expansion is polyclonal (data not shown).

In contrast to the enlarged spleen in KRR mice, the peripheral lymphnodes (PLN; inguinal, axillary, cervical) and mesenteric lymphnodes (MLN) of KRR mice were visibly smaller than those of normal controls. Accordingly, the average number of resident lymphocytes per lymphnode was significantly lower (50–80%) in KRR mice

Fig. 4. Altered T-cell count and distribution in KRR mice. The statistical significance of wild-type versus homozygous Tg was calculated using the Student's *t*-test; *P* values are indicated above each graph. (A) Flow cytometric analyses of thymocytes, splenocytes and lymphnode cells from wild-type and homozygous KRR mice. (B) Increased CD3⁺ T cell in spleens of KRR mice. Results of wild-type (−/−), hemizygous (−/KRR) and homozygous (KRR/KRR) mice are shown. The bars represent mean values (±SD) determined from five mice for each group. (C) Increased CD4⁺ and CD8⁺ cell counts in spleens of KRR mice. (D) Decreased cell counts in LN and PP of KRR mice. Single-cell suspensions were prepared from peripheral LN (PLN; cervical, inguinal, axillary), mesenteric LN (MLN) and Peyer's patches (PP). (E) Decreased CD4⁺ and CD8⁺ cell counts in KRR lymphnodes.

compared with litter-mate controls (Figure 4D). FACS analysis revealed that the reduction is mostly due to a decrease in CD3⁺ T cells. Among T cells, decrease in CD8⁺ cells was more prominent than that of CD4⁺ cells

(Figure 4A and E). KRR mice also exhibited overall hypoplasia of Peyer's patches (PP), with a 40% reduction in the number of PP >1 mm in diameter (Table II). As for the peripheral blood of KRR mice, there was consistent

Table I. Cell counts in spleen and peripheral blood

Total spleen cell counts ($\times 10^7$)			
Genotype	6 week	10 week	16 week
–/–	4.5 $\pm$ 0.39	7.7 $\pm$ 0.63	8.0 $\pm$ 0.91
–/KRR	6.2 $\pm$ 0.60	10.2 $\pm$ 0.82	10.9 $\pm$ 0.98
KRR/KRR	8.1 $\pm$ 0.65 ($P < 0.001$)	12.6 $\pm$ 1.08 ($P < 0.001$)	13.9 $\pm$ 1.12 ($P < 0.001$)
Peripheral blood T-cell counts ($\times 10^4$ /ml)			
Genotype	CD3	CD4	CD8
–/–	275.1 $\pm$ 21.1	131.6 $\pm$ 11.2	140.0 $\pm$ 12.1
–/KRR	302.1 $\pm$ 31.0	171.5 $\pm$ 15.2	125.3 $\pm$ 11.6
KRR/KRR	328.5 $\pm$ 31.2 ($P = 0.073$)	204.0 $\pm$ 18.2 ($P < 0.001$)	110.3 $\pm$ 10.2 ($P < 0.005$)

Single-cell suspensions were prepared from spleen and peripheral blood of wild and KRR mice, and red cells were hemolysed. More than six mice per group were analyzed and the mean ($\pm$ SD) values were shown. P values for wild versus homozygous Tg were calculated using Student's t -test.

Table II. Numbers of Peyer's patch >1 mm in diameter ($n = 5$ in each group)

Genotype	Counts (mean $\pm$ SD)
–/–	11.35 $\pm$ 2.52
–/KRR	9.25 $\pm$ 2.31
KRR/KRR	7.31 $\pm$ 1.94

Small intestine from wild-type or mutant mice were soaked in 10% acetic acid for 10 min and the number of Peyer's patch >1 mm in diameter per intestine was counted. $P = 0.009$ for wild-type versus homozygous Tg.

increase in CD4⁺ cells and decrease in CD8⁺ cells, resulting in a total increase of CD3⁺ T cells (Table I). In summary, the T-cell count for KRR mice is increased in spleen and peripheral blood, and is decreased in LN and PP. As a whole, the net change in total T-cell count is a nearly 2-fold increase for the CD4⁺ cells and remains almost unchanged for the CD8⁺ cells.

KRR T cells have defective homing to systemic lymphnodes and Peyer's patches

Alteration in T-cell distribution observed in KRR mice suggests that a defect lies in KRR T cells for appropriate homing to these organs. To determine the effect of KRR-GATA3 on T-cell homing, we performed an *in vivo* trafficking assay (Figure 5A). At 1 h after injection of the fluorescence-labeled cells, the Ro:Ri ratio was significantly lower for KRR T cells than for control T cells, clearly indicating that the short term trafficking of KRR T cells to PLN, MLN and PP is impaired (Figure 5A, left). At 48 h post-injection, the accumulation of KRR T cells in these organs was also decreased, whereas those trafficking to spleen and circulating in PB were increased (Figure 5A, right). Although the differences in Ro:Ri values were somewhat compromised when compared with those at 1 h post-injection, they more faithfully reflect actual changes in the organ cell counts. In addition, the more exaggerated

contrast at 1 h post-injection indicates that it is the incoming migration to LN and PP, rather than outgoing, which is distorted. Therefore, these results strongly indicate that KRR T cells have defective homing to PLN, MLN and PP, causing decreased cell count in these organs. The distribution of fluorescence-labeled KRR T cells after injection was also examined. For spleen, the marginal zone was stained with MoAb specific for metallophilic macrophages to identify the white pulp (Figure 5B). While pertussis toxin pre-treated T cells were observed outside the marginal zone (PTX), both wild-type and KRR T cells accumulated normally in the periaarterial lymphoid sheath in the white pulp (Wild and KRR). Though less efficient, the labeled KRR T cells also distributed normally in LN (data not shown). Pathologically, the tissue section of LN from KRR mice showed normal architecture with respect to primary follicles (Figure 5C). These results suggest that while KRR T cells are defective in migrating into the LN, they do distribute normally once they have entered.

Normal primary response but prolonged cell survival in KRR T cells

A recent report on CCR7^{–/–} mice showed that impaired migration of lymphocytes to secondary lymphoid organs results in a delayed primary immune response (Förster *et al.*, 1999). Therefore we tested KRR mice for the primary T-cell response to antigen stimulation. Both wild-type and KRR T cells showed a similar increase in number of staphylococcal enterotoxin A (SEA)-reactive V β 11⁺ T cells, reaching the maximum on day 2 after SEA immunization (Figure 6A, upper left and upper middle). This indicates that the primary T-cell response is not impaired in KRR mice. However, when an elimination phase was examined on day 8, litter-mate control mice showed characteristic deletion of CD4⁺V β 11⁺ cells down to the level of unimmunized control, whereas KRR mice retained many more SEA-reactive CD4⁺V β 11⁺ cells (Figure 6A, upper left). For CD8⁺ cells, however, elimination of the SEA-reactive V β 11⁺ cells was almost indistinguishable between wild-type and KRR mice (Figure 6A, upper middle). The SEA-non-reactive V β 8⁺ T cells showed compensatory change in each case, confirming the specificity of the results (Figure 6A, lower left and middle). A similar reduction in elimination of preactivated CD4⁺ cells was also observed for the staphylococcal enterotoxin B (SEB)-reactive V β 8⁺CD4⁺ cells (Figure 6A, upper right), with specificity confirmed by reciprocal change of the SEB-non-reactive V β 6⁺CD4⁺ T cells (Figure 6A, lower right). Therefore, the process of eliminating antigen-activated T cells, namely designated 'activation-induced cell death' (AICD) is somehow impaired in CD4⁺ KRR T cells, while those of the CD8⁺ T cells are less affected.

To confirm the above findings, the primary T-cell response was tested *in vitro* for various stimulations including anti-CD3, Concanavalin A (ConA) and PMA/ionomycin. Both wild-type and KRR T cells showed indistinguishable proliferation in response to these reagents, confirming the intact primary response in KRR T cells (Table III). *In vitro* evaluation of the compromised cell death was also performed in a simple cell culture system (Figure 6B). CD4⁺ or CD8⁺ T cells from KRR mice

Fig. 5. Impaired homing of KRR T cell to all lymphnodes and Peyer's patches. **(A)** *In vivo* trafficking assay. FITC-labeled KRR T cells migrate less efficiently to PLN, MLN or PP compared with wild-type T cells. The migration capacities are shown as Ro/Ri values (Ro/Ri; mean $\pm$ SD), with the greater value indicating the higher migration capacity to the organ (see Materials and methods). **(B)** BCECF-labeled (green) wild-type T cells, KRR T cells or pertussis-toxin (PTX)-treated T cells were injected into recipient mice and the splenic section was prepared. The marginal zone was stained with MOMA-1 MoAb (red). **(C)** Tissue section of representative mesenteric lymphnodes from control and KRR mice stained with hematoxylin and eosin. Scale bar, 1 mm.

and litter-mate controls were purified and cultured in medium, and their viability was assessed by propidium iodide staining. There was a modest but consistent increase in survival of CD4⁺ KRR T cells compared with that of control CD4⁺ T cells. A less distinguished change in survival was observed for CD8⁺ KRR T cells. The longer survival observed for the CD4⁺ cells than for the CD8⁺ cells may represent the increased expansion of CD4⁺ cells *in vivo*.

The Fas- and TNF-mediated pathways are known to play pivotal roles in the death of activated T cells (Singer and Abbas, 1994; Dhein *et al.*, 1995; Ju *et al.*, 1995; Zheng *et al.*, 1995; Wong and Choi, 1997). To investigate the candidate gene that might cause impaired cell death in KRR mice, we checked for the expression of these molecules in activated T cells. The induction of Fas and FasL after activation was identical between the wild-type and KRR T cells (data not shown). However, while control

Table III. Primary T-cell response to various stimulations			
Genotype	Anti-CD3	ConA	PMA-Ionomycin
-/-	13936 $\pm$ 1236.5	16387 $\pm$ 1256.3	25698 $\pm$ 2351.5
-/KRR	15365 $\pm$ 1196.3	15332 $\pm$ 1795.2	24956 $\pm$ 2121.6
KRR/KRR	14125 $\pm$ 1415.8	16963 $\pm$ 2693.5	27953 $\pm$ 3251.6

Purified T cells from wild-type or KRR mice were stimulated with anti-CD3 (10 μ g/ml), ConA (2 μ g/ml), or PMA (10 ng/ml) plus ionomycin (0.5 μ g/ml) for 48 h. Shown is the mean ($\pm$ SD) [³H]thymidine uptake.

T cells showed the previously documented patterns of TNF α and LT α mRNA induction, where TNF α mRNA reached its peak at 4 h and LT α was maximally induced at 24 h post-stimulation, the induction of TNF α and LT α was substantially diminished in KRR T cells (Figure 6C). The expression of LT β was indistinguishable between wild-type and KRR T cells, verifying the specificity of the

reduced TNF α and LT α induction. These results strongly indicate that the induction of TNF α and LT α in T cell is suppressed by KRR-GATA3. Such a defect in TNF α and LT α production could lead to insufficient cell death after activation, which would provide a possible explanation for the impaired AICD and increased T-cell population in KRR mice.

KRR-GATA3 enhances the IL-2-induced IL-2R α expression and proliferation

To investigate further the mechanism of prolonged cell survival in KRR T cells, we checked for the IL-2 production and IL-2 response in KRR T cells. Given the normal IL-2 production in KRR T cells (data not shown), the possibility of augmented cellular sensitivity to IL-2 was tested. Purified T cells from control and KRR mouse spleens were preactivated with anti-CD3 antibody, washed and subsequently cultured with different concentrations of IL-2. As shown in Figure 7A, T cells from KRR mice proliferated 3- to 4-fold more compared with those from litter-mate control mice, clearly indicating the increased sensitivity to IL-2. A transgene-specific dose dependency was also observed, with more proliferation seen for the homozygous Tg than the heterozygous mice.

This increased sensitivity to IL-2 could be due to the sustained expression of IL-2R α , which comprises a high affinity IL-2 receptor complex (Sugamura *et al.*, 1995). Stimulation through the TCR triggers IL-2R α expression, while its expression is known to be sustained by IL-2 secreted from the stimulated T cells themselves (Soldaini *et al.*, 1995; Sperisen *et al.*, 1995). While anti-CD3-induced levels of surface IL-2R α expression were nearly identical between wild-type and KRR T cells [Figure 7B, see CD3 (24 h) in each panel], there was substantial increase in the IL-2-induced IL-2R α expression in KRR T cells [Figure 7B, see IL-2 (48 h) in each panel]. A similar up-regulation of surface IL-2R α was seen for the CD8 $^{+}$ T cells (Figure 7B, CD8 cells). These up-regulations seem specific to IL-2R α , since CD69 expression, another activation marker for T cells, was not affected by similar IL-2 stimulation (data not shown).

To see whether increased surface expression of IL-2R α protein correlates with enhanced IL-2R α mRNA expression, northern blot analysis was performed for the purified T cells, which were preactivated and stimulated in the same way. When the preactivated T cells were further stimulated with IL-2 for 12 h, KRR T cells showed robust expression of IL-2R α mRNA compared with wild-type

Fig. 6. Normal primary immune response but impaired deletion in antigen-stimulated KRR T cells. **(A)** Time course of expansion and deletion of superantigen-reactive T cells in LNs of control and homozygous KRR mice. Control (open symbols) and homozygous KRR mice (filled symbols) were injected with 10 μ g of SEA or SEB on day 0. Two or 8 days after immunization, the percentage of SEA-reactive V β 11 $^{+}$ or SEB-reactive V β 8 $^{+}$ cells in CD4 $^{+}$ or CD8 $^{+}$ T cells were quantified along with SEA-non-reactive V β 8 $^{+}$ cells or SEB-non-reactive V β 6 $^{+}$ cells. For SEB experiments, KRR mice were backcrossed to BALB/c for four generations to obtain mice homozygous for H2-K d and subsequently intercrossed to obtain mice homozygous for KRR-GATA3. Shown are the results (mean $\pm$ SD) with four mice in each group, and the results were essentially reproducible. **(B)** Prolonged survival for CD4 $^{+}$ KRR T cells *in vitro*. Purified CD4 $^{+}$ or CD8 $^{+}$ T cells from wild-type (open square), heterozygous (filled triangle) or homozygous KRR (filled square) mice were cultured in RPMI medium supplemented with 10% FCS and 2-mercaptoethanol, at 10×10^5 cells/ml. On days indicated, cells were stained with propidium iodide (PI) to determine viable cell counts by flow cytometry. **(C)** Impaired TNF α and LT α mRNA induction after T-cell stimulation. Purified splenic T cells from wild-type or homozygous KRR mice ($n = 5$ for each) were stimulated with ConA (2 μ g/ml) for 0, 4 or 24 h, and RNA was extracted. Fifteen micrograms of total RNAs on each lane were hybridized with mouse TNF α , LT α or LT β probes. Twenty-four hour stimulation was not done for LT β .

Fig. 7. Increased IL-2-dependent proliferation and IL-2R α expression in preactivated KRR T cells. (A) Increased IL-2-dependent proliferation of preactivated KRR T cells. Purified T cells (1×10^6 /ml) from wild-type or KRR mice were stimulated in plate coated with 10 μ g/ml anti-CD3 mAb for 24 h and washed. Subsequently, cells (2×10^5 /well) were cultured in the presence of IL-2 at the indicated concentrations for 48 h, with [3 H]thymidine added for the last 12 h. (B) Prolonged surface IL-2R α expression in IL-2-dependent phase. Splenocytes from wild-type or KRR mice were stimulated in plates coated with 10 μ g/ml anti-CD3 MAb for 24 h [CD3 (24 h)]. Subsequently, cells were washed with PBS and cultured in medium containing 100 U/ml of recombinant human IL-2 for another 24 or 48 h [IL-2 (24 h) and IL-2 (48 h)]. The histograms of IL-2R α expression and fluorescent intensity (mean) in each stage were shown for CD4 and CD8 cells. More than five trios of mice were analyzed and the results were essentially reproducible. (C) Enhanced IL-2R α mRNA expression in response to IL-2 in CD3-preactivated KRR T cells. Purified T cells from wild-type or homozygous KRR mice were activated and stimulated as in (A). Total RNA was isolated from each culture and the RNA blot was hybridized with mouse IL-2R α or control GAPDH probe. The intensities of IL-2R α mRNA were normalized with the control signals in each lane and the result is shown as a bar chart.

T cells (Figure 7C). The difference is most prominent at 12 h, but persists until 48 h post-stimulation. Therefore, it is likely that the up-regulation in IL-2-mediated IL-2R α transcription leads to sustained cell surface expression of IL-2R α , causing exaggerated proliferation in response to IL-2. The enhanced IL-2R α expression in the IL-2-dependent phase could also account for the prolonged cell survival after antigen stimulation *in vivo*.

Discussion

Acetylation state affects transactivation ability of GATA-3

KRR-GATA3 was originally discovered during an attempt to generate a dominant-negative form of GATA-3 (Smith *et al.*, 1995). However, the molecular mechanism of the suppressive effect brought by the KRR $\rightarrow$ AAA substitution has long been a mystery. The KRR motif, located in the lysine-rich region of GATA-3, is the most conserved acetylation-target motif among the GATA-family proteins

(Hung *et al.*, 1999). Accordingly, a tempting speculation was made that mutations introduced into certain acetylation target motifs would yield a potent negative form of protein. Some mutants exhibited low transactivation ability, but the KRR mutant (M2) exhibited the lowest activity and showed dominant-negative effect over wild-type GATA-3. Hence, we assigned the KRR motif as the most transcriptionally relevant acetylation motif. This conclusion is in line with two previous independent studies on GATA-1 acetylation, where GATA-1 harboring a corresponding mutation loses its cooperation with p300 (Boyes *et al.*, 1998) or exhibits the lowest hemoglobin synthesis (Hung *et al.*, 1999). However, whether the same mutation in GATA-1 functions as a dominant-negative form is uncertain.

To probe for the molecular event that is associated with KRR mutation, we performed the *in vitro* acetylation assay and showed that KRR mutation not only disrupts its own acetylation but also interferes with acetylation of adjacent lysines. This indicates that KRR-GATA3 renders a

hypocetylated form of GATA-3, without introducing actual mutations into Lys293 (M1) and 347 (M3). From the acetylation point of view, therefore, KRR mutation is almost equivalent to mutant M123. Thus, de-acetylation is not just a single lysine but in multiple target lysines by the KRR mutation accounts for the low transactivation by KRR-GATA3. The acetylation of this portion may affect protein-protein interaction. Given the strong suppressive effect of KRR-GATA3, it is possible that de-acetylated GATA-3 may preferentially interact with some transcriptional co-repressor molecules.

KRR mice reveal novel roles of GATA-3 in the immune system

Expressing KRR-GATA3 in mature T cells has revealed the novel roles of GATA-3 in T cells. Several findings observed in KRR mice are attributed to the KRR mutation in GATA-3 but not to the increase of GATA-binding protein, because overexpressing wild-type GATA-3 in CD4⁺ T cell with CD4-GATA-3 transgenic mice is reportedly causing no obvious changes in T-cell population or distribution (Zheng and Flavell, 1997). Whether all the functions of GATA-3 are disrupted by the KRR mutation must be carefully evaluated. It is conceivable that among the GATA-3 target genes, only those whose expressions are effected by the KRR mutation manifest themselves phenotypically. Hence, the phenotype of KRR mice could partly be different from those future coming GATA-3^{-/-} mice by the conditional null mutation approach.

Recently, Zhang *et al.* have established the pivotal role of GATA-3 in asthma development by expressing KRR-GATA3 using Lck proximal-promoter-driven dox-inducible system (Zhang *et al.*, 1999). While their mice showed significant induction of KRR-GATA3 in thymus, our mice exhibited strong expression in spleen, with only a small amount of it in thymus. The strong and constitutive expression of KRR-GATA3 in mature peripheral T cells may account for the observed phenotypes in our mice.

GATA-3 is involved in T-cell homing

A striking feature of the mice in this experiment is that KRR T cells have defective homing to all the LN and PP. The reduction of CD4⁺ cells in KRR-LN seems less severe than that of CD8⁺ cells (Figure 4E). This could be due to the net increase of CD4⁺ cells offsetting the gross reduction of CD4⁺ cells in KRR-LN, and that CD4⁺ as well as CD8⁺ cells are impaired for their homing. The result of the trafficking assay indicates that the impaired homing is attributed to some defect in KRR T cells, but not to those in the stromal elements. However, the nature of this defect is unknown. The expression of molecules that are involved in T-cell homing, including CCR7, L-selectin, LFA-1, CD44, and integrin- α 4, - β 1 and - β 7, was essentially normal in KRR T cells (data not shown). Therefore, it is conceivable that some unidentified cell surface molecule or intracellular signaling pathway, essential for appropriate T cell homing, is disrupted in KRR T cells. Whatever the mechanism, it is worthwhile to note that the KRR mouse is the first transgenic or knockout mouse of a transcription factor that shows impaired lymphocyte homing, and that GATA-3

is the first transcription factor shown to be involved in this process.

KRR-GATA3 positively regulates the survival of T cells by affecting transcriptions from the relevant genes

Generally, the peripheral T-cell compartment maintains a constant size over time, despite persistent stimulation by environmental antigens. This peripheral homeostasis is achieved by a balance between proliferative expansion and elimination of activated cells by AICD (Kawabe and Ochi, 1991; McHeyzer-Williams and Davis, 1995). The polyclonal expansion of peripheral T cells in KRR mice suggests that GATA-3 plays a general role in maintaining this homeostatic balance, which could be disturbed by impaired cell death, sustained proliferation or both.

The impaired deletion of the superantigen-activated T cells in KRR mice indicates that GATA-3 is involved in regulation of AICD (Figure 6A). The compromised passive cell death in the simple culture system may reflect the same event *in vitro* (Figure 6B). TNF α and LT α have been shown to sufficiently mediate the apoptotic death of activated T cells (Sarin *et al.*, 1995; Körner and Sedgwick, 1996; Sytwu *et al.*, 1996). Therefore, although the possibility remains that KRR-GATA3 effects expression of other AICD-related genes, the diminished expressions of TNF α and LT α in KRR T cells could explain the reduced elimination of activated cells.

Another explanation for the prolonged cell survival in KRR T cells could be proliferation assistance. Addition of IL-2R α converts the intermediate-affinity ($K_d = 10^{-9}$ M) receptor, composed of IL-2R β and γ_c , into the high-affinity receptor ($K_d = 10^{-11}$ M). The signal from IL-2R itself supports the transcription from IL-2R α gene, bringing maximal cellular response to the proliferation signal (Soldaini *et al.*, 1995; Sperisen *et al.*, 1995). The prolonged IL-2R α expression in late phase of activation not only assists proliferation but also supports survival after activation. Therefore, this could also contribute to the prolonged cell survival in KRR mice.

Our study shows that the KRR mutation creates local hypoacetylation in GATA-3, and that KRR-GATA3 negatively regulates the TNF α and LT α gene while positively affecting the IL-2R α gene transcription, leading to expansion of the T-cell population. Our findings not only shed a light on the novel roles of GATA-3 in the immune system, but also provide evidence that altering the state of acetylation in a transcription factor affects endogenous gene transcriptions and causes relevant physiological changes. Similar acetylation-mutant models of transcription factors will be a powerful tool in probing the yet unknown roles of the gene regulators.

Materials and methods

***In vitro* acetyltransferase assays**

The DNA fragment from the p300 HAT domain, corresponding to amino acids 1194–1812, was amplified by PCR and subcloned into pFLAG[®]-MAC (Kodak). The FLAG-p300(1194–1812) protein was expressed using the IBI *Escherichia coli* FLAG[®] Expression System (Kodak) and purified on anti-FLAG M2 affinity gel. Protein acetyltransferase assays for the GST fusion proteins were performed as described previously (Gu and Roeder, 1997).

In vivo sodium [¹⁴C]acetate labeling and immunoprecipitation

Fresh splenocytes (1×10^8 cells), or splenocytes stimulated with ConA (2 µg/ml) for 24 h, were resuspended in 2.5 ml of medium containing 0.05 mCi/ml of sodium [¹⁴C]acetate (55 mCi/mmol; NEN™) and incubated for 3 h. The solvent ethanol of sodium [¹⁴C]acetate was evaporated to minimize the cytotoxic effect of the ethanol in the culture medium. The cells were washed, resuspended in lysis buffer and sonicated to destroy cells. Following centrifugation, the supernatants were incubated with pre-immune serum or anti-GATA-3 serum (G3N5; Yamagata *et al.*, 1995) and with protein G-Sepharose to immunoprecipitate endogenous GATA-3. After fixation, the gel was dried and subjected to image analyzing using a BAS2000 Image Analyzer (Fuji Film).

Transgene construction and transgenic mice

C57BL/6 (B6) mice were purchased from Clea Inc. (Tokyo, Japan). The KRR-GATA3 was provided by Dr A.Winoto (Smith *et al.*, 1995). HA-tag was attached to the N-terminus of KRR-GATA3 to create KRR-GATA3(HA). The KRR-GATA3(HA) fragment was subcloned into the *Bam*HI site of the transgene expression vector pW120 (Wildin *et al.*, 1995; Hashimoto *et al.*, 1996) to create pW120-KRR-GATA3(HA). The KRR-GATA3 mice (KRR mice) were produced by injection of the purified fragment into the pronucleus of fertilized single cell embryos of B6 mice. All the mice were kept inbred on C57BL/6 background. Homozygous transgenic mice were also generated by breeding heterozygous litter-mates to obtain maximal level of expression and dose-dependent nature of the results.

Superantigen-induced clonal expansion and deletion

Three- to 4-month-old mice were intraperitoneally injected with phosphate-buffered saline (PBS) or 10 µg of SEA or SEB (Sigma). Lymphnode cells were stained with anti-CD4- or anti-CD8-Cy-Chrome plus anti-Vβ11-PE and anti-Vβ8.1,2-FITC for SEA-injected mice, and anti-Vβ8.1,2-FITC and anti-Vβ6-PE for SEB-injected mice, respectively. For the SEB injection experiment, Tg mice were backcrossed to BALB/c for four generations, and those homozygous for H2-K^d alleles were used for the experiments.

IL-2-dependent cell surface IL-2Rα (CD25) expression

Splenocytes (1×10^6 /ml) were stimulated in Falcon 3047 plates coated with 10 µg/ml anti-CD3ε MAb (145-2C11) for 24 h in culture medium. Subsequently, the cells were collected, washed and resuspended in the culture medium supplemented with 100 U/ml of IL-2. Cells were harvested and stained with anti-IL-2Rα (CD25)-FITC (7D4) and anti-CD4-PE (RM4-5) or anti-CD8-PE (Ly-2) at the times indicated.

Proliferation assay

For proliferation of fresh T cells, purified T cells (2×10^5 /well) were cultured in 96-well plates coated with 10 µg/ml anti-CD3ε MAb, or in the presence of 2 µg/ml ConA or PMA (10 ng/ml) plus Ionomycin (0.5 µg/ml) for 48 h, with 1 µCi of [³H]thymidine added for the last 12 h. For the IL-2-dependent proliferation, purified T cells were preactivated with anti-CD3ε MAb for 24 h, washed and cultured (2×10^5 /well) with indicated concentrations of recombinant human IL-2 for 48 h, with [³H]thymidine pulsed for the final 12 h.

Lymphocyte migration assay

FITC-labeled splenocytes (green fluorescent) from wild-type (–/–), heterozygous (–/KRR) or homozygous (KRR/KRR) mice were mixed with an equal number of wild-type splenocytes labeled with PKH-26 (red fluorescent), and a total of 5×10^7 cells were injected intravenously into C57BL/6 wild-type recipient mice. An aliquot of the injected mixtures was stained with TCRαβ-Cy-Chrome (H57-597) and analyzed by FACS to calculate the ratio of FITC- versus PKH26-labeled cells (Ri) among the TCRαβ-positive populations. After 1 or 48 h, single-cell suspensions from each organ were prepared from the recipient mice, and the ratios of FITC- versus PKH26-labeled cells (Ro) among the TCRαβ-positive populations were determined by FACS. Non-fluorescent cells and cells presenting light scattering properties of dead cells were excluded from the analysis. At least 200 000 TCRαβ-Cy-Chrome-positive cells were counted for each tissue sample. Results were expressed as Ro value divided by Ri value (Ro:Ri ratio). For confocal laser microscopic analysis of the injected cells, BECEF-AM-labeled T cells were injected into wild-type recipient mice, and the tissue sections were stained with MOMA-1(BMA)/anti-rat IgG-Cy3 (Amersham) to visualize the marginal zone.

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