

SUMO-defective c-Maf preferentially transactivates *Il21* to exacerbate autoimmune diabetes

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SUMOylation is involved in the development of several inflammatory diseases, but the physiological significance of SUMO-modulated c-Maf in autoimmune diabetes is not completely understood. Here, we report that an age-dependent attenuation of c-Maf SUMOylation in CD4⁺ T cells is positively correlated with the IL-21-mediated diabetogenesis in NOD mice. Using 2 strains of T cell-specific transgenic NOD mice overexpressing wild-type c-Maf (Tg-WTc) or SUMOylation site-mutated c-Maf (Tg-KRc), we demonstrated that Tg-KRc mice developed diabetes more rapidly than Tg-WTc mice in a CD4⁺ T cell-autonomous manner. Moreover, SUMO-defective c-Maf preferentially transactivated *Il21* to promote the development of CD4⁺ T cells with an extrafollicular helper T cell phenotype and expand the numbers of granzyme B-producing effector/memory CD8⁺ T cells. Furthermore, SUMO-defective c-Maf selectively inhibited recruitment of Daxx/HDAC2 to the *Il21* promoter and enhanced histone acetylation mediated by CREB-binding protein (CBP) and p300. Using pharmacological interference with CBP/p300, we illustrated that CBP30 treatment ameliorated c-Maf-mediated/IL-21-based diabetogenesis. Taken together, our results show that the SUMOylation status of c-Maf has a stronger regulatory effect on IL-21 than the level of c-Maf expression, through an epigenetic mechanism. These findings provide new insights into how SUMOylation modulates the pathogenesis of autoimmune diabetes in a T cell-restricted manner and on the basis of a single transcription factor.

Introduction

The conjugation of small ubiquitin-like modifier (SUMO) to specific lysine residues of cellular proteins is a dynamic and reversible posttranslational modification (1). Deficiencies in SUMOylation enzymes have been reported to affect the pathogenesis and severity of inflammatory diseases (2–4). Transcription factors are major SUMOylation substrates (5), and SUMOylation-regulated transcription factors are involved in the pathogenesis of type 1 diabetes (T1D) (6, 7). However, it is not known whether T cell-restricted SUMOylation based on a single transcription factor modulates autoimmune diabetes, and if so, what mechanism is involved.

Autoimmune diabetes is a T cell-mediated inflammatory disease caused by the destruction of insulin-producing β cells. Studies in animal models of spontaneous autoimmune diabetes, particularly in NOD mice, have demonstrated that disease pathogenesis depends on the effector function of islet antigen-specific CD4⁺ and CD8⁺ T cells (8). IL-21, produced mainly by activated CD4⁺ T cells, is critical for enhancing the effector functions of CD8⁺ T cells (9). Moreover, systemic deletion of IL-21 or its receptor renders NOD mice resistant to diabetes, while β cell-specific overexpression of IL-21 elicits diabetes in diabetes-resistant C57BL/6 mice (10–12). Importantly, memory CD4⁺ T cells from patients with

T1D have been reported to express high levels of IL-21 and surface molecules associated with follicular helper T (Tfh) cells (13, 14), further supporting the concept that IL-21-producing Tfh cells play a crucial role in the pathogenesis of T1D.

The transcription factor c-Maf was previously reported to be a Th2 cell-specific factor that transactivates the *Il4* gene (15). However, expression of transgenic c-Maf in NOD T cells has a minimal enhancing effect on IL-4 expression and does not protect NOD mice from autoimmune diabetes (16). Subsequent studies have revealed that SUMOylation at the lysine 33 residue reduces the ability of c-Maf to bind the *Il4* promoter and decreases its transactivating activity in a luciferase reporter assay (7, 17). Moreover, an association of c-Maf SUMOylation with autoimmune diabetogenesis has been suggested, because the level of SUMOylated c-Maf in CD4⁺ T cells is significantly higher in NOD mice than in diabetes-resistant B10.D2 mice (7). In addition, recent reports indicated that c-Maf binds directly to the *Il21* promoter in CD4⁺ T cells (18) and is critical for the development of Th17 and Tfh cells (19). Furthermore, a subset of IL-21⁺CCR9⁺CD4⁺ T cells with a Tfh-like phenotype and containing abundant c-Maf contributed to CD8⁺ T cell-dependent diabetes progression in NOD mice (20). However, the effect of c-Maf SUMOylation on IL-21 production and the development of disease in NOD mice is not well defined. In this study, we used transgenic NOD mice overexpressing wild-type c-Maf (Tg-WTc) or K33R c-Maf, which has a mutated SUMOylation site (Tg-KRc), to demonstrate that the SUMOylation status of c-Maf possesses a stronger effect than its level of expression on the

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initiation and early development of autoimmune diabetes. These experiments provide new insights into the underlying mechanisms linking the SUMOylation status of a single transcription factor with the pathogenesis of autoimmune diabetes.

Results

c-Maf SUMOylation in CD4⁺ T cells is inversely correlated with the severity of insulinitis and IL-21 production in NOD mice. Recent studies have shown that deficiencies in SUMOylation enzymes have significant impacts on the pathogenesis and severity of inflammatory diseases (2–4). However, the physiological significance and detailed mechanism(s) by which SUMOylation modulates autoimmune diabetes are not completely understood. Since the SUMOylation-regulated transcription factor c-Maf has been reported to be associated with the pathogenesis of autoimmune diabetes (7), we first determined the SUMOylation status of c-Maf in CD4⁺ T cells of NOD mice at different stages of diabetogenesis. We activated CD4⁺ T cells from 6- to 8-week-old and 12- to 14-week-old NOD mice in vitro using anti-CD3 and anti-CD28 for 36 hours, then immunoprecipitated cell lysates with anti-c-Maf followed by Western blotting with anti-SUMO-1 or anti-c-Maf. In these samples, in addition to the typical c-Maf species (~50 kDa), a higher-molecular mass species (~70 kDa) was detected (Figure 1A), consistent with the previously reported size of SUMOylated c-Maf (7, 17). Notably, the amount of SUMOylated c-Maf in CD4⁺ T cells was at least 50% lower in 12- to 14-week-old mice than in 6- to 8-week-old mice, whereas the amount of unSUMOylated c-Maf was comparable (Figure 1A and Supplemental Table 1A; supplemental material available online with this article; <https://doi.org/10.1172/JCI98786DS1>), suggesting an inverse correlation between c-Maf SUMOylation and the severity of insulinitis in NOD mice. Since c-Maf SUMOylation was catalyzed by a specific enzyme cascade, including SAE1/SAE2 (SUMO E1 activating enzymes), UBC9 (a SUMO E2 conjugating enzyme), and PIAS1 (a SUMO E3 ligase) (1, 5, 7, 17), we next examined the expression of SUMOylation enzymes in NOD CD4⁺ T cells from mice of different ages. Our results demonstrated that the transcript levels of *Sae1*, *Sae2*, *Ubc9*, and *Pias1* in CD4⁺ T cells were significantly lower in 12- to 14-week-old mice than in 6- to 8-week-old mice (Figure 1B), implying that downregulation of the c-Maf-associated SUMO enzymatic cascade in CD4⁺ T cells was positively associated with the diabetogenic process and its severity in NOD mice. Besides, we observed that the amount of SUMOylated or unSUMOylated c-Maf in CD4⁺ T cells was comparable between 6- to 8-week-old C57BL/6 (B6) and NOD mice (Supplemental Figure 1A), suggesting that the SUMOylation status of c-Maf in CD4⁺ T cells from 6- to 8-week-old mice was similar in both non-diabetes-prone and spontaneous diabetic mouse strains. However, the ratio of SUMOylated to unSUMOylated c-Maf was significantly decreased by approximately 50% in 12- to 14-week-old NOD CD4⁺ T cells compared with that in 6- to 8-week-old NOD CD4⁺ T cells, whereas this SUMOylated/unSUMOylated ratio was comparable between 6- to 8-week-old and 12- to 14-week-old B6 CD4⁺ T cells (Supplemental Figure 1A and Supplemental Table 1B). These results provided evidence that there was a significant age-related downregulation in SUMOylation status of c-Maf from NOD CD4⁺ T cells rather than B6 CD4⁺ T cells, implying that this age-dependent

attenuation of c-Maf SUMOylation underlies the development of autoimmune diabetes in NOD mice.

It has been reported that c-Maf is critical for transactivating *Il4* (15), *Il10* (21), and *Il21* (18). We examined the expression of IL-4, IL-10, and IL-21 in CD4⁺ T cells of NOD mice at different ages. Our results revealed that expressions of IL-21 mRNA and protein were significantly higher in 12- to 14-week-old NOD CD4⁺ T cells than in 6- to 8-week-old NOD CD4⁺ T cells, whereas expressions of IL-4 or IL-10 mRNA and protein were indistinguishable between the 2 groups (Figure 1, C and D, respectively), suggesting that IL-21 production in CD4⁺ T cells of NOD mice was positively correlated with diabetogenesis. Since c-Maf transactivates *Il4*, *Il10*, and *Il21* through a direct interaction with the c-Maf response element (MARE) motif in these promoters (15, 18, 21), we next performed a ChIP assay to examine whether the SUMOylation status of c-Maf affects its interaction with the MARE motifs in these promoters. Our results indicated that the binding of c-Maf was not detected in the regions devoid of c-Maf response element (non-MARE) of the *Il4* promoter (*Il4p*), the *Il10* promoter (*Il10p*), or the *Il21* promoter (*Il21p*) of NOD CD4⁺ T cells (Figure 1E and Supplemental Figure 1, B and C, respectively). Moreover, we observed that c-Maf enrichment in the *Il21p* MARE was significantly greater in 12- to 14-week-old NOD CD4⁺ T cells than in 6- to 8-week-old NOD CD4⁺ T cells (Figure 1E), whereas c-Maf enrichment in the *Il4p* MARE or the *Il10p* MARE was comparable in both groups (Supplemental Figure 1, B and C, respectively). These findings suggested that c-Maf is recruited more efficiently to the *Il21p* than to the *Il4p* or to the *Il10p* in 12- to 14-week-old NOD CD4⁺ T cells and that this age-dependent increase in the *Il21p* recruitment is negatively correlated with the level of c-Maf SUMOylation.

To evaluate whether pharmacological intervention in the SUMOylation process affects c-Maf-modulated IL-21 production, we treated CD4⁺ T cells with anacardic acid, which is reported as a SUMOylation inhibitor (22). Our results indicated that the amount of SUMOylated c-Maf was significantly decreased in anacardic acid-treated CD4⁺ T cells compared with that in solvent-treated cells, whereas the amount of unSUMOylated c-Maf was comparable (Figure 1F and Supplemental Table 1C). Notably, we observed that levels of IL-21 mRNA and protein were significantly higher in anacardic acid-treated CD4⁺ T cells than in solvent-treated cells, whereas levels of IL-4 or IL-10 mRNA and protein were indistinguishable between these 2 groups of CD4⁺ T cells (Figure 1, G and H, respectively). Moreover, a ChIP assay revealed that c-Maf enrichment in the *Il21p* MARE was significantly higher in anacardic acid-treated CD4⁺ T cells than in solvent-treated cells (Figure 1I), whereas c-Maf enrichment in the *Il4p* MARE or the *Il10p* MARE was comparable in the 2 groups (Supplemental Figure 1, D and E, respectively). This indicated that pharmacological downregulation of c-Maf SUMOylation preferentially promotes its transactivation of *Il21* in NOD CD4⁺ T cells. Besides, we observed that the level of IL-21 mRNA was significantly higher in anacardic acid-treated B6 CD4⁺ T cells than in solvent-treated B6 CD4⁺ T cells (Supplemental Figure 1F), suggesting that anacardic acid has a similar effect of promoting IL-21 expression of CD4⁺ T cells from non-diabetes-prone mice. Taken together, our results suggest that an age-dependent attenuation of c-Maf SUMOylation in CD4⁺ T cells is positively correlated with an IL-21-mediated diabetogenic process in NOD mice.

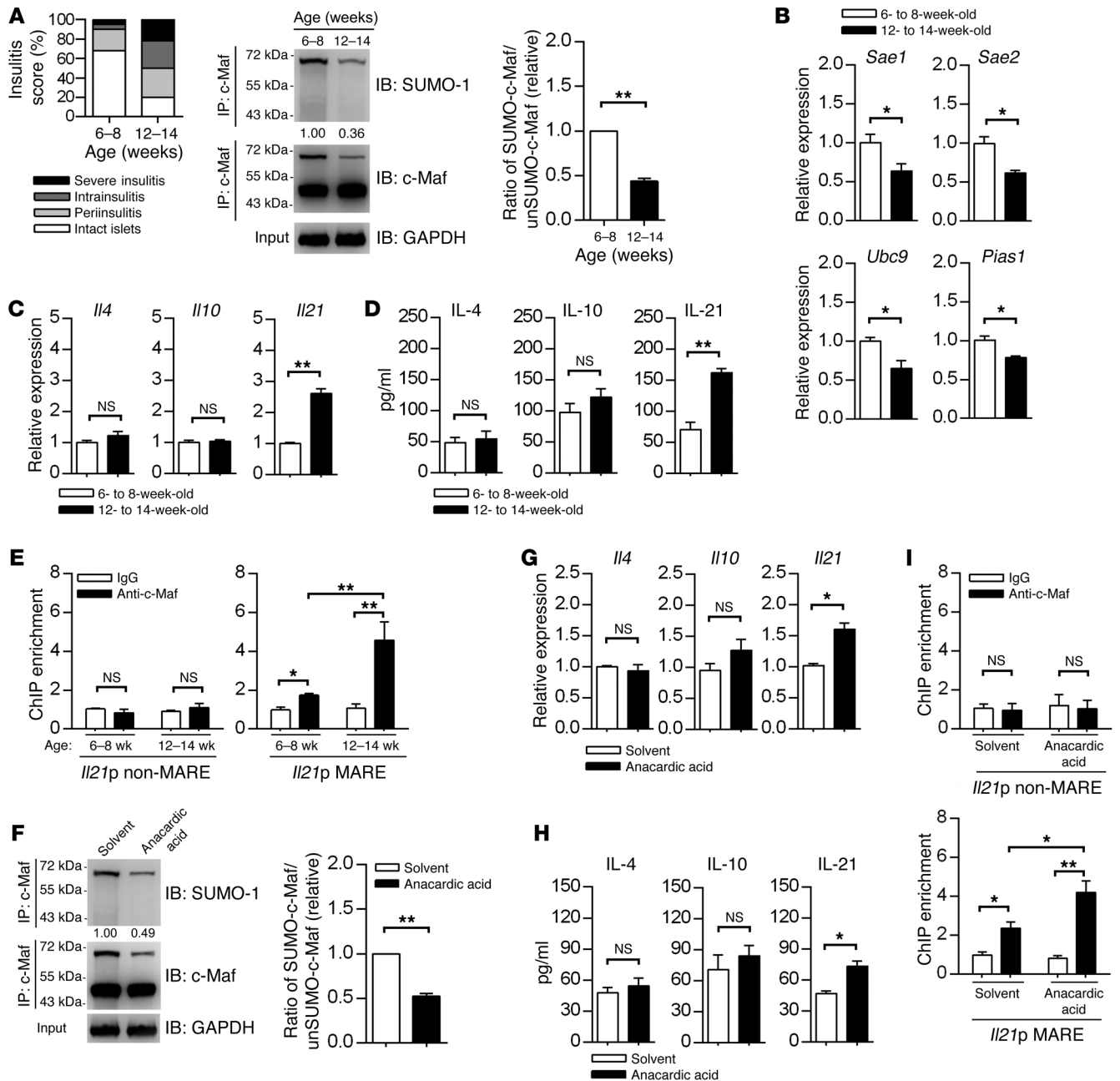


Figure 1. c-Maf SUMOylation in CD4⁺ T cells is inversely correlated with the severity of insulinitis and IL-21 production in NOD mice. (A) The severity of insulinitis was classified and scored on 100 islets from 10 NOD mice per group. Immunoprecipitation analysis of c-Maf SUMOylation in 6- to 8-week-old and 12- to 14-week-old NOD CD4⁺ cells cultured with anti-CD3 and anti-CD28 for 36 hours. (B and C) Expressions of *Sae1*, *Sae2*, *Ubc9*, and *Pias1* mRNA (B) or *Il4*, *Il10*, and *Il21* mRNA (C) in CD4⁺ cells cultured for 36 hours as described in A. (D) ELISA of indicated cytokines in supernatants of CD4⁺ T cells cultured as described in A for 48 hours. (E) ChIP analysis of the interaction of c-Maf with the *Il21p* promoter (*Il21p*) in CD4⁺ cells cultured for 36 hours as described in A. (F) Immunoprecipitation analysis of c-Maf SUMOylation in 6- to 8-week-old NOD CD4⁺ cells cultured for 36 hours with anti-CD3 and anti-CD28 in the presence of anacardic acid (3 μM) or its solvent (DMSO), which were added after 18 hours of culture. (G) Expressions of indicated cytokine mRNA in CD4⁺ T cells cultured for 36 hours as described in F. (H) ELISA of indicated cytokines in supernatants of CD4⁺ cells cultured as described in F for 48 hours. (I) ChIP analysis of the interaction of c-Maf with the *Il21p* promoter in CD4⁺ cells cultured for 36 hours as described in F. For E and I, isotype-matched IgG was used as a control. See complete unedited blots in the supplemental material. Data represent the mean ± SEM; n = 5 mice (A and F), n = 3 mice (B–E and G–I) per group; 3 independent experiments. *P < 0.05; **P < 0.01; 2-tailed Student’s t test (A–D and F–H) or 1-way ANOVA with Tukey’s post-test (E and I).

Generation of transgenic NOD mice with wild-type or SUMO-defective c-Maf. To investigate further whether T cell-restricted and c-Maf-based SUMOylation modulates the pathogenesis of autoimmune diabetes, we generated 2 transgenic NOD mouse

models to additionally express wild-type or SUMO-defective c-Maf in their T cells by injecting the distal *Lck* promoter construct fused with wild-type c-Maf (dLck-WTc) or K33R c-Maf, which was mutated at the lysine 33 residue of the SUMO conjugation site

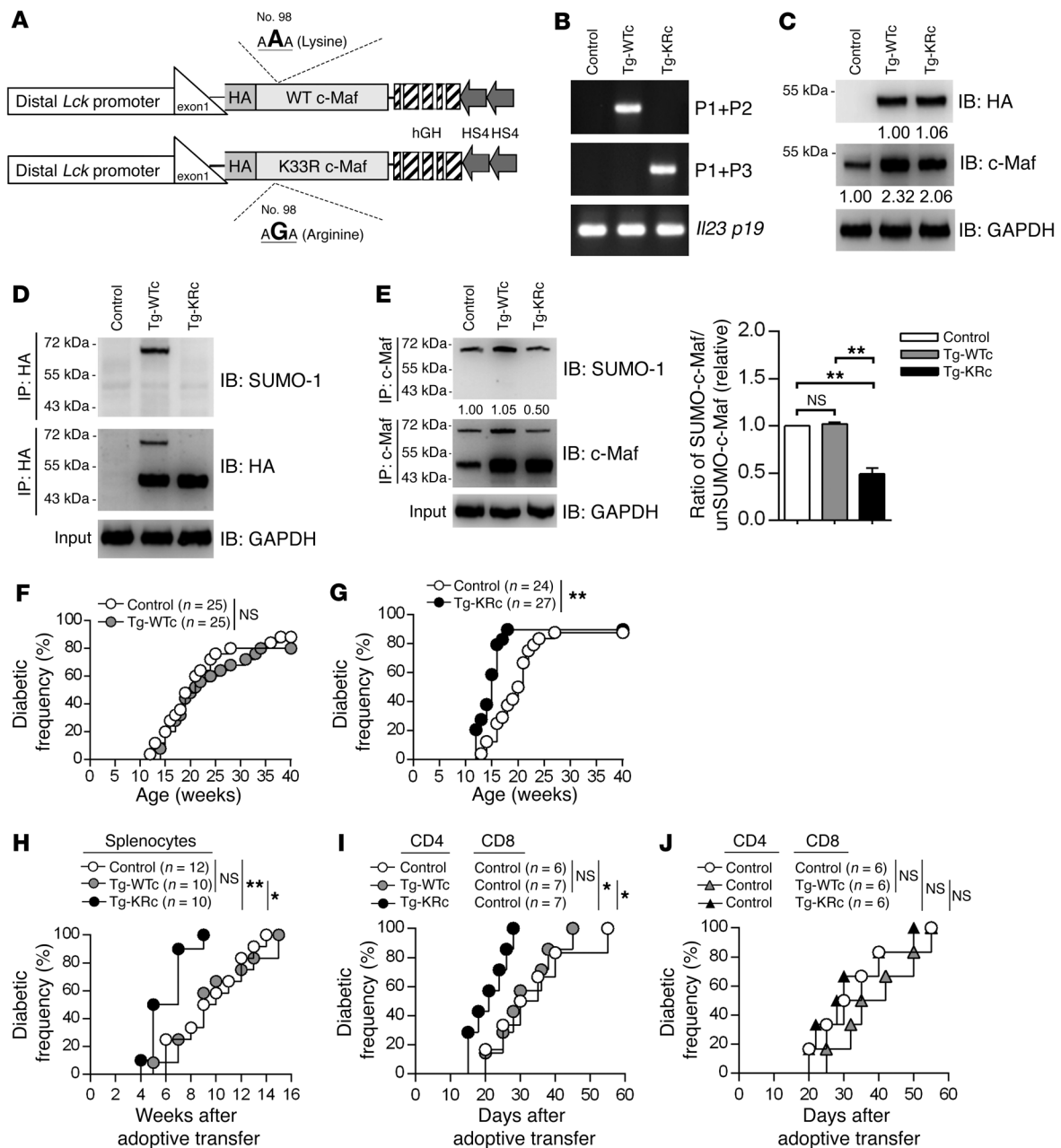


Figure 2. Accelerated kinetics of autoimmune diabetes in SUMO-defective *c-Maf*-transgenic NOD mice. (A) Schematic diagram of distal *Lck* promoter-driven wild-type *c-Maf* or K33R *c-Maf* transgenes. (B) PCR detection of transgenic *c-Maf* in genomic DNA extracted from control, Tg-WTc, and Tg-KRc mice. *Il23 p19* was used as an internal control. (C) Western blot analysis of c-Maf in control, Tg-WTc, and Tg-KRc CD4⁺ T cells. (D and E) Immunoprecipitation analysis of c-Maf SUMOylation in control, Tg-WTc, and Tg-KRc CD4⁺ T cells cultured with anti-CD3 and anti-CD28 for 36 hours. (F) Diabetes incidence in Tg-WTc NOD mice and their littermate controls. (G) Diabetes incidence in Tg-KRc NOD mice and their littermate controls. (H) Diabetes incidence in NOD-SCID recipients of splenocytes from 12- to 14-week-old control, Tg-WTc, or Tg-KRc NOD mice. (I and J) CD25⁺CD4⁺ plus CD8⁺ T cells from 12- to 14-week-old indicated NOD mice were transferred into NOD.*Rag1*^{-/-} mice; all groups were treated simultaneously in 2 independent experiments. The control CD4/control CD8 (white circles) group is presented in both I and J. Diabetic incidences among groups that received control CD8⁺ T cells plus different CD4⁺ T cells (I) and diabetic incidences in among groups that received control CD4⁺ T cells plus different CD8⁺ T cells (J) were compared with each other by a log-rank test. See complete unedited blots in the supplemental material. For E, data represent the mean \pm SEM; $n = 3$ mice (B and C) or $n = 5$ mice (D and E) per group; 3 independent experiments (B-E) or 2 independent experiments (H). * $P < 0.05$; ** $P < 0.01$; 1-way ANOVA with Tukey's post-test (E) or log-rank test (F-J).

(dLck-KRc), into fertilized NOD eggs. The construct contained the mouse distal *Lck* promoter, part of the first noncoding exon of *Lck*, HA-tagged mouse wild-type *c-Maf* or K33R *c-Maf* cDNA, the human growth hormone (hGH) gene with a poly(A) signal, and 2 chicken 5' terminus β -globin hypersensitivity site 4 (HS4) insu-

lator elements (Figure 2A). With 2 independent microinjections, we obtained the transgenic founders of dLck-WTc, denoted as Tg-WTc, and of dLck-KRc, denoted as Tg-KRc.

To determine the presence of the transgene and to distinguish K33R *c-Maf* from wild-type *c-Maf* by PCR, we designed a forward

primer P1 that locates on the distal *Lck* promoter and 2 reverse primers P2 and P3 specifically to recognize wild-type or K33R *c-Maf* cDNA at nucleotide 98 (A and G, respectively) (Figure 2A). The Tg-WTc and Tg-KRc mice can thus be distinguished by PCR genotyping with primers P1/P2 or P1/P3, respectively (Figure 2B). Western blot analysis indicated that expression of *c-Maf* in Tg-WTc and Tg-KRc CD4⁺ cells was much higher than that in control CD4⁺ cells and was similar in both transgenic lines (Figure 2C). To examine whether K33R *c-Maf* in CD4⁺ T cells was able to resist SUMO modification, we prepared cell lysates in the presence of the deSUMOylation inhibitor NEM from NOD CD4⁺ T cells stimulated with anti-CD3/CD28, and immunoprecipitated them with anti-HA followed by anti-SUMO-1 or anti-HA blotting. The SUMOylated form of transgenic *c-Maf* was detected in Tg-WTc cells but was barely detectable in Tg-KRc cells (Figure 2D), demonstrating that transgenic K33R *c-Maf* is resistant to SUMOylation *in vivo*.

In addition, the results of immunoprecipitation with anti-*c-Maf* followed by blotting with anti-SUMO-1 or anti-*c-Maf* demonstrated that the amounts of both SUMOylated and unSUMOylated *c-Maf* were augmented in Tg-WTc CD4⁺ T cells compared with control cells (Figure 2E and Supplemental Table 2). However, the ratio of SUMOylated to unSUMOylated *c-Maf* in Tg-WTc CD4⁺ T cells was similar to that in control cells (Figure 2E), suggesting that the SUMOylation machinery in CD4⁺ T cells is initiated and regulated in a *c-Maf* dosage-dependent manner. In contrast, the amount of SUMOylated *c-Maf* in Tg-KRc CD4⁺ T cells was comparable to that in control cells (Figure 2E and Supplemental Table 2), indicating that the level of SUMOylated *c-Maf* in Tg-KRc CD4⁺ T cells mainly reflects the SUMOylation of endogenous *c-Maf*. Since the total amount of unSUMOylated *c-Maf* in Tg-KRc CD4⁺ T cells was much higher than that in control cells (Figure 2E and Supplemental Table 2), the ratio of SUMOylated to unSUMOylated *c-Maf* was significantly decreased by approximately 50% in Tg-KRc CD4⁺ T cells compared with control cells (Figure 2E). This unique “low ratio” of SUMOylated/unSUMOylated *c-Maf* in Tg-KRc CD4⁺ T cells may physiologically mimic the SUMOylation status of *c-Maf* in CD4⁺ T cells of 12- to 14-week-old NOD mice, providing a feasible and available model to explore the modulatory role of *c-Maf* SUMOylation in the diabetogenic process in NOD mice.

Accelerated kinetics of autoimmune diabetes in SUMO-defective *c-Maf*-transgenic NOD mice. To investigate whether the SUMOylation status of *c-Maf* in CD4⁺ T cells modulates the pathogenesis of autoimmune diabetes in NOD mice, we first compared the disease kinetics and severity in *c-Maf*-transgenic mice and their littermate controls. The diabetes kinetics and incidence in Tg-WTc and control mice were indistinguishable, indicating that overexpression of wild-type *c-Maf* in T cells does not influence the progress and severity of diabetes in NOD mice (Figure 2F). In contrast, Tg-KRc mice showed accelerated development of diabetes before 13 weeks of age compared with control mice (30% vs. 4%, $P = 0.0236$; Supplemental Figure 2A). At 18 weeks of age, the incidence of diabetes in Tg-KRc mice reached 90%, but in control mice was 37% ($P = 0.0001$; Supplemental Figure 2B). Although the overall diabetic rate at 40 weeks of age was similar in Tg-WTc and Tg-KRc mice, the diabetic kinetics in Tg-KRc mice was significantly faster than that of control mice ($P = 0.005$; Figure 2G), implying that *c-Maf* SUMOylation may negatively modulate the initial stages of the develop-

ment of autoimmune diabetes in NOD mice. To address whether SUMO-defective *c-Maf* mediates this accelerated diabetes development in a T cell-autonomous and/or immune cell-dependent manner, we performed adoptive transfer experiments by injecting pre-diabetic splenocytes from 12- to 14-week-old mice into NOD-SCID mice and monitored their diabetes phenotype. Mice that received Tg-KRc splenocytes showed faster diabetes development than mice that received control or Tg-WTc splenocytes (Figure 2H), indicating that the enhanced immunopathogenesis seen in Tg-KRc mice was immune cell dependent. Since a distal *Lck* promoter-driven transgene can be expressed in all T cell subsets, we further dissected whether CD4⁺ and/or CD8⁺ T cells are mainly responsible for this Tg-KRc-modulated diabetogenesis. We cotransferred effector CD4⁺ T cells (CD4⁺CD25⁻) and CD8⁺ T cells from the relevant mice into NOD.*Rag1*^{-/-} mice and monitored their disease kinetics. Mice that received Tg-KRc CD4⁺ cells plus control CD8⁺ cells developed diabetes more rapidly than mice that received control or Tg-WTc CD4⁺ cells plus control CD8⁺ cells (Figure 2I); the latter group developed diabetes at a rate that was indistinguishable from that in recipients of control CD4⁺ cells plus transgenic CD8⁺ cells (Figure 2J). This result further indicates that the SUMO-defective *c-Maf*-mediated acceleration of diabetes development in Tg-KRc mice is CD4⁺ T cell autonomous. Taken together, our results provide *in vivo* evidence that the SUMOylation status of *c-Maf* is more critical than its level of expression for the initiation and early development of disease in NOD mice, implying the effect of SUMO-defective *c-Maf* on the pathogenesis of autoimmune diabetes.

To investigate whether transgenic SUMO-defective *c-Maf* accelerates diabetic kinetics by modulating the development of peripheral CD4⁺ T cells, we first determined the numbers of CD4⁺ T cells in the spleens of these transgenic mice and observed that the number of splenic CD4⁺ T cells in Tg-WTc or Tg-KRc mice was lower than that in control mice (Supplemental Figure 3A), whereas the number of splenic CD8⁺ T cells was indistinguishable between Tg and control mice (Supplemental Figure 3A), consistent with a previous report that overexpression of *c-Maf* in T cells resulted in a reduced CD4⁺ T cell number in the spleen (23). However, the number of splenic CD4⁺ T cells was comparable between Tg-WTc and Tg-KRc mice (Supplemental Figure 3A), suggesting that the SUMOylation status of *c-Maf* does not additionally impact on CD4⁺ splenocyte survival. Moreover, we observed that the frequency of splenic CD69⁺CD4⁺ or CD62L^{hi}CD4⁺ T cells was indistinguishable among control, Tg-WTc, and Tg-KRc mice (Supplemental Figure 3, B and C, respectively), suggesting that overexpression of *c-Maf*, either wild-type or SUMO-defective, has minimal effect on the expressions of activation and homing molecules of CD4⁺ T cells in NOD mice. Besides, we observed that the frequency of splenic Foxp3⁺CD4⁺ T cells and their expression level of Foxp3 or CD25 were comparable among control, Tg-WTc, and Tg-KRc mice (Supplemental Figure 3, D and E, respectively), suggesting that overexpression of *c-Maf*, either wild-type or SUMO-defective, has minimal effect on the development or the phenotype/function of Tregs in NOD mice.

Overexpression of SUMO-defective *c-Maf* preferentially augments the development of IL-21-producing CD4⁺ T cells. It has been reported that *c-Maf* is required to enhance *Il4* transactivation in Th2 cells (15) and to promote IL-10 and/or IL-21 expression in Th17 (19, 21),

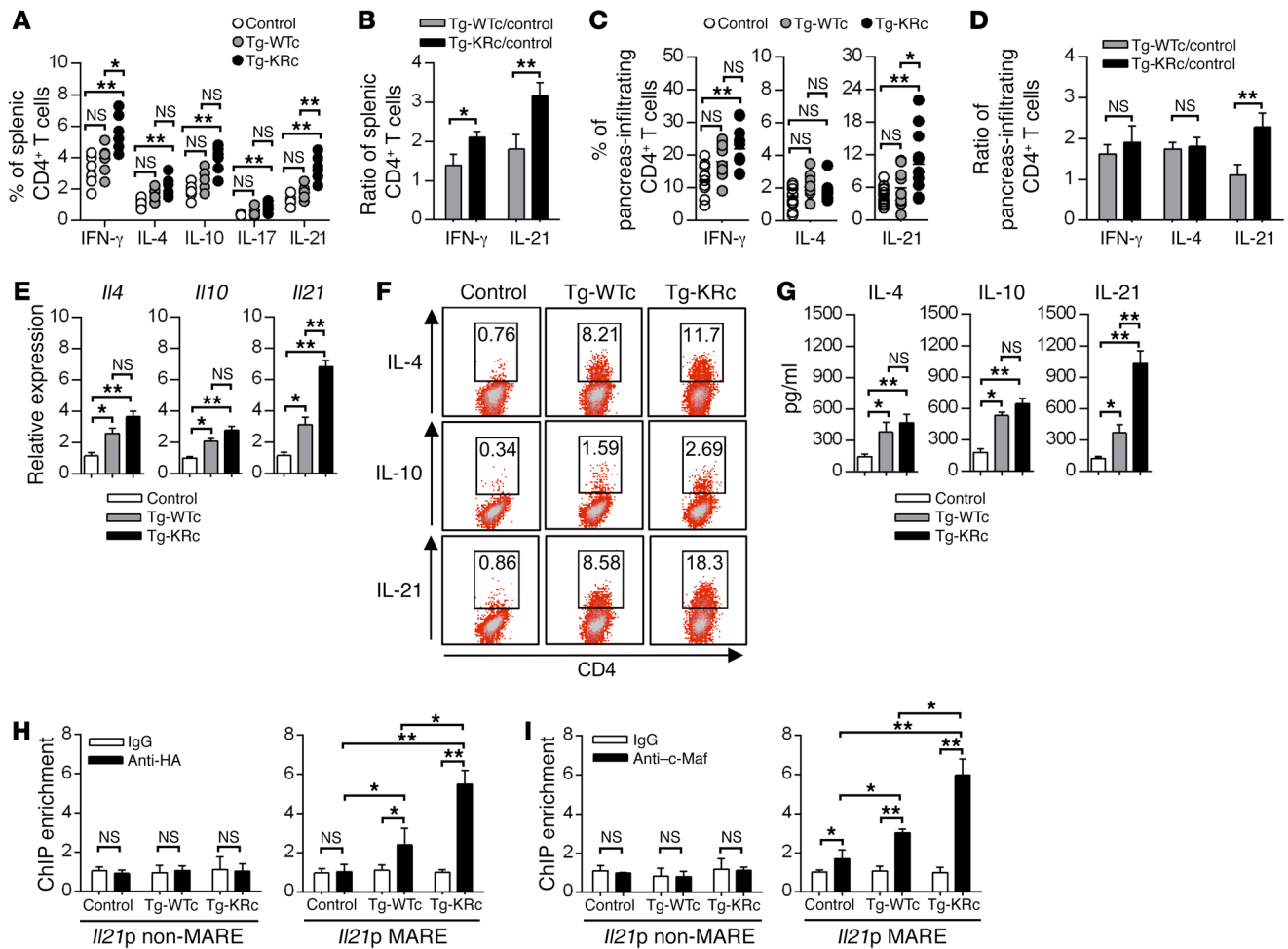


Figure 3. Overexpression of SUMO-defective c-Maf preferentially augments IL-21-producing CD4⁺ T cells by enhancing its recruitment to the //21p. (A and B) Frequencies of IFN- γ ⁺, IL-4⁺, IL-10⁺, IL-17⁺, and IL-21⁺ cells (A) and Tg/control ratio of IFN- γ ⁺, IL-4⁺, and IL-21⁺ cells (B) in splenic CD4⁺ T cells from 12- to 14-week-old control, Tg-WTc, and Tg-KRc NOD mice were analyzed by flow cytometry. (C and D) Frequencies (C) and Tg/control ratio (D) of IFN- γ ⁺, IL-4⁺, and IL-21⁺ cells in pancreas-infiltrating CD4⁺ T cells from 12- to 14-week-old control, Tg-WTc, and Tg-KRc NOD mice were analyzed by flow cytometry. (E) Expressions of *Il4*, *Il10*, and *Il21* mRNA in naive control, Tg-WTc, and Tg-KRc NOD CD4⁺ T cells cultured with anti-CD3 and anti-CD28 for 36 hours. (F) Intracellular staining for IL-4, IL-10, and IL-21 in naive CD4⁺ T cells cultured as described in E for 48 hours. Numbers in outlined areas indicate the percentages of the gated populations. (G) ELISA of IL-4, IL-10, and IL-21 in supernatants of naive CD4⁺ T cells cultured as described in E for 48 hours. (H and I) ChIP analysis of the interaction of c-Maf with the //21p in naive CD4⁺ T cells cultured for 36 hours as described in E. Isotype-matched IgG was used as a control. Data represent the mean \pm SEM; $n = 6$ mice (A and B), $n = 10$ mice (C and D), $n = 3$ mice (E–G), or $n = 5$ mice (H and I) per group; 3–4 independent experiments. * $P < 0.05$; ** $P < 0.01$; 1-way ANOVA with Tukey’s post-test (A, C, E, and G–I) or 2-tailed Student’s *t* test (B and D).

Tfh (19), and type 1 regulatory (24, 25) cells. We first sorted CD4⁺ T cells from 12- to 14-week-old Tg-WTc and Tg-KRc NOD mice and performed RNA sequencing-based transcriptome analyses. Our results revealed that the levels of *Il21* and *Ifng* transcripts were obviously enhanced in the Tg-KRc CD4⁺ T cells compared with Tg-WTc CD4⁺ T cells (Supplemental Figure 4A). Moreover, we observed that overexpression of SUMO-defective c-Maf, but not wild-type c-Maf, significantly expanded the populations of splenic IFN- γ ⁺, IL-4⁺, IL-10⁺, IL-17⁺, and IL-21-producing CD4⁺ T cells in comparison with control mice (Figure 3A). Interestingly, although the frequencies of IFN- γ ⁺CD4⁺ and IL-21⁺CD4⁺ cells in the spleens of Tg-KRc mice were both significantly higher than those in Tg-WTc mice (Figure 3A), the degree of enhancement of IL-21⁺CD4⁺ T cells (1.8 times higher) was greater than that of IFN- γ ⁺CD4⁺ T cells (1.5 times higher) (Figure 3B). These results

suggest that the SUMOylation status of c-Maf has a stronger modulatory effect on the development of cytokine-producing Th cell subsets than the amount of c-Maf and that it is dominant in the regulation of development of IL-21⁺CD4⁺ T cells in the periphery. Moreover, we observed that the splenic populations of CD4⁺ T cells coexpressing IL-21/IFN- γ , IL-21/IL-4, or IL-21/IL-17 were less than 0.5% and the percentage of IL-21⁺/CXCR5⁺ CD4⁺ T cells was less than 1% in both Tg-WTc and Tg-KRc mice (Supplemental Figure 5, A–D). Apparently, these populations were significantly lower, compared with IL-21⁺/IFN- γ , IL-21⁺/IL-4, IL-21⁺/IL-17, or IL-21⁺/CXCR5⁺ CD4⁺ T cells, respectively, in Tg-WTc or Tg-KRc mice (Supplemental Figure 5, A–D). These results suggest that increased population of IL-21⁺CD4⁺ T cells is not overlapped with Th1, Th2, Th17, or Tfh cells in Tg-WTc or Tg-KRc mice. Next, we analyzed the percentages of CD4⁺ T cells expressing IFN- γ , IL-4,

or IL-21 in pancreatic infiltrates from transgenic and control mice. Our results clearly indicated that the IL-21⁺CD4⁺ T cell population in pancreatic infiltrates was markedly increased in Tg-KRc mice compared with that in Tg-WTc mice, whereas the frequencies of pancreas-infiltrating IFN- γ - or IL-4-producing CD4⁺ T cells were comparable between Tg-WTc and Tg-KRc mice (Figure 3, C and D), implying that SUMO-defective c-Maf preferentially augments IL-21-producing CD4⁺ T cells in the pancreas of Tg-KRc mice. Taken together, our results suggested that the SUMOylation status of c-Maf has a stronger modulatory effect on the c-Maf/IL-21 axis in CD4⁺ T cells than the level of expression of c-Maf, and consequently that an additional expression of SUMO-defective c-Maf accelerates the development of diabetes in NOD mice.

SUMO-defective c-Maf preferentially augments the transactivation of Il21 by enhancing its recruitment to the Il21p. To investigate whether SUMO-defective c-Maf expands the IL-21⁺CD4⁺ T cell population in Tg-KRc mice (as shown in Figure 3, A–D) by intrinsically and transcriptionally regulating IL-21 expression in CD4⁺ T cells, we purified naive CD4⁺ T cells and activated them with anti-CD3/CD28, then determined their expression of IL-4, IL-10, and IL-21 in both RNA and protein levels. Our results revealed that the expression of *Il21* transcripts was significantly higher in Tg-KRc cells than in Tg-WTc cells, whereas their levels of *Il4* or *Il10* transcripts were indistinguishable (Figure 3E). Similarly, the percentage of IL-21-producing cells and their levels of IL-21 production were markedly higher in Tg-KRc cells than in Tg-WTc cells (Figure 3F, bottom, and Figure 3G, right), demonstrating that SUMO-defective c-Maf preferentially augments IL-21 but not IL-4 or IL-10 expression in activated CD4⁺ T cells. A ChIP assay revealed that the binding of c-Maf to the *Il21p* MARE, detected using either anti-HA or anti-c-Maf, was significantly higher in Tg-KRc CD4⁺ T cells than in Tg-WTc CD4⁺ T cells (Figure 3, H and I), whereas the levels of binding to the *Il4p* MARE or to the *Il10p* MARE were comparable (Supplemental Figure 5, E–H). This indicates that c-Maf, either transgenic or total, is more efficiently recruited to the *Il21p* than to the *Il4p* or to the *Il10p* in Tg-KRc CD4⁺ T cells. Besides, we observed that transgenic expression of c-Maf, either wild-type or SUMO-defective, upregulated the level of IL-21 mRNA in B6 CD4⁺ T cells (Supplemental Figure 5I). Moreover, the level of IL-21 mRNA was significantly higher in B6.Tg-KRc CD4⁺ T cells than in B6.Tg-WTc CD4⁺ T cells (Supplemental Figure 5I), suggesting that SUMO-defective c-Maf is able to augment IL-21 expression in CD4⁺ T cells regardless of the susceptibility of mouse strains to autoimmune diabetes. Taken together, these results suggest that the SUMOylation status of c-Maf has a stronger effect than its level of expression on transactivation of *Il21* and that this phenomenon is more marked for *Il21* than for *Il4* and *Il10* in NOD CD4⁺ T cells.

SUMO-defective c-Maf promotes differentiation of CD4⁺ T cells with an extrafollicular helper T cell phenotype. It has been reported that IL-21 is critical for the generation of Tfh cells (26, 27) and that impaired production of this cytokine limits the development of Tfh cells in c-Maf-deficient mice after immunization (19). To investigate whether SUMO-defective c-Maf modulates the generation of Tfh cells in NOD mice, we analyzed the expression on CD4⁺ T cells from Tg-KRc mice of a panel of Tfh-associated surface molecules, including ICOS, PD-1, and CXCR5. Our results indicated that overexpression of SUMO-defective c-Maf significantly expanded

the populations of splenic ICOS^{hi}CD4⁺ and PD-1^{hi}CD4⁺ T cells in comparison with control or Tg-WTc mice, whereas the frequency of CXCR5⁺CD4⁺ splenocytes was indistinguishable between control and Tg mice (Figure 4A). Moreover, although the population of CD4⁺ T cells coexpressing ICOS^{hi} and PD-1^{hi} was moderately increased in Tg-WTc mice compared with controls, it was significantly augmented in Tg-KRc mice (Figure 4B), suggesting that CD4⁺ T cells with SUMO-defective c-Maf possess a similar phenotype to Tfh cells. Moreover, we observed that the expression levels of ICOS and PD-1 in ICOS^{lo}PD-1^{lo}CD4⁺ T cells from Tg-KRc mice were significantly higher than those from control and Tg-WTc mice, whereas those in ICOS^{hi}PD-1^{hi}CD4⁺ T cells were comparable (Figure 4C). This phenomenon that preferential upregulation of ICOS and PD-1 in ICOS^{lo}PD-1^{lo}CD4⁺ T cells by SUMO-defective c-Maf signifies the endogenous potential of these cells contributing to the development of the ICOS^{hi}PD-1^{hi}CD4⁺ T cell population.

Tfh cells are typically characterized by CXCR5 expression, which enables them to migrate to B cell follicles and reside within germinal centers (28, 29). Recently, a distinct subset of IL-21-producing extrafollicular helper T (Teffh) cells, which was classified by high expression of ICOS and/or PD-1 but lacked CXCR5, was identified and contributed to pathogenesis in mouse models of autoimmune or autoimmune-like diseases (20, 30–32). We observed that the frequency of splenic Tfh cells (CXCR5⁺ICOS^{hi}PD-1^{hi}CD4⁺) was indistinguishable in all mouse strains (Figure 4D), implying that overexpression of wild-type or SUMO-defective c-Maf has minimal effect on the development of Tfh cells. In contrast, we observed that the population of Teffh cells (CXCR5⁺ICOS^{hi}PD-1^{hi}CD4⁺) was significantly higher in Tg-KRc mice than in control and Tg-WTc mice (Figure 4D). We next determined the level of IL-21 expression in these populations and observed that the levels of *Il21* transcripts consistently increased in a stepwise fashion from non-Tfh cells to Teffh cells to Tfh cells in all strains (Figure 4E), indicating that IL-21 production was highest in Tfh cells, second highest in Teffh cells, and lowest in non-Tfh cells. We also observed that the level of *Il21* transcripts in Tfh cells was comparable in all strains (Figure 4F, right), suggesting that overexpression of c-Maf, either wild-type or SUMO-defective, is unable to augment *Il21* expression in these Tfh cells. Interestingly, the level of *Il21* transcripts in Teffh cells was significantly higher in Tg-KRc mice than in control or Tg-WTc mice (Figure 4F, middle), indicating that SUMO-defective c-Maf predominantly augments *Il21* expression in Teffh cells. Taken together, our results provides the insight that the SUMOylation status of c-Maf has a stronger effect than its level of expression on enhancement of IL-21 expression in Teffh cells rather than the Tfh cells, potentially orchestrating the autoimmune process involved in the destruction of β cells.

SUMO-defective c-Maf expands effector/memory CD8⁺ T cells and enhances their granzyme B production and diabetogenic activity in an IL-21-dependent manner. A previous study demonstrated that a subset of CCR9⁺CD4⁺ T cells with a Tfh-like phenotype contributes to CD8⁺ T cell-dependent autoimmune diabetes in an IL-21-specific manner (20). To evaluate whether augmented IL-21 promotes diabetogenesis of CD8⁺ T cells in Tg-KRc mice, we first analyzed the surface molecules on CD8⁺ T cells, including CD69 (activation), KLRG1 (short-lived effector cells), and CD44 (effector/memory cells). Our results revealed that the frequencies of CD69⁺CD8⁺ and

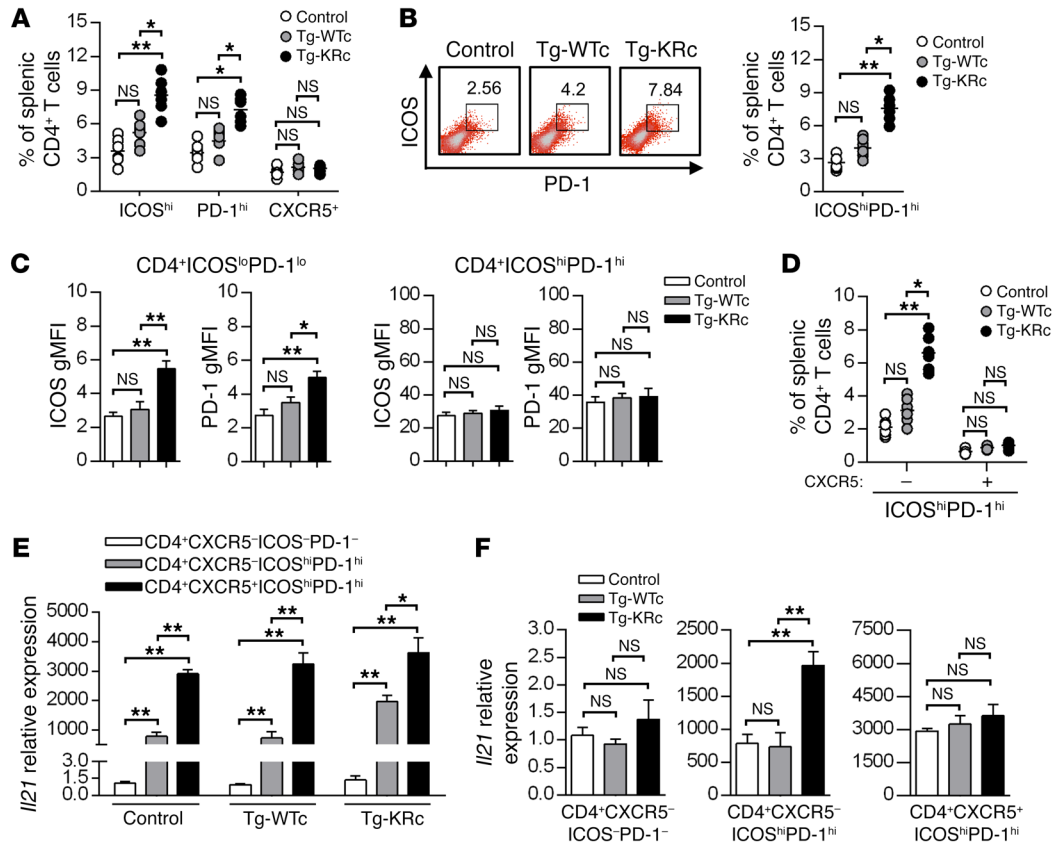


Figure 4. SUMO-defective c-Maf promotes the differentiation of CD4⁺ T cells with an extrafollicular helper T cell phenotype. (A) Flow cytometry analysis of the expression of ICOS, PD-1, and CXCR5 in splenic CD4⁺ T cells from 12- to 14-week-old control, Tg-WTc, and Tg-KRc NOD mice. Summary of the frequencies of ICOS^{hi}CD4⁺, PD-1^{hi}CD4⁺, and CXCR5⁺CD4⁺ T cells. (B) Flow cytometry analysis of the expression of ICOS and PD-1 in splenic CD4⁺ T cells as described in A. Numbers adjacent to outlined areas indicate the percentages of ICOS^{hi}PD-1^{hi} cells (left panel). Right panel: Summary of the frequencies of ICOS^{hi}PD-1^{hi}CD4⁺ T cells. (C) Flow cytometry analysis of ICOS or PD-1 expression in ICOS^{lo}PD-1^{lo}CD4⁺ and ICOS^{hi}PD-1^{hi}CD4⁺ T cells from 12- to 14-week-old control, Tg-WTc, and Tg-KRc NOD mice. Summary of the geometric mean fluorescence intensity (gMFI) of ICOS or PD-1 in ICOS^{lo}PD-1^{lo}CD4⁺ or ICOS^{hi}PD-1^{hi}CD4⁺ T cells. (D) Flow cytometry analysis of the expression of ICOS, PD-1, and CXCR5 in splenic CD4⁺ T cells as described in A. Summary of the frequencies of CXCR5⁺ICOS^{lo}PD-1^{hi}CD4⁺ and CXCR5⁺ICOS^{hi}PD-1^{hi}CD4⁺ T cells. (E and F) Expression of *I/21* mRNA in CXCR5⁺ICOS^{lo}PD-1^{lo}CD4⁺, CXCR5⁺ICOS^{hi}PD-1^{hi}CD4⁺, and CXCR5⁺ICOS^{hi}PD-1^{hi}CD4⁺ T cells from control, Tg-WTc, and Tg-KRc NOD mice. Data represent the mean ± SEM; n = 6 mice (A–D) or n = 3 mice (E and F) per group; 3–4 independent experiments. *P < 0.05; **P < 0.01; 1-way ANOVA with Tukey’s post-test.

KLRG1⁺CD8⁺ splenocytes were indistinguishable between control and Tg mice (Figure 5A), implying that neither wild-type nor SUMO-defective c-Maf affects the activation status or short-lived effector population of CD8⁺ T cells in NOD mice. Interestingly, we observed that the level of *Cd44* transcripts was enhanced in Tg-KRc CD8⁺ T cells compared with Tg-WTc CD8⁺ T cells (Supplemental Figure 4B) and the population of splenic CD44^{hi}CD8⁺ T cells was significantly higher in Tg-KRc mice than in control and Tg-WTc mice (Figure 5A), suggesting that SUMO-defective c-Maf expands effector/memory CD8⁺ T cells in Tg-KRc mice; this is consistent with a previous report that overexpression of IL-21 promotes CD44^{hi}CD8⁺ T cell accumulation (33). We further determined the steady-state expression of cytotoxic T lymphocyte-related genes and observed that the level of *Gzmb* transcripts in CD8⁺ T cells was significantly increased in Tg-KRc mice compared with control and Tg-WTc mice, whereas the levels of *Ifig* and *Prfl* transcripts were comparable in all strains (Figure 5B). Moreover, in all mice, *Gzmb* was more strongly expressed in CD44^{hi}CD8⁺ T cells compared with CD44^{lo}CD8⁺ T cells (Figure 5C). Furthermore, the expression level of granzyme B protein in CD8⁺ T cells was remarkably

higher in Tg-KRc mice compared with that in control or Tg-WTc mice (Supplemental Figure 6A), suggesting that granzyme B, produced at high levels in CD44^{hi}CD8⁺ T cells of Tg-KRc mice, acts as a key molecule in their diabetogenic activity. To investigate further whether enhanced granzyme B expression in CD8⁺ T cells is the result of augmented IL-21 production by Tg-KRc CD4⁺ T cells, we cocultured CD4⁺ and CD8⁺ T cells in a Transwell system. Our results revealed that the percentage of granzyme B-producing CD8⁺ T cells significantly increased in a stepwise fashion from those cells cocultured with Tg-WTc CD4⁺ to those cocultured with Tg-KRc CD4⁺ T cells (Figure 5D, top panel). Moreover, a significant reduction in granzyme B⁺CD8⁺ T cells was observed following IL-21R.Fc administration in cells cocultured with Tg-WTc CD4⁺ or Tg-KRc CD4⁺ T cells (Figure 5D, bottom panel). These in vitro data supported that augmented IL-21 production by Tg-KRc CD4⁺ T cells enhanced more granzyme B expression in CD8⁺ T cells, compared with that of Tg-WTc CD4⁺ T cells.

It has been reported that IL-21/IL-21R signaling in CD8⁺ T cells is critical for the development of autoimmune diabetes in NOD mice (20). To characterize the impact of IL-21R signaling on

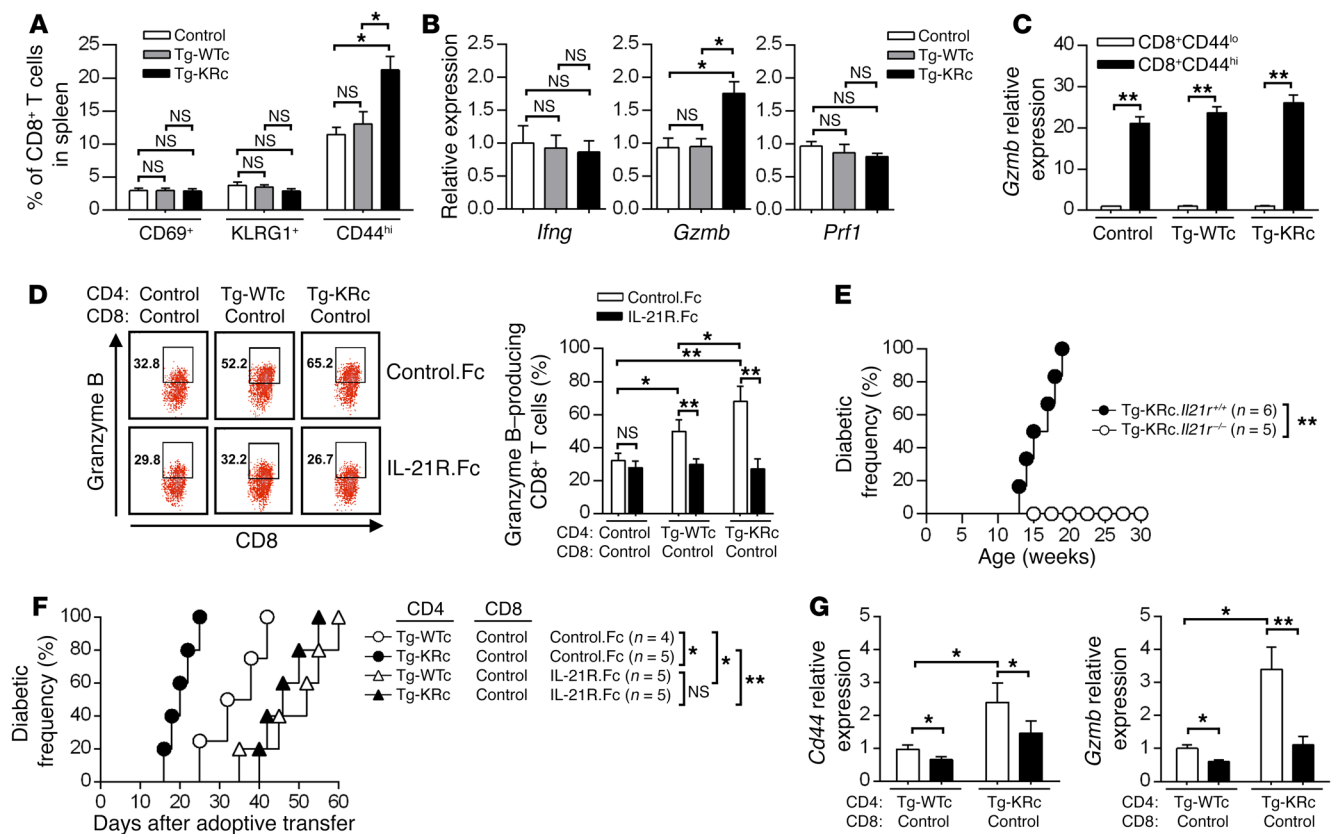


Figure 5. SUMO-defective c-Maf expands effector/memory CD8⁺ T cells and enhances their granzyme B production and diabetogenic activity in an IL-21-dependent manner. (A) Flow cytometry analysis of CD69⁺, KLRG1⁺, and CD44^{hi} in splenic CD8⁺ T cells from 12- to 14-week-old control, Tg-WTc, and Tg-KRc NOD mice. Summary of the frequencies of CD69⁺CD8⁺, KLRG1⁺CD8⁺, and CD44^{hi}CD8⁺ T cells. (B) Expression of *Ifng*, *Gzmb*, and *Prf1* mRNA in splenic CD8⁺ T cells from 12- to 14-week-old control, Tg-WTc, and Tg-KRc NOD mice. (C) Expression of *Gzmb* mRNA in CD44^{lo}CD8⁺ and CD44^{hi}CD8⁺ T cells from 12- to 14-week-old control, Tg-WTc, and Tg-KRc NOD mice. (D) Intracellular staining for granzyme B in control CD8⁺ T cells cocultured with control, Tg-WTc, and Tg-KRc CD4⁺ cells in Transwell chambers for 48 hours with anti-CD3 and anti-CD28 in the presence of control.Fc and IL-21R.Fc (2 μg/ml). Numbers adjacent to outlined areas indicate the percentages of granzyme B⁺ cells. Summary of the frequency of granzyme B⁺CD8⁺ T cells. (E) Diabetes incidence in Tg-KRc.*Il21r*^{+/+} and Tg-KRc.*Il21r*^{-/-} NOD mice. (F) Diabetes incidence in NOD.*Rag1*^{-/-} recipients injected with Tg-WTc and Tg-KRc CD25⁺CD4⁺ T cells plus control CD8⁺ T cells on day 0, and then injected with control.Fc or IL-21R.Fc (10 μg) every 2 days from day 1 to day 13. (G) On day 14, quantitative reverse transcription PCR (RT-qPCR) analysis of *Cd44* or *Gzmb* mRNA expression in CD8⁺ T cells from NOD.*Rag1*^{-/-} recipients reconstituted as described in F. Data represent the mean ± SEM; *n* = 6 mice (A), *n* = 4 mice (B–D), *n* = 3 mice (G) per group; 3 independent experiments (A–D) or 2 independent experiments (F and G). **P* < 0.05; ***P* < 0.01; 1-way ANOVA with Tukey's post-test (A, B, D, and G), 2-tailed Student's *t* test (C), or log-rank test (E and F).

SUMO-defective c-Maf-mediated pathogenesis in vivo, we crossed Tg mice and *Il21r*^{-/-} NOD mice to generate Tg-WTc.*Il21r*^{-/-} mice or Tg-KRc.*Il21r*^{-/-} mice and monitored their diabetic phenotype. Our results revealed that Tg-WTc and Tg-KRc mice deficient in IL-21R were completely protected from diabetic development up to 30 weeks of age, whereas the incidence of diabetes in Tg-WTc.*Il21r*^{+/+} mice reached 75% at 30 weeks of age and all Tg-KRc.*Il21r*^{+/+} mice developed diabetes before 20 weeks of age (Figure 5E and Supplemental Figure 6B, respectively). This result demonstrated the critical role of IL-21 in facilitating diabetic progression in NOD mice. To expound the necessity of IL-21/IL-21R signaling for the pathogenicity of CD4⁺ and/or CD8⁺ T cells, we cotransferred effector CD4⁺ T cells (CD4⁺CD25⁺) from Tg-KRc.*Il21r*^{+/+} or Tg-KRc.*Il21r*^{-/-} mice with CD8⁺ T cells from *Il21r*^{+/+} or *Il21r*^{-/-} mice into NOD.*Rag1*^{-/-} mice and monitored their diabetic kinetics. Our results revealed that the onset of diabetes in mice that received Tg-KRc.*Il21r*^{-/-} CD4⁺/*Il21r*^{+/+} CD8⁺ T cells was delayed compared with that in mice that received Tg-KRc.*Il21r*^{+/+} CD4⁺/*Il21r*^{+/+} CD8⁺

T cells (Supplemental Figure 6C), suggesting that the defect of IL-21 signaling on CD4⁺ T cells diminished their pathogenic activity. Furthermore, NOD.*Rag1*^{-/-} mice that received Tg-KRc.CD4⁺ T cells, either from *Il21r*^{+/+} or *Il21r*^{-/-} background, with *Il21r*^{-/-} CD8⁺ T cells were completely resistant to diabetic development (Supplemental Figure 6C), indicating that prevention of CD8⁺ T cells from receiving IL-21 signals effectively abrogated the pathogenic ability of CD8⁺ T cells and restrained autoimmune diabetes in NOD mice. These results provide evidence that there is differential requirement of IL-21 signaling for the pathogenicity of CD4⁺ and CD8⁺ T cells, implying that SUMO-defective c-Maf-mediated augmented IL-21 has a stronger impact on the pathogenicity of CD8⁺ T cells than on that of CD4⁺ T cells.

To evaluate further whether IL-21 blockage inhibits c-Maf/IL-21/CD8 axis-mediated pathogenicity, we cotransferred effector CD4⁺ T cells (CD4⁺CD25⁺) from transgenic mice and control CD8⁺ T cells into NOD.*Rag1*^{-/-} mice and treated them with IL-21R.Fc protein. Our results revealed that after control.Fc treatment,

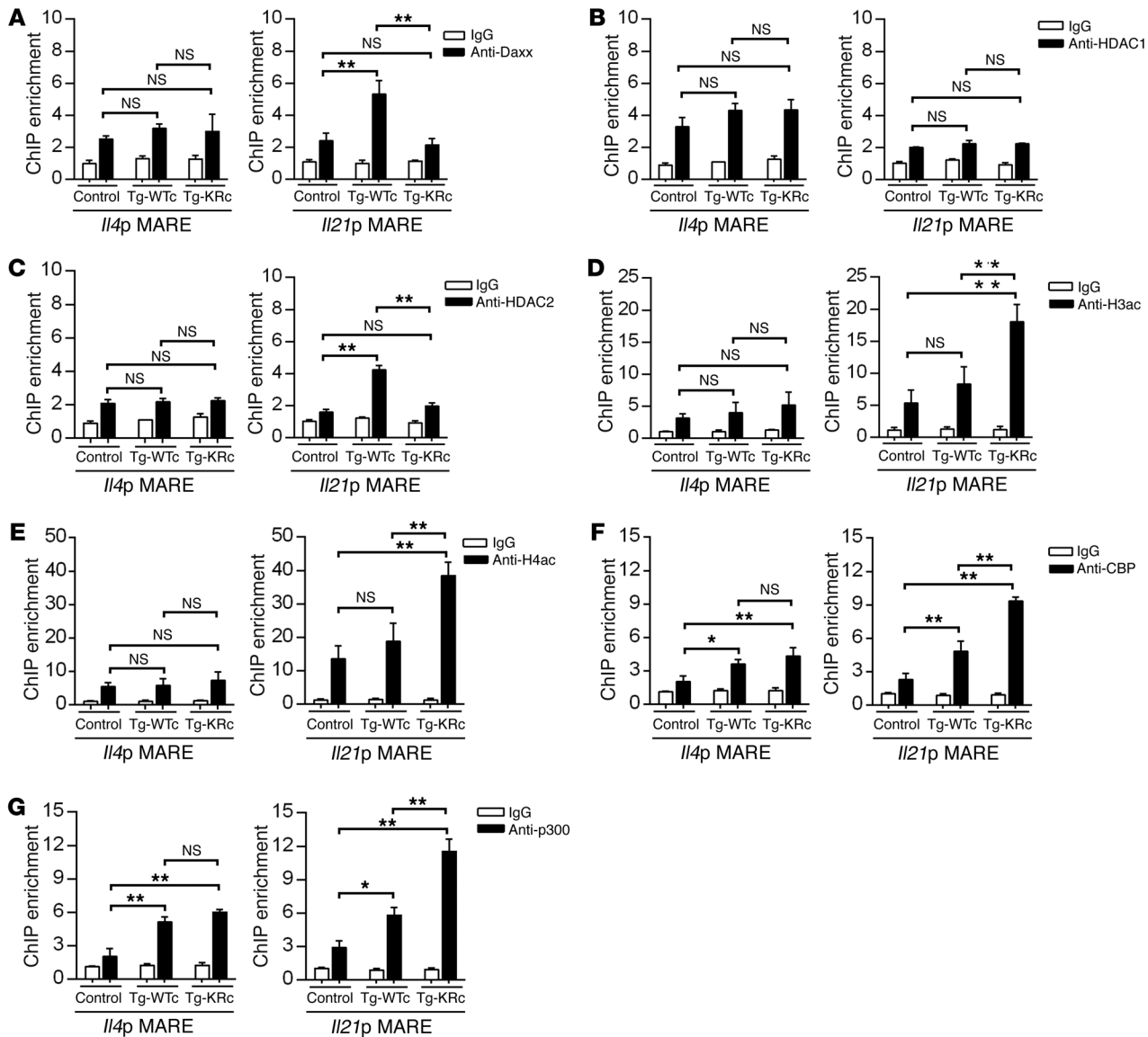


Figure 6. SUMO-defective c-Maf prevents Daxx/HDAC2 recruitment to the *I/21p* and enhances CBP/p300-mediated histone acetylation. (A–C) ChIP analysis of the interaction of Daxx (A), HDAC1 (B), and HDAC2 (C) with the c-Maf-binding sites of the *I/4p* and the *I/21p* in naive control, Tg-WTc, and Tg-KRc CD4⁺ T cells cultured with anti-CD3 and anti-CD28 for 36 hours. (D and E) ChIP analysis of the abundance of H3ac (D) and H4ac (E) in the c-Maf-binding site of the *I/4p* or the *I/21p* in naive CD4⁺ T cells cultured for 36 hours as in A–C. (F and G) ChIP analysis of the interaction of CBP (F) and p300 (G) with the c-Maf-binding site of the *I/4p* or the *I/21p* in naive CD4⁺ T cells cultured for 36 hours as in A–C. Isotype-matched IgG was used as a control. Data represent the mean ± SEM; *n* = 3–5 mice per group; 3 independent experiments. **P* < 0.05; ***P* < 0.01; 1-way ANOVA with Tukey's post-test.

NOD.*Rag1*^{-/-} mice that received Tg-KRc CD4⁺ and control CD8⁺ T cells developed diabetes more rapidly than mice that received Tg-WTc CD4⁺ and control CD8⁺ T cells (Figure 5F and Supplemental Table 3). In contrast, diabetic development in the IL-21R.Fc-treated groups, which was attenuated compared with that in the control.Fc-treated groups, was comparable in recipients of Tg-WTc CD4⁺/control CD8⁺ or Tg-KRc CD4⁺/control CD8⁺ T cells (Figure 5F and Supplemental Table 3), indicating that SUMO-defective c-Maf accelerates diabetes development via an IL-21-dependent crosstalk between CD4⁺ and CD8⁺ T cells. In addition, the levels of *Cd44* and *Gzmb* transcripts in CD8⁺ T cells were significantly higher in mice treated with Tg-KRc CD4⁺/control CD8⁺ cells than in mice treated with Tg-WTc CD4⁺/control CD8⁺ cells, and levels

of these transcripts were significantly decreased following IL-21.Fc administration (Figure 5G). Moreover, we observed that the expression levels of CD44 or granzyme B mRNA and protein in CD8⁺ T cells were significantly decreased on day 14 and day 28 in IL-21.Fc-treated groups, compared with those on day 14 in control.Fc-treated groups, respectively (Supplemental Figure 6, D–G, respectively). Moreover, the expression levels of CD44 or granzyme B in CD8⁺ T cells on day 14 and day 28 were comparable in IL-21R.Fc-treated groups (Supplemental Figure 6, D–G, respectively). However, the expression levels of CD44 or granzyme B in CD8⁺ T cells on day 42 in IL-21R.Fc-treated groups were significantly increased, compared with those on day 14 and day 28, and were similar to those on day 14 in control.Fc-treated groups (Supplemental Figure

6, D–G, respectively). These results provide evidence that the suppressive effect of the IL-21R.Fc treatment on the activation status of CD8⁺ T cells was sustained for up to 14 days and was abolished at 28 days after the final administration. Taken together, these results indicate an IL-21-dependent promotion of CD44 and granzyme B expression in CD8⁺ T cells in vivo and further support the hypothesis that SUMO-defective c-Maf regulates the CD4/IL-21/CD8 axis to accelerate the pathogenesis of diabetes in Tg-KRc mice.

SUMO-defective c-Maf prevents Daxx/HDAC2 recruitment to the Il21p and enhances CBP/p300-mediated histone acetylation. It has been reported that SUMOylation of transcription factors triggers the recruitment of corepressors, such as death domain-associated protein 6 (Daxx), to suppress gene expression (34, 35). To investigate whether the SUMOylation status of c-Maf is involved in Daxx recruitment to the MARE motif, we performed a ChIP assay and observed that Daxx was detected on the *Il4p* MARE and the *Il21p* MARE in control, Tg-WTc, and Tg-KRc CD4⁺ T cells (Figure 6A), indicating its recruitment to the *Il4p* and the *Il21p*. We observed that the level of Daxx binding to the *Il4p* was similar in all cells (Figure 6A), suggesting that Daxx recruitment to the *Il4p* is independent of the SUMOylation status of c-Maf or its level of expression. In contrast, the level of Daxx binding to the *Il21p* was significantly higher in Tg-WTc cells than in control cells (Figure 6A), suggesting a c-Maf dose-dependent enhancement of Daxx recruitment to the *Il21p*. Interestingly, the level of Daxx recruitment to the *Il21p* was significantly lower in Tg-KRc cells than in Tg-WTc cells (Figure 6A), suggesting that SUMO-defective c-Maf may inhibit the recruitment of Daxx to the *Il21p*. Our findings also indicate that SUMOylation-associated Daxx recruitment/detachment is promoter dependent. Since Daxx represses gene transcription by cooperation with histone deacetylases (HDACs) such as HDAC1 (36) and HDAC2 (35), we next evaluated whether HDAC(s) were recruited to the MARE motif. Our results revealed that both HDAC1 and HDAC2 were detected on the MARE motifs in the *Il4p* and the *Il21p* (Figure 6, B and C, respectively), implying that HDAC1 and HDAC2 are involved in Daxx-associated transcriptional repression of c-Maf-targeted promoters. We observed that HDAC1 enrichment to both the *Il4p* and the *Il21p* was comparable in CD4⁺ T cells from control and the 2 transgenic mouse lines (Figure 6B), suggesting that the recruitment of HDAC1 to these motifs is independent of the amount or degree of SUMOylation of c-Maf. Interestingly, similarly to that of Daxx, the level of HDAC2 recruitment to the *Il21p* was markedly increased in comparison with control cells in Tg-WTc cells but not in Tg-KRc cells, whereas its recruitment to the *Il4p* was similar in all cells (Figure 6C). These results imply that HDAC2 is involved in the formation of a Daxx-associated repression complex on the *Il21p* that is initiated in a dose-dependent manner by c-Maf SUMOylation. The acetylation of histones, counterregulated by histone acetyltransferases (HATs) and HDACs, is usually associated with open chromatin and active promoters that enable transcription factors to interact with target sequences (37). We next analyzed the extent of acetylation of histone H3 (H3ac) and H4 (H4ac) on the MARE motifs. Our results revealed that the acetylation level of both H3 and H4 in the *Il21p* was significantly higher in Tg-KRc cells than in control and Tg-WTc cells, whereas their acetylation levels in the *Il4p* were indistinguishable in all cells (Figure 6, D and E, respectively). This

suggested a SUMO-defective c-Maf-associated enhancement of histone acetylation in the *Il21p*. Since HATs such as CREB-binding protein (CBP) or p300 can directly interact with c-Maf and enhance its transactivation on crystallin genes (38), we evaluated the recruitment of CBP and p300 to the *Il4p* and the *Il21p*. Our results revealed that the levels of CBP and p300 binding to the *Il4p* MARE were both increased in Tg-WTc and Tg-KRc CD4⁺ T cells compared with control cells, but were comparable between Tg-WTc and Tg-KRc cells (Figure 6, F and G, respectively), suggesting that the SUMOylation status of c-Maf has a minimal effect on modulation of CBP/p300 recruitment to the *Il4p*. However, CBP and p300 recruitment to the *Il21p* significantly increased in a stepwise fashion from control to Tg-WTc to Tg-KRc CD4⁺ T cells (Figure 6, F and G, respectively). These results suggested that SUMO-defective c-Maf may augment CBP/p300 recruitment and subsequently enhance histone acetylation in the *Il21p* of Tg-KRc CD4⁺ T cells. Besides, we observed that the acetylation levels of both H3 and H4 in the *Il21p* MARE were significantly higher in 12- to 14-week-old NOD CD4⁺ T cells compared with 12- to 14-week-old B6 CD4⁺ T cells, whereas they were comparable between 6- to 8-week-old B6 and NOD CD4⁺ T cells (Supplemental Figure 7, A and B, respectively). The finding of an age-associated increase of histone acetylation in NOD CD4⁺ T cells supported the idea that enhanced histone acetylation was inversely correlated with the attenuation of c-Maf SUMOylation and was involved in the pathogenesis of autoimmune diabetes. Taken together, our results provide an interesting clue that SUMO-defective c-Maf deters Daxx/HDAC2 recruitment to the *Il21p* and promotes CBP/p300-mediated histone acetylation, implying the existence of a c-Maf SUMOylation-based epigenetic regulation of the *Il21p*.

A CBP/p300 inhibitor attenuates SUMO-defective c-Maf-mediated transactivation of Il21 and ameliorates autoimmune diabetes in NOD mice. To evaluate whether pharmacological targeting of CBP/p300 affects SUMO-defective c-Maf-mediated transactivation of *Il21* in CD4⁺ T cells, we treated these T cells with CBP30, which has been reported to inhibit CBP/p300 via specifically binding to their bromodomains (39). Our results revealed markedly reduced levels of *Il21* transcripts in CBP30-treated control, Tg-WTc, or Tg-KRc cells, compared with the respective solvent-treated groups (Figure 7A). Moreover, the levels of c-Maf binding to the *Il21p* were significantly decreased in the CBP30-treated groups compared with solvent-treated groups (Figure 7B), indicating that CBP30 inhibits c-Maf-mediated transactivation of *Il21* in NOD CD4⁺ T cells. Furthermore, we observed that CBP/p300 recruitment to the *Il21p* was much lower in CBP30-treated groups than in solvent-treated groups (Figure 7, C and D, respectively), whereas the acetylation levels of both histones H3 and H4 in the *Il21p* were significantly decreased in CBP30-treated groups compared with solvent-treated groups (Figure 7, E and F, respectively). These results suggest that CBP30 attenuates c-Maf-mediated transactivation of *Il21* by downregulating CBP/p300-mediated histone acetylation in NOD CD4⁺ T cells. To evaluate whether CBP30 has the potential to modulate SUMO-defective c-Maf-mediated diabetogenesis in vivo, we cotransferred effector CD4⁺ T cells (CD4⁺CD25⁻) from transgenic mice with control CD8⁺ T cells into NOD.*Rag1*^{-/-} mice that were then treated with CBP30. Our results indicated that after solvent

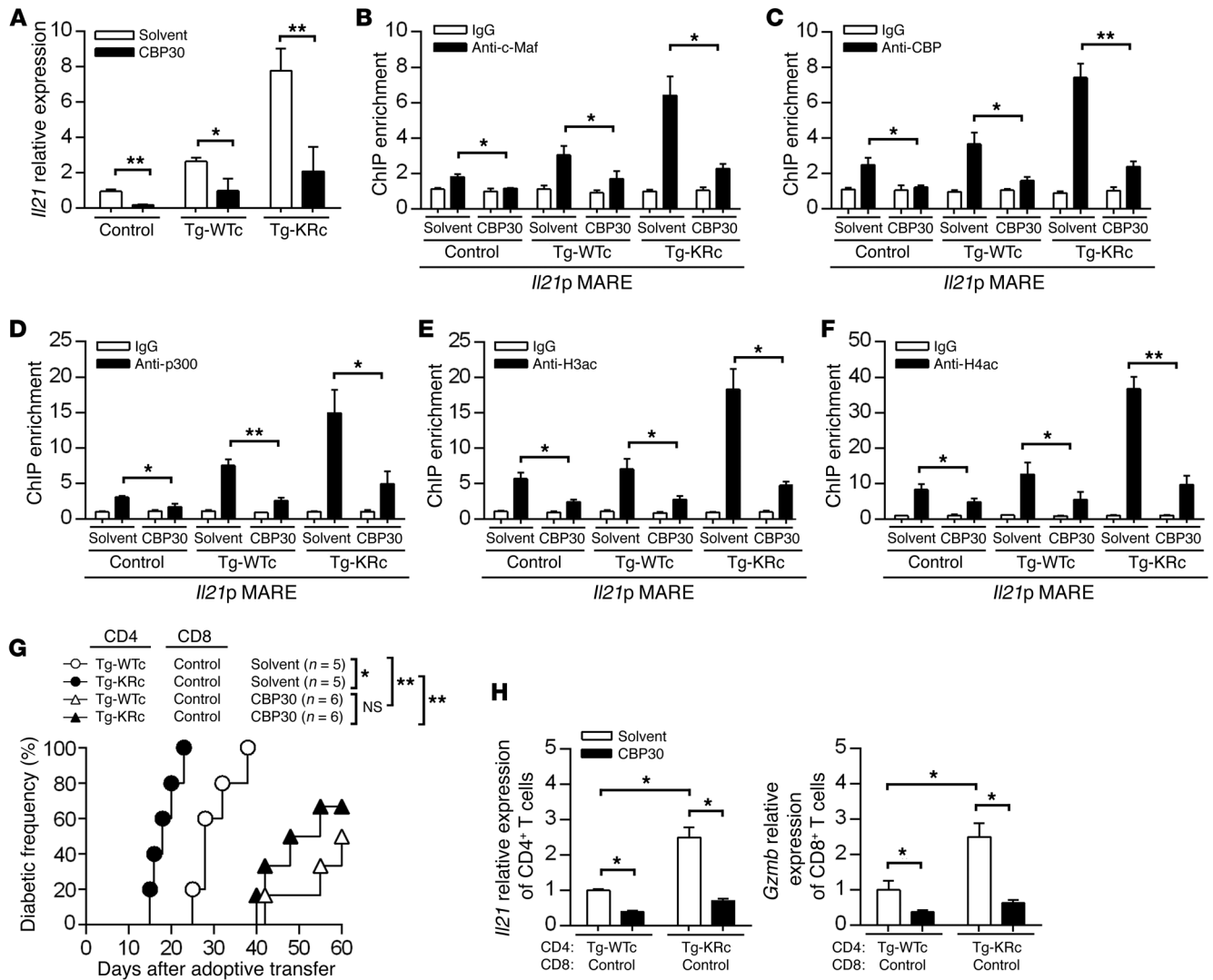


Figure 7. A CBP/p300 inhibitor attenuates SUMO-defective c-Maf-mediated transactivation of *Il21* and ameliorates autoimmune diabetes in NOD mice. (A) Expression of *Il21* mRNA in naive control, Tg-WTc, and Tg-KRc CD4⁺ T cells cultured for 36 hours with anti-CD3 and anti-CD28 in the presence of CBP30 (2 μM) or its solvent (DMF), which were added after 18 hours of culture. (B–D) ChIP analysis of the interaction of c-Maf (B), CBP (C), and p300 (D) with the c-Maf-binding site in the *Il21* pMARE in naive CD4⁺ T cells cultured for 36 hours as in A. (E and F) ChIP analysis of the abundance of H3ac (E) and H4ac (F) in the c-Maf-binding site of the *Il21* pMARE in naive CD4⁺ T cells cultured for 36 hours as in A. Isotype-matched IgG was used as a control. (G) Diabetic incidence in NOD.*Rag1*^{-/-} recipients injected with effector Tg-WTc and Tg-KRc CD4⁺ T cells (CD4⁺CD25⁻) plus control CD8⁺ T cells on day 0, and then injected with 2 mg/kg CBP30 or its solvent (DMF) every 2 days from day 1 to day 13. (H) On day 14, RT-qPCR analysis of *Il21* mRNA expression in CD4⁺ T cells and *Gzmb* mRNA expression in CD8⁺ T cells from NOD.*Rag1*^{-/-} recipients reconstituted as in G. Data represent the mean ± SEM; n = 3 mice (A and H), n = 5 mice (B–F), n = 5–6 mice (G) per group; 3 independent experiments (A–F) or 2 independent experiments (G and H). *P < 0.05; **P < 0.01; 2-tailed Student’s t test (A–F), log-rank test (G), or 1-way ANOVA with Tukey’s post-test (H).

treatment, NOD.*Rag1*^{-/-} mice that received Tg-KRc CD4⁺/control CD8⁺ cells developed diabetes more rapidly than mice that received Tg-WTc CD4⁺/control CD8⁺ cells (Figure 7G and Supplemental Table 4). Strikingly, the onset of diabetes was significantly delayed and its incidence was lower in CBP30-treated mice that received either Tg-WTc CD4⁺/control CD8⁺ or Tg-KRc CD4⁺/control CD8⁺ cells than in solvent-treated mice (Figure 7G and Supplemental Table 4). Moreover, the levels of transcripts of *Il21* in CD4⁺ cells and *Gzmb* in CD8⁺ cells were significantly reduced in CBP30-treated mice compared with solvent-treated mice (Figure 7H). Furthermore, we observed that the expression levels of IL-21 mRNA and protein in CD4⁺ T cells and granzyme B mRNA

and protein in CD8⁺ T cells were significantly decreased on day 14, day 28, and day 42 in CBP30-treated groups, compared with those on day 14 in solvent-treated groups (Supplemental Figure 8, A–D), suggesting that the impairment of the c-Maf-mediated IL-21/granzyme B signaling axis by CBP30 was sustained up to 28 days after the end of treatment. These results indicated that CBP30 markedly impairs the c-Maf-mediated IL-21/granzyme B signaling axis in vivo and may protect NOD mice from autoimmune diabetes. Taken together, our results provide the new insight that pharmacological intervention targeting CBP/p300 is a possible therapeutic strategy to ameliorate the c-Maf-mediated/IL-21-based pathogenesis of autoimmune diabetes.

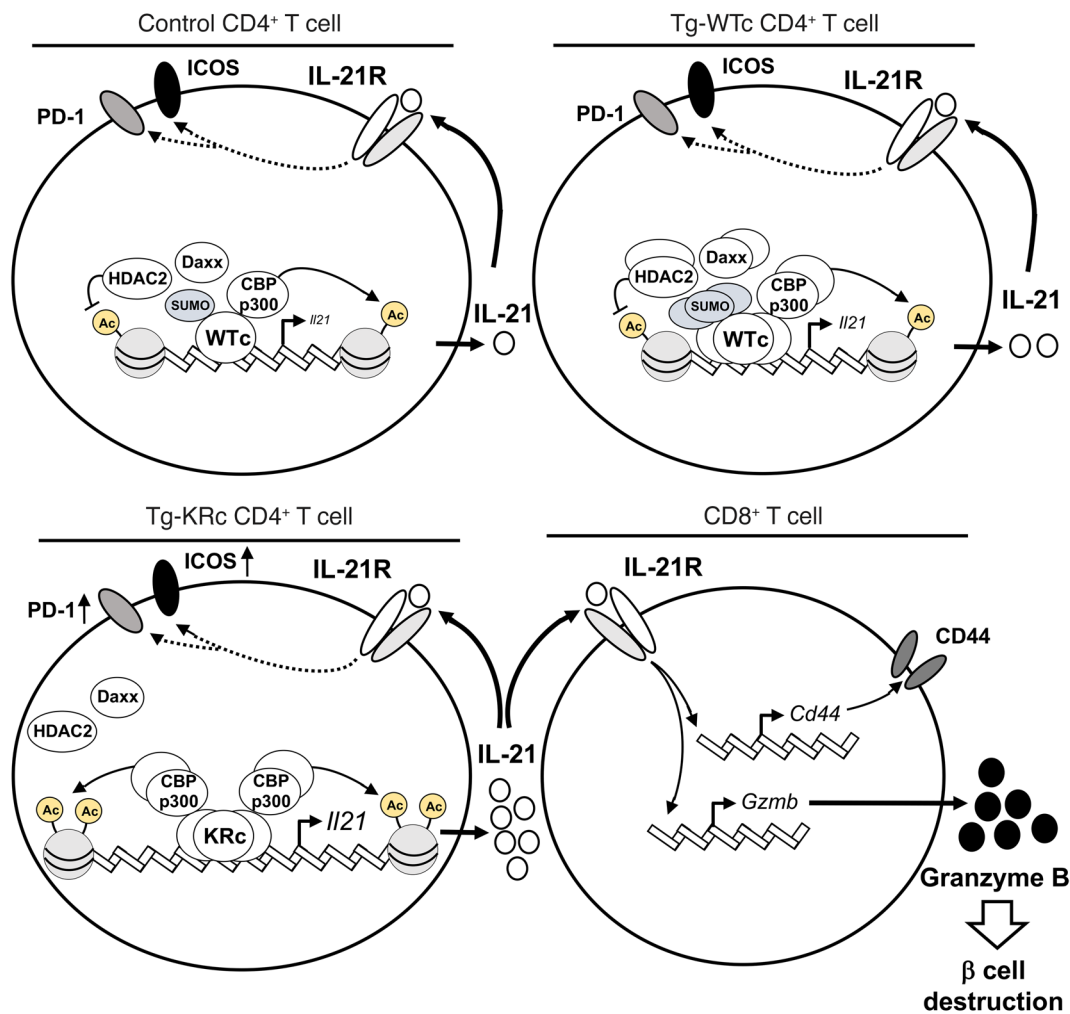


Figure 8. Schematic diagrams illustrating the critical role of SUMOylation in the regulation of c-Maf-mediated IL-21 production.

In this study, we demonstrated that the SUMOylation status of c-Maf possessed a stronger effect than its level of expression on the initiation and early development of autoimmune diabetes in NOD mice. This unique process promotes the differentiation of CD4⁺ T cells toward a Tefh cell phenotype and expands the numbers of granzyme B-producing effector/memory CD8⁺ T cells to accelerate the onset of diabetes in an IL-21-dependent manner. Furthermore, SUMO-defective c-Maf selectively deterred recruitment of Daxx/HDAC2 to the *Il21p* and enhanced CBP/p300-mediated histone acetylation, revealing a mechanism of c-Maf SUMOylation-based epigenetic regulation in NOD mice (Figure 8). These findings provide new insights into the underlying mechanisms linking the SUMOylation status of a single transcription factor with the pathogenesis of autoimmune diabetes.

Discussion

Global deficiencies in SUMOylation enzymes such as UBC9 and PIAS1 have been reported to impact significantly on the pathogenesis and severity of inflammatory diseases (2, 3). Accumulating evidence also indicates that selective deletion of *Ubc9* within Tregs results in fatal early-onset autoimmunity (4). These reports indicated the physiological significance of SUMOylation in inflammatory diseases. It has been reported that reactive oxygen species

(ROS) are the key regulators that control the SUMO-deSUMOylation equilibrium in cells (40). Hydrogen peroxide-induced (H₂O₂-induced) ROS attenuated SUMOylation by interfering with the formation of disulfide bonds in SUMO E1 and E2 (41). Moreover, a high concentration of ROS impaired T cell receptor-driven Myc upregulation in CD4⁺ T cells (42). Myc has been reported to be a master regulator of the SUMOylation system, which acts by inducing transcription of the genes of the SUMO enzymatic cascades (43). These results suggest that the levels of ROS may inversely correlate with the SUMOylation status of proteins in CD4⁺ T cells. A recent study demonstrated that both exogenous and endogenous sources of ROS are involved in initiating cytokine responses of CD4⁺ T cells (44). The loss of ROS synthesis significantly alters the effector function of T cells and ameliorates autoimmune diabetes in NOD mice (45). Interestingly, we also observed that the level of ROS in CD4⁺ T cells was moderately higher in 12- to 14-week-old mice than in 6- to 8-week-old mice (data not shown), suggesting that an age-dependent upregulation of ROS may impair the expression and function of SUMO enzymes and thereby attenuate c-Maf SUMOylation in NOD CD4⁺ T cells, resulting in an increased pathogenic effector function.

A previous report has revealed that any single-lysine mutation (K to R) of c-Maf was not able to prevent its ubiquitination,

suggesting that ubiquitination of this transcription factor was mediated by multiple lysine residues (46). Subsequent studies demonstrated that polyubiquitination of c-Maf was mediated by the ubiquitin-conjugating enzyme UBE2O (47) and the ubiquitin ligase HERC4 (48). UBE2O mediated ubiquitination at K331 and K345 of c-Maf (47), and HERC4 modulated ubiquitination at K85 and K297 of c-Maf (48). Moreover, the study of Xu et al. indicated that UBE2O-mediated c-Maf polyubiquitination was comparable between wild-type c-Maf and K33/34R c-Maf (47). Together, these results suggest that the mutation of K33 residue alone in c-Maf is less likely to affect its ubiquitination.

Previous results from Pauza and colleagues demonstrated that transgenic expression of c-Maf controlled by a modified *Cd4* promoter, which allowed its expression in both CD4⁺ and CD8⁺ T cells, had minimal effect on the development of autoimmune diabetes in NOD mice (16). However, this modified CD4 promoter has been reported to drive transgene expression at a very early stage of thymocyte development and may potentially affect the maturation and selection of autoreactive T cells (49). To avoid this possible influence, we generated c-Maf-transgenic mice in which expression was driven by the distal *Lck* promoter, which allows transgene expression from the late stages of thymocyte development (50). Nonetheless, similarly to previous reports, our data from these Tg-WTc mice revealed that overexpression of wild-type c-Maf in T cells does not influence the progress and severity of diabetes in NOD mice. This indicates that transgenic c-Maf-mediated modulation of autoimmune diabetes in NOD mice is very limited and is independent of the stage at which it is expressed and its quantity. In contrast, the results from our Tg-KRc mice provided *in vivo* evidence that, compared with a quantitative increase of c-Maf in CD4⁺ T cells, the SUMOylation status of this transcriptional factor has a much greater effect on the initiation and early development of autoimmune diabetes in NOD mice.

Previous studies reported that memory CD4⁺ T cells from T1D patients express high levels of IL-21 (13, 14), consistent with the observation that NOD mice deficient in IL-21 completely resist diabetic development (12). These results support a critical role of IL-21 in the pathogenesis of T1D. However, there is no direct evidence to support that c-Maf SUMOylation-modulated IL-21 expression is involved in the pathogenesis of human autoimmune diabetes. Whether the pathogenic mechanism of c-Maf SUMOylation-regulated IL-21 expression elaborated in the NOD mouse model is similar in human T1D patients needs to be further explored.

It has been reported that genetic deletion of granzyme B did not confer resistance to the development of spontaneous diabetes in NOD mice (51, 52). Kawakami and colleagues had observed that the levels of *Gzma* and *Fas* transcripts were significantly increased in the islets of NOD.*Gzmb*^{-/-} mice compared with those of NOD mice (52), suggesting that deficiency of granzyme B may result in upregulations of other cytolytic molecules, such as granzyme A and Fas, to induce β cell apoptosis in NOD.*Gzmb*^{-/-} mice. In our results, other than granzyme B, we observed that the transcript of *Tnfa*, one of the key inflammatory cytokine genes in NOD mice, was enhanced in Tg-KRc CD8⁺ T cells compared with Tg-WTc CD8⁺ T cells (Supplemental Figure 4B). Moreover, the transcripts of proinflammatory or inflammatory cytokine receptor genes, such as *Il1r1*, *Il7ra*, *Il12rb1*, *Il15r*, and *Il18r*, were also upregulated in Tg-KRc CD8⁺ T cells (Supplemental Figure 4B). These results suggested that oth-

er cytotoxic T lymphocyte-associated molecules besides granzyme B may also be involved in acceleration of diabetes in Tg-KRc mice.

In summary, we demonstrated that the SUMOylation status of c-Maf possesses a stronger effect than its level of expression to regulate IL-21 expression in T cells through an epigenetic mechanism. These data provide new insights into how SUMOylation modulates the pathogenesis of autoimmune diabetes in a T cell-restricted manner and single transcription factor-based manner.

Methods

Mice. NOD/Sytwu mice (K^d, D^b, I-A^{g7}, I-E^{mutl}), NOD-SCID mice, NOD.*Rag1*^{-/-} mice, and *Il21r*^{-/-} mice were originally purchased from The Jackson Laboratory. C57BL/6 mice were obtained from the National Laboratory Animal Center, Taipei, Taiwan. *Il21r*^{-/-} mice were backcrossed to the NOD strain for 5 generations. The protocol to generate transgenic mice was as described previously (53). Briefly, HA-tagged mouse wild-type *c-Maf* or K33R *c-Maf* cDNA, a gift from Shi-Chuen Miaw (College of Medicine, National Taiwan University, Taipei, Taiwan), was subcloned into the distal *Lck* promoter expression vector to generate the dLck-HA-WTc or dLck-HA-KRc constructs. The linearized DNA fragment was purified and microinjected into the pronuclei of 1-cell NOD embryos. These injected embryos were then implanted into pseudopregnant (BALB/c \times FVB) F₁ females. Tg-WTc and Tg-KRc NOD mice were backcrossed to the C57BL/6 strain for 6 generations. All mice were bred in the specific pathogen-free facility of the animal center at the National Defense Medical Center (Taipei, Taiwan).

Statistics. The log-rank (Mantel-Cox) test was used for the comparison of survival curves. A 2-tailed Student's unpaired *t* test was used for comparisons between 2 groups, and 1-way ANOVA with Tukey's post-test was used for multigroup comparisons. A *P* value less than 0.05 was considered significant.

Study approval. All protocols using live animals were carried out in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee at the National Defense Medical Center.

Additional methods can be found in Supplemental Methods, available online.

Author contributions

CYH performed experiments and analyzed data. LTY, SHF, MWC, and YWL performed experiments. SCM and DMC gave advice. CYH and HKS wrote the manuscript.

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