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Research Article **Immunology**

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The type III histone deacetylase Sirt1 is essential for maintenance of T cell tolerance in mice

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Although many self-reactive T cells are eliminated by negative selection in the thymus, some of these cells escape into the periphery, where they must be controlled by additional mechanisms. However, the molecular mechanisms underlying peripheral T cell tolerance and its maintenance remain largely undefined. In this study, we report that sirtuin 1 (Sirt1), a type III histone deacetylase, negatively regulates T cell activation and plays a major role in clonal T cell anergy in mice. In vivo, we found that loss of Sirt1 function resulted in abnormally increased T cell activation and a breakdown of $CD4^+$ T cell tolerance. Conversely, upregulation of Sirt1 expression led to T cell anergy, in which the activity of the transcription factor AP-1 was substantially diminished. Furthermore, Sirt1 interacted with and deacetylated c-Jun, yielding an inactive AP-1 factor. In addition, Sirt1-deficient mice were unable to maintain T cell tolerance and developed severe experimental allergic encephalomyelitis as well as spontaneous autoimmunity. These findings provide insight into the molecular mechanisms of T cell activation and anergy, and we suggest that activators of Sirt1 may be useful as therapeutic agents for the treatment and/or prevention of autoimmune diseases.

Introduction

Many self-reactive T cells are eliminated by negative selection during development in the thymus (central tolerance), but leaking of autoreactive T cells into the periphery can occur. One of the additional mechanisms to inactivate self-reactive T cells in the periphery is clonal anergy (peripheral tolerance), which is induced by partial or suboptimal stimulation (1–3). A breakdown of peripheral tolerance is considered an important mechanism in autoimmunity. Activation of T cells requires the cooperative interactions of several transcription factors, including AP-1, NF-κB, and NFAT. Among these transcription factors, AP-1 is selectively inhibited in peripheral T cell tolerance (4). However, the molecular mechanisms by which AP-1 transcriptional activity is inhibited in tolerized autoreactive T cells remain largely unknown.

Sirtuin 1 (Sirt1) is the human ortholog of the yeast Sir2 protein, which is the prototypic class III histone deacetylase (HDAC) (5). This protein contains one HDAC domain that has the deacetylation activity, one nuclear localization sequence, and a coiled-coil–like domain. Sirt1 is highly expressed in the heart, brain, and skeletal muscle and is expressed at very low levels in the kidney and lung (6). In vitro studies indicated that Sirt1 deacetylates a variety of proteins including histones H1, H3, and H4 and may mediate heterochromatin formation (7). Several other proteins besides histones can serve as substrates for Sirt1 (8). Indeed, Sirt1 regulates the tumor suppressor proteins p53 and FOXO3 to suppress apoptosis and promote cell survival. Also, it plays a role in several biological processes including stress resistance, metabolism, differentiation, and aging (5). Mice carrying 2 null alleles of the *Sirt1* gene are significantly smaller than wild-type animals at birth and exhibit notable developmental defects of the retina and heart, and both sexes are sterile (9, 10).

Sirt1 is expressed in all tissues but is abundant in the thymus, particularly in $CD4^+CD8^+$ thymocytes, suggesting an involvement of Sirt1 in T cell development. $CD4^+CD8^+$ thymocytes from *Sirt1*$^{-/-}$ mice exhibit increased sensitivity to γ irradiation–induced apoptosis (10). Moreover, several studies suggest that Sirt1 may negatively regulate T cell activation. Indeed, treatment of T cells with resveratrol, a Sirt1 activator, suppresses proliferation and cytokine production in vitro (11). Resveratrol suppresses immune functions by inducing lymphocyte apoptosis (12, 13). Downregulation of APC functions is another possible mechanism for the immune-suppressive functions of resveratrol (14). While the mechanisms of resveratrol action remain debatable, its interference with immune function is well established and provides a potential avenue for treatment of autoimmune diseases as well as allograft rejections.

In the present study, we demonstrate that Sirt1 functions as an anergic factor in peripheral $CD4^+$ T cell tolerance. *Sirt1*$^{-/-}$ mice have elevated immune responses and fail to maintain peripheral tolerance to autoantigens, as exemplified by the presence of anti-nuclear antibodies, systemic lymphocyte infiltration, and increased susceptibility to experimental autoimmune encephalomyelitis (EAE). Sirt1 suppression of AP-1 transcriptional activity likely represents a central mechanism for control of T cell activation and induction of anergy. Indeed, we found that Sirt1 inhibits AP-1 transcriptional activity by deacetylating the AP-1 family transcription factor c-Jun. This previously unrecognized observation

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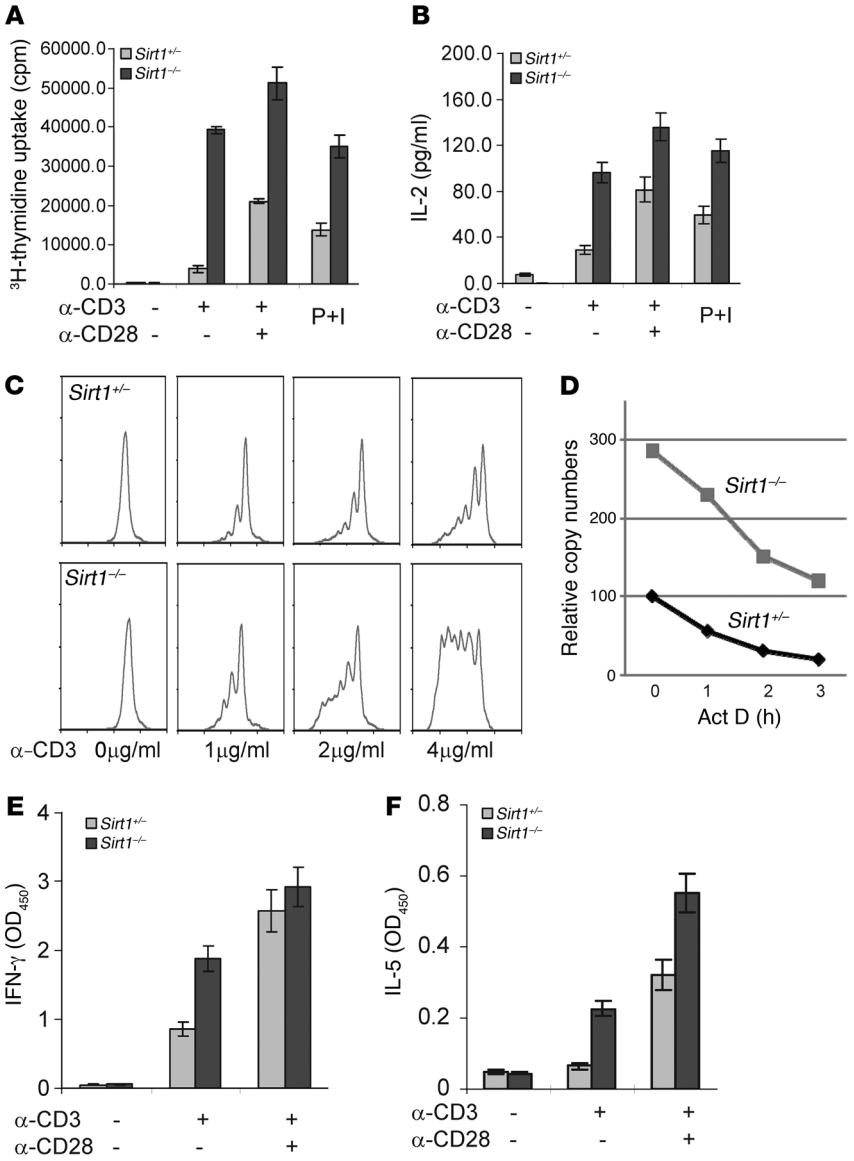

Figure 1
Sirt1 inhibits T cell activation in vitro**.** Primary CD4$^+$ T cells were isolated from splenocytes of *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice. Cells were stimulated with anti-CD3 or anti-CD3 plus anti-CD28, or with PMA plus ionomycin (P+I) as a control, for 3 days. (**A**) Proliferation was analyzed with a ^{3}H-thymidine incorporation assay**.** (**B**) The production of IL-2 was analyzed by ELISA. In **A** and **B**, data are from 3 independent experiments (mean ± SD). (**C**) Flow cytometry of purified CD4$^+$ T cells labeled with CFSE and left unstimulated or stimulated with anti-CD3 at the concentrations for 5 days. (**D**) Mouse primary T cells were isolated from *Sirt1*$^{-/-}$ and *Sirt1*$^{+/-}$ mice and cultivated with anti-CD3 plus anti-CD28 for 24 hours. Cells were then treated with 10 μg/ml of actinomycin D (Act D) for different amounts of time as indicated. The levels of mouse *Il2* mRNA were analyzed by real-time RT-PCR. The production of IFN-γ (**E**) and IL-5 (**F**) by CD4$^+$ T cells after 3-day stimulation was analyzed by ELISA. In **E** and **F**, data are from 3 independent experiments (mean ± SD).

provides a molecular mechanism for modulation of T cell activation and manifestation of anergy.

Results

Sirt1 inhibits T cell activation. Sirt1 was highly expressed in lymphoid tissues including the thymus, bone marrow, lymph nodes, and spleen (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38902DS1). However, disruption of Sirt1 expression in mice appeared not to affect T cell development, because the cell surface expression of CD4 or CD8 in *Sirt1*$^{-/-}$ thymocytes was comparable to that in heterozygous mice (Supplemental Figure 2A). Similarly, the percentage of CD4$^+$ and CD8$^+$ mature T cells did not change in peripheral lymphoid tissues such as the spleen. The ratios of B220$^+$ B cells to CD3$^+$ T cells in the spleens and lymph nodes were also comparable in *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice (Supplemental Figure 2A).

After stimulation with anti-CD3 or anti-CD3 plus anti-CD28 antibodies, *Sirt1*$^{-/-}$ T cells showed dramatically increased proliferation and produced more IL-2 compared with *Sirt1*$^{+/-}$ T cells (Figure 1, A–C), suggesting that Sirt1 suppresses T cell activation. Sirt1 appeared to inhibit IL-2 transcription without affecting *Il2* mRNA stability in T cells because the *Il2* mRNA level, but not its half-life, was increased in *Sirt1*$^{-/-}$ T cells during activation (Figure 1D). The enhanced activation of *Sirt1*$^{-/-}$ T cells was not due to preexisting activated T cells because the percentages of T cells bearing the activation markers CD69, CD44, and CD25 were similar in heterozygous and mutant mice (Supplemental Figure 2B). Also, the enhanced activation was not due to a higher level of cell surface TCR, as heterozygous and mutant mice displayed similar TCR expression (Supplemental Figure 2C). Thus, we determined that Sirt1 intrinsically inhibits T cell activation. Indeed, deletion of *Sirt1* gene expression in vitro by tamoxifen treatment of CD4$^+$ T cells from Sirt1$^{loxp/loxp}$ESR-Cre^{TG} mice (Supplemental Methods) resulted in a dramatically increased CD4$^+$ T cell proliferation (Supplemental Figure 3A), and ecotropic expression of Sirt1 inhibited both *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ T cell activation (Supplemental Figure 3B).

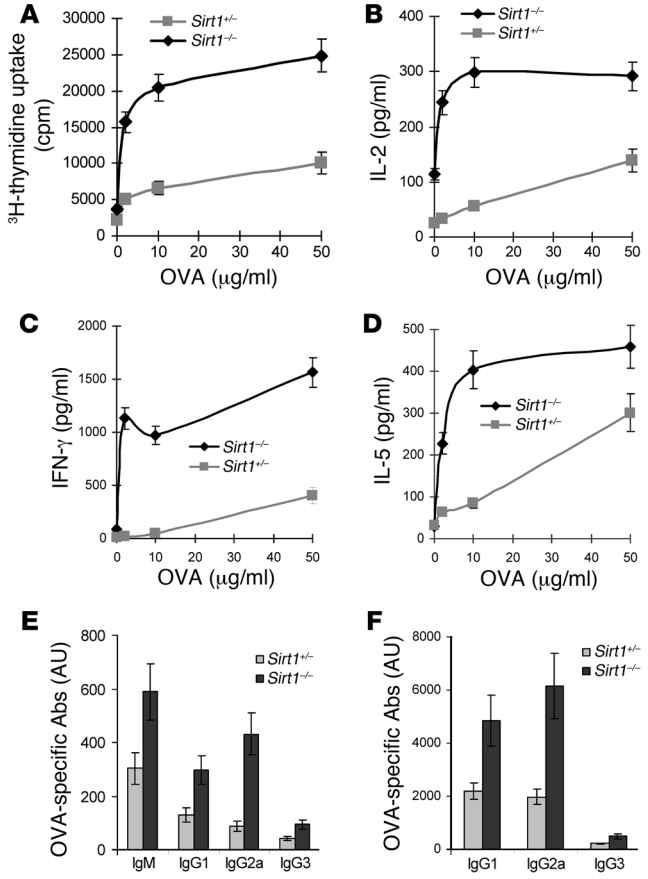

Figure 2
Analysis of T cell–mediated immune responses. *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice were immunized with 200 μg OVA protein in CFA. (**A**–**D**) Seven days later, mice were euthanized and total splenocytes were cultured with different amounts of OVA. (**A**–**D**) Proliferation (**A**) and production of IL-2 (**B**), IFN-γ (**C**), and IL-5 (**D**) were determined. Experiments were repeated 3 times using 6 *Sirt1*$^{+/-}$ and 6 *Sirt1*$^{-/-}$ mice. Representative data are shown. (**E** and **F**) Sera were obtained 10 days after immunization with OVA/CFA (**E**) or 7 days after a second immunization with OVA/IFA (**F**). The concentrations of each isotype of immunoglobulin were determined by ELISA. The arbitrary units of IgG1 shown in the figure were determined after 1:1,000 dilution. Data are from 5 *Sirt1*$^{+/-}$ and 5 *Sirt1*$^{-/-}$ mice (mean ± SD).

It is likely that T cell hyperresponsiveness driven by Sirt1 deficiency is due to signals downstream of the TCR. This hypothesis was drawn from the observation that upon stimulation with PMA plus ionomycin, *Sirt1*$^{-/-}$ T cells exhibited a significant increase in proliferation and IL-2 production (Figure 1, A and B). Since PMA directly activates protein kinase (15) and ionomycin forms a lipid-soluble calcium complex to convey Ca^{2+} across the hydrocarbon region of the cell membrane (16), Sirt1 likely targets signaling molecules or transcription factors downstream of the TCR. Furthermore, similar to *Cbl-b*$^{-/-}$ T cells (17), the *Sirt1*$^{-/-}$ T cells exhibited a full-scale activation when stimulated with anti-CD3 antibody alone, whereas *Sirt1*$^{+/-}$ T cells showed only minimal activation under the same stimulation conditions (Figure 1, A–C). These results suggest that TCR signaling without costimulation is sufficient for activation of *Sirt1*$^{-/-}$ T cells.

Next, we analyzed the effect of Sirt1 on the production of both Th1 and Th2 cytokines by CD4$^+$ T cells. When stimulated with anti-CD3 or anti-CD3 plus anti-CD28 in vitro, *Sirt1*$^{-/-}$ CD4$^+$ T cells produced more Th1 cytokine IFN-γ and Th2 cytokine IL-5 than did *Sirt1*$^{+/-}$ CD4$^+$ T cells (Figure 1, E and F). The recall experiments using lymphocytes from mice immunized with OVA indicated substantial increases of both IFN-γ and IL-5 production by *Sirt1*$^{-/-}$ T cells compared with *Sirt1*$^{+/-}$ T cells (Figure 2, C and D). These results suggest that Sirt1 inhibits the productions of both Th1 and Th2 cytokines by CD4$^+$ T cells.

Sirt1 suppresses T cell–dependent immunity in mice. To determine the effect of Sirt1 deficiency on T cell activation in vivo, 6- to 8-week-old *Sirt1*$^{-/-}$ mice and their heterozygous littermates were immunized subcutaneously with chicken OVA protein emulsified in CFA, and their OVA-specific T cell responses were analyzed 7 days later. The results indicated that proliferation as well as IL-2 production by *Sirt1*$^{-/-}$ T cells were dramatically increased compared with *Sirt1*$^{+/-}$ T cells (Figure 2, A and B), suggesting that Sirt1 functions as a negative regulator of antigen-specific T cell activation in vivo.

To determine the effects of Sirt1 on T cell–dependent humoral immune responses, OVA-specific antibodies were measured after the primary immunization with OVA plus CFA as well as after boosting with OVA plus incomplete Freund's adjuvant (IFA). The results indicated that *Sirt1*$^{-/-}$ mice had increased antigen-specific antibodies of both IgM and IgG isotypes in the primary and secondary responses, suggesting that Sirt1 deficiency sustained a more vigorous T cell–dependent humoral response (Figure 2, E and F).

The elevated immune response of *Sirt1*$^{-/-}$ T cells was not the consequence of altered APC function in *Sirt1*$^{-/-}$ mice, because the proliferation of *Sirt1*$^{-/-}$ T cells showed comparable levels when they were stimulated with *Sirt1*$^{+/-}$ or *Sirt1*$^{-/-}$ APCs (Supplemental Figure 4A). In addition, expression of costimulatory molecules such as CD80 and CD86 on APCs was not affected by Sirt1 deficiency (Supplemental Figure 4B). Overall, these findings suggest that Sirt1 is a negative regulator of T cell activation.

Sirt1 is required for peripheral CD4$^+$ T cell tolerance. TCR ligation in the absence of costimulation gives rise to a state of long-term functional unresponsiveness known as anergy (1–3). Similar to *Cbl-b*$^{-/-}$ T cells (17), *Sirt1*$^{-/-}$ T cells were fully activated when they were stimulated with anti-CD3 alone without any costimulations (Figure 1, A–C). This suggests that loss of Sirt1 function overrides costimulation, leading to breakdown of peripheral T cell tolerance. To test whether loss of Sirt1 leads to a breakdown of tolerance in vivo, we bred the *Sirt1*$^{+/-}$ mice with OT-II TCR transgenic mice (18) and generated OT-II TCR *Sirt1*$^{+/-}$ and OT-II TCR *Sirt1*$^{-/-}$ mice. The animals (with 90% or higher TCR Vβ5 chain expression) were then given $OVA_{323–339}$ peptide in PBS intravenously, and their splenic T cells were tested for antigen-induced proliferation and IL-2 production. The results showed that while *Sirt1*$^{+/-}$ OT-II T cells were unresponsive, the *Sirt1*$^{-/-}$ OT-II cells had significant proliferative responses and increased IL-2 production (Figure 3, A and B, and Supplemental Figure 5, A and B). Similar results were obtained when purified CD4$^+$ T cells from $OVA_{323–339}$ peptide–treated mice were cocultured with APC and OVA peptide (Supplemental Figure 6). Therefore, we concluded that Sirt1 deficiency causes a breakdown of CD4$^+$ T-cell tolerance in vivo.

Using an in vitro T cell anergy induction assay (19) we show that ionomycin treatment failed to induce anergy of *Sirt1*$^{-/-}$ T cells. In contrast, proliferation and IL-2 production of *Sirt1*$^{+/-}$ T cells

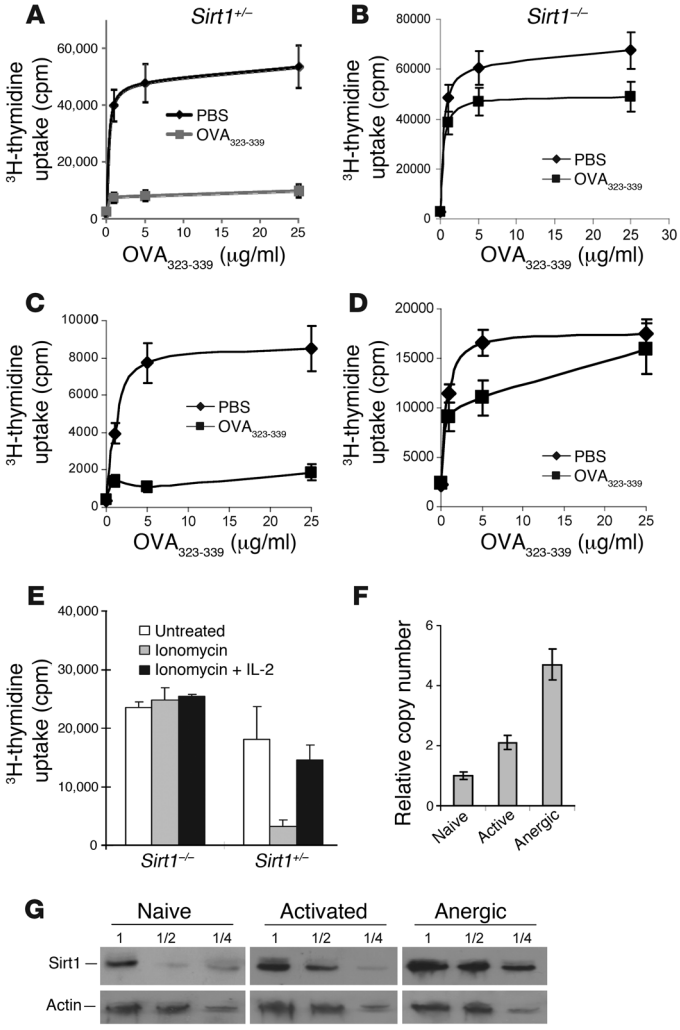

Figure 3
Sirt1 is required for CD4+ T cell tolerance. (**A** and **B**) In vivo tolerance was induced by treatment of *Sirt1*$^{+/-}$OTII (left) and Sirt1$^{-/-}$OTII (right) mice with $OVA_{323–339}$ peptides, or with PBS as a control, by tail vein injection. Total lymphocytes from lymph nodes were isolated from treated mice, and proliferation was analyzed. Data are from 5 *Sirt1*$^{+/-}$ and 5 *Sirt1*$^{-/-}$ mice. (**C** and **D**) CD4+ T cells were purified from *Sirt1*$^{+/-}$OTII (left) and *Sirt1*$^{-/-}$OTII (right) mice and adoptively transferred into T cell–null mice. CD4+ T cell tolerance in the recipient mice was analyzed as described in **A** and **B**. (**E**) The proliferation of *Sirt1*$^{-/-}$ and *Sirt1*$^{+/-}$ CD4+ T cells that underwent in vitro anergy induction was determined. (**F** and **G**) Freshly purified CD4+ T cells (naive), CD4+ T cells after 24 hours of stimulation with anti-CD3 plus anti-CD28 (active), and CD4+ T cells tolerized in vitro by ionomycin treatment (anergic) were used. (**F**) The mRNA levels of *Sirt1* in naive, activated, and anergic T cells were analyzed with real-time RT-PCR, using β-actin as a reference standard. Data are from 3 independent experiments. (**G**) Whole cell lysates of naive, activated, and anergic T cells were serially diluted to 1:2 and 1:4. The protein expression of Sirt1 was analyzed by Western blot, and the same membrane was reprobed with anti–β-actin antibody. Data are from 3 independent experiments (mean ± SD).

were inhibited by ionomycin treatment, and this was reversible by adding exogenous IL-2 (Figure 3E and Supplemental Figure 5C). Ecotropic expression of Sirt1, as described in the Supplemental Methods, inhibited the activation and restored the anergic induction of *Sirt1*$^{-/-}$ CD4+ T cells (Supplemental Figures 3B and 7A). In addition, in vitro deletion of Sirt1 from CD4+ T cells using an inducible Cre expression system resulted in the breakdown of T cell tolerance (Supplemental Figure 7B). Therefore, Sirt1 appears to regulate tolerance independent of its function in T cell development. The resistance of *Sirt1*$^{-/-}$ CD4+ T cells to tolerance was probably not the result of decreased cell death because annexin V staining revealed comparable percentages of apoptotic cells among *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ CD4+ T cells (Supplemental Figure 8). Together, these results indicate that Sirt1 is required for CD4+ T cell tolerance in vitro.

To further assess the role Sirt1 deficiency plays in the breakdown of T cell tolerance, CD4+ T cells were isolated from *Sirt1*$^{-/-}$ and *Sirt1*$^{+/-}$ OT-II mice and adoptively transferred into T cell–null mice, and CD4+ T cell tolerance was analyzed. As shown in Figure 3, C and D, injection of $OVA_{323–339}$ peptide into the host mice inhibited *Sirt1*$^{+/-}$ but not *Sirt1*$^{-/-}$ OT-II T cell activation. To determine whether this breakdown of *Sirt1*$^{-/-}$ CD4+ T cell tolerance was due to increased homeostatic proliferation in the lymphopenic host, we adoptively transferred CFSE-labeled CD4+ T cells into T cell–null mice and analyzed cell division. The findings indicate that homeostatic proliferation of *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ CD4+ T cells in the hosts was indistinguishable 7 days after transfer (Supplemental Figure 9).

Increased Sirt1 expression in anergic CD4+ T cells. The fact that CD4+ T cells of *Sirt1*$^{-/-}$ mice were unable to be tolerized suggests that Sirt1 could function as an anergic factor in T cells. Recent studies suggest that anergy induction requires upregulation of the expression of inhibitory proteins such as the E3 ligase Cbl-b (20). We therefore determined whether expression of Sirt1 changes in anergic T cells. Real-time PCR analysis revealed that expression of *Sirt1* mRNA was increased 4- to 5-fold in anergic versus naive T cells but increased only slightly in activated T cells (Figure 3F). Western blotting analysis also showed a similar increase at the protein level (Figure 3G). Thus, Sirt1 is upregulated in anergic T cells. In support of this is the finding that TCR stimulation alone, which presumably induces tolerance in vivo, is sufficient for Sirt1 upregulation (data not shown).

Previous studies have identified several genes that are upregulated in anergic CD4+ T cells when there is NFAT but not AP-1 transcriptional activation (20–27). We therefore determined whether Sirt1, as a HDAC, induces and/or maintains CD4+ T cell tolerance by altering the expression of these anergic genes. Real-time PCR analysis indicated that the expression levels of Cbl-b, DGK-a, EGR2, and EGR3 were comparable between *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ CD4+ T cells even when treated with ionomycin. Interestingly, the transcription of Grail and IKAROS-1 in *Sirt1*$^{-/-}$ T cells was reduced, suggesting a functional linkage of Sirt1 with Grail and IKAROS-1 in anergic T cells (Supplemental Figure 10).

Sirt1 inhibits AP-1 transcriptional activity in T cells. The transcription factor AP-1, usually made of c-Jun homodimers or c-Jun/Fos heterodimers, has been identified as a molecular target in T cell clonal anergy (4). However, the underlying molecular mechanism remains largely unknown. Sirt1 is a nuclear protein and has been found to suppress the transcriptional activity of several transcription factors, such as p53. We thus asked whether Sirt1 induces anergy by suppressing AP-1 transcriptional activity in T cells.

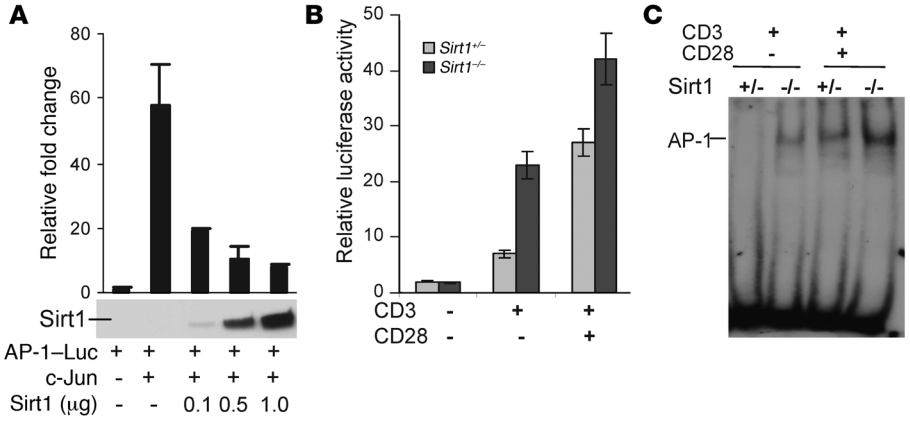

Figure 4

Sirt1 inhibits AP-1 transcriptional activity. (**A**) AP-1 luciferase, control luciferase, and c-Jun expression plasmids were cotransfected into HEK-293 cells with different amounts of Sirt1 expression plasmid DNA. The ratios of AP-1 luciferase activities to control luciferase activity (fold change) are shown. (**B**) Primary T cells were isolated from *Sirt1*$^{+/-}$AP-1luc and *Sirt1*$^{-/-}$AP-1luc mice and left unstimulated or stimulated with anti-CD3 or anti-CD3 plus anti-CD28 for 24 hours. The luciferase activity of stimulated cells was analyzed. (**C**) Increased DNA-binding activity in *Sirt1*$^{-/-}$ T cells. Nuclear extracts from primary T cells stimulated with anti-CD3 or anti-CD3 plus anti-CD28 were used for gel-shift analysis. Data are from 3 independent experiments (mean ± SD).

Indeed, using a luciferase AP-1 reporter system we demonstrated that overexpression of Sirt1 inhibited AP-1 transcriptional activity in a dose-dependent manner (Figure 4A). This suggests that Sirt1 can function as a suppressor of AP-1 transcription factor. To determine whether Sirt1 inhibits AP-1 transcriptional activity in primary T cells, we bred *Sirt1*$^{+/-}$ mice with AP-1 luciferase transgenic (AP-1luc^{TG}) mice (28) and used this specific reporter to evaluate the effect of *Sirt1*$^{-/-}$ on AP-1 transcriptional activity. As shown in Figure 4B, after stimulation with anti-CD3 or anti-CD3 plus anti-CD28 for 24 hours, AP-1 luciferase activities were increased in *Sirt1*$^{-/-}$AP-1luc^{TG} T cells compared with *Sirt1*$^{+/-}$AP-1^{TG} cells. Also, gel shift experiments demonstrated a significant increase of AP-1 promoter DNA-binding activity in *Sirt1*$^{-/-}$ T cells after TCR/CD28 stimulation (Figure 4C). This increased AP-1 transcriptional activation does not appear to result from the elevated activation of the upstream MAPK pathway because both JNK and Erk activation were indistinguishable between *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ T cells (Supplemental Figure 11). These results indicate that Sirt1 functions as a suppressor of AP-1 in T cells.

Sirt1 inhibits c-Jun acetylation to sustain T cell anergy. Since Sirt1 functions as a deacetylase and c-Jun requires acetylation for its activity, it is possible that Sirt1 operates T cell anergy by suppressing c-Jun acetylation. To test this premise, c-Jun acetylation was compared in activated versus anergic T cells. Indeed, c-Jun was highly acetylated in activated CD4$^+$ T cells, while its acetylation was diminished in anergic T cells (in which anergy was induced either in vitro [Figure 5A] or in mice [Figure 5B]) to levels comparable to those of naive T cells. These results provide a direct link between the regulation of c-Jun acetylation and T cell anergy.

The fact that Sirt1 expression is upregulated in anergic T cells implies that Sirt1 may be responsible for impaired c-Jun acetylation. If this hypothesis were correct, increased c-Jun acetylation would be observed in *Sirt1*$^{-/-}$ compared with *Sirt1*$^{+/-}$ CD4$^+$ T cells. Indeed, acetylation of c-Jun was detected in *Sirt1*$^{+/-}$ T cells under stimulation with anti-CD3 plus anti-CD28 but not with anti-CD3 alone. In contrast, c-Jun acetylation was increased in *Sirt1*$^{-/-}$ T cells (Figure 5C). These results indicate that Sirt1 inhibits T cell activation by suppressing c-Jun acetylation. In particular, anti-CD3 stimulation is sufficient to induce c-Jun acetylation in *Sirt1*$^{-/-}$ T cells, indicating that TCR-mediated c-Jun acetylation is inhibited by Sirt1 (Figure 5C). To support this conclusion, we further demonstrated that expression of Sirt1 in T cells inhibited c-Jun acetylation (Figure 5D). TCR stimulation alone usually induces

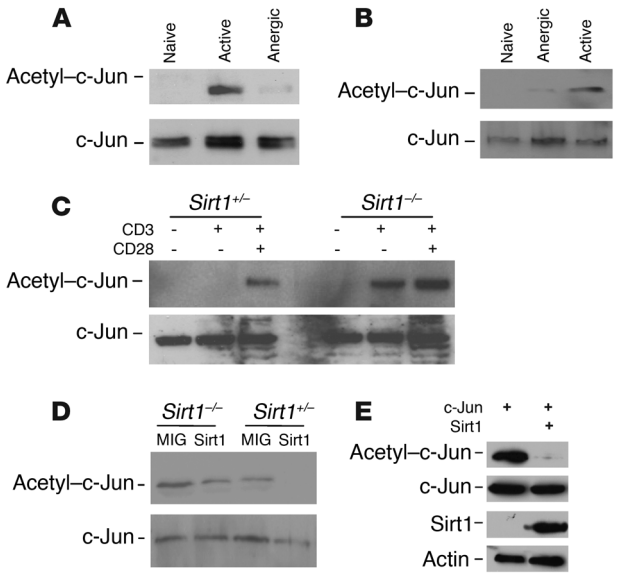

Figure 5

Sirt1 deacetylates c-Jun in T cells. (**A**) c-Jun protein was immunoprecipitated from the lysates of T cells activated or anergized in vitro. The acetylation of c-Jun was detected by anti–acetyl-lysine antibody (top). The same membrane was reprobed with anti–c-Jun antibody (bottom). (**B**) In vivo T cell anergy was induced by treatment of OT-II TCR transgenic mice with OVA$_{323–339}$ peptide, using PBS as a control. CD4$^+$ T cells from peptide-treated (anergic) and control (naive and activated with anti-CD3 plus anti-CD28) mice were isolated and restimulated with anti-CD3 plus anti-CD28 for 2 hours. c-Jun acetylation was analyzed as described in **A**. (**C**) Primary T cells were isolated from *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice. Purified T cells were stimulated with anti-CD3 or anti-CD3 plus anti-CD28 for 24 hours. c-Jun acetylation in the stimulated *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ T cells was determined as described in **A**. (**D**) T cells from *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice were infected with a retrovirus that carries an empty vector (MIG) or the *Sirt1* gene. c-Jun acetylation in these infected T cells was analyzed. (**E**) HEK-293 cells were transfected with c-Jun, p300 with Sirt1 expression plasmids. c-Jun acetylation in the transfected HEK-293 cells was analyzed as described in **A**.

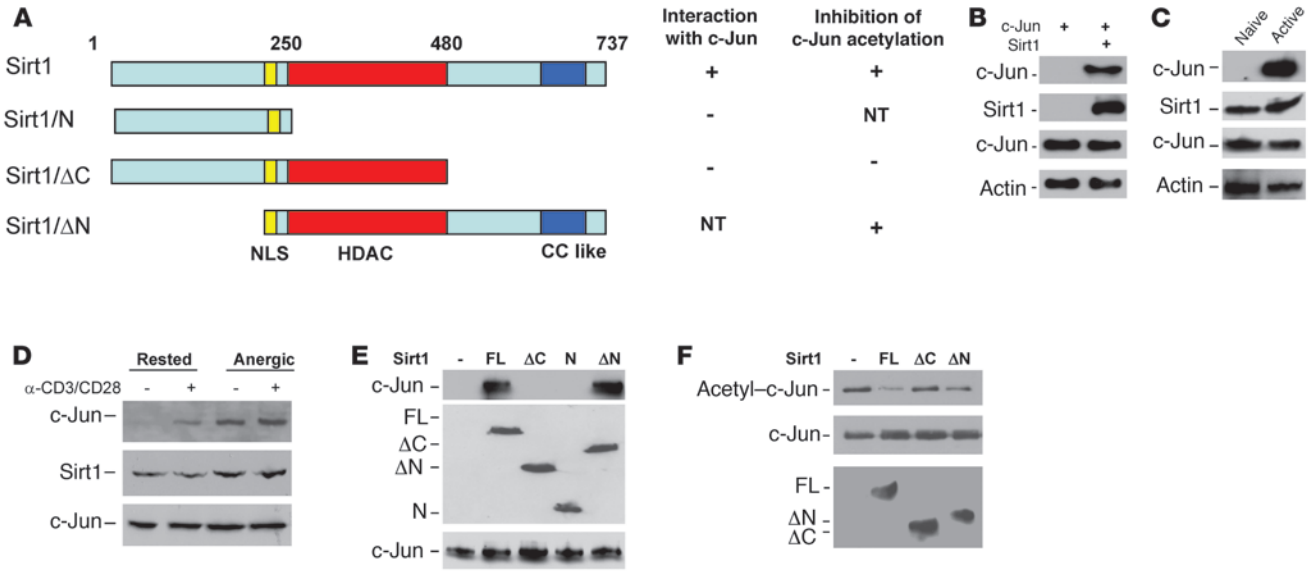

Figure 6

The Sirt1 C terminus mediates its interaction with c-Jun. (**A**) Schematic representations of Sirt1 and Sirt1 mutants. The c-Jun interaction patterns of Sirt1 and its mutants as well as their deacetylase activity to inhibit c-Jun acetylation are summarized. (**B**) Xpress–c-Jun was cotransfected with or without Flag-Sirt1 in HEK-293 cells. Sirt1 protein in the lysates was immunoprecipitated with an anti-Flag antibody. The interaction of c-Jun with Sirt1 was determined with an anti-Xpress antibody (first panel). The expression levels of Sirt1 (second panel), c-Jun (third panel), and actin (fourth panel) in whole cell lysates were analyzed. (**C**) Primary T cells were activated by anti-CD3 plus anti-CD28 for 16 hours. The interaction of Sirt1 with c-Jun was analyzed as described in **B**. (**D**) Expanded T cells were treated with (anergic) or without (rested) ionomycin for 16 hours, followed with 30 minutes of stimulation with anti-CD3 plus anti-CD28. The interaction of Sirt1 and c-Jun was analyzed as described in **B**. (**E**) The interaction of c-Jun with Flag-tagged Sirt1 or each of its deletion mutants was analyzed as described in **A**. (**F**) c-Jun, p300, and Sirt1 or Sirt1 mutants were cotransfected into HEK-293 cells. c-Jun acetylation was analyzed by immunization of c-Jun and Western blotting with anti–acetyl-lysine antibodies (top panel). The expression levels of c-Jun and Sirt1 or Sirt1 mutants in the whole cell lysates were detected with anti–c-Jun (middle panel) and anti-Flag (bottom panel), respectively.

T cell tolerance, which is a state of anergy. Given the observation that Sirt1 expression is upregulated in anergic T cells and that the lack of Sirt1 function causes breakdown of T cell tolerance, these results suggest that Sirt1 maintains T cell tolerance by suppressing c-Jun acetylation. In support of this statement is the observation that overexpression of Sirt1 inhibited c-Jun acetylation in transiently transfected HEK-293 cells (Figure 5E).

Sirt1 interacts with c-Jun independently of JNK. To gain further insight into the interaction of Sirt1 with c-Jun in mouse primary T cells, we analyzed the binding of Sirt1 with c-Jun by coimmunoprecipitation. HEK-293 cells were transfected with Flag-tagged Sirt1 plasmid and/or c-Jun, and anti-flag immunoprecipitation was performed. The findings indicate that c-Jun protein was detected in anti-Flag immunoprecipitates when the cells were transfected with Sirt1 and c-Jun but not in cells transfected with c-Jun alone (Figure 6, A and B). This suggests that Sirt1 interacts with c-Jun. Furthermore, when mouse primary T cells were stimulated with anti-CD3 plus anti-CD28, Sirt1/c-Jun interaction was detectable (Figure 6C). However, Sirt1/c-Jun interaction could not be detected in naive unstimulated T cells. Consistent with this finding, colocalization of Sirt1 with c-Jun in the nuclei was observed in the activated but not naive T cells (Supplemental Figure 13). These results indicate that Sirt1 interacts with c-Jun both in transiently transfected HEK-293 cells and in mouse primary T cells. More interestingly, a constitutive and significantly increased interaction of Sirt1 with c-Jun was detected in anergic T cells (Figure 6D), indicating that Sirt1 constitutively suppresses AP-1 transcription to maintain $CD4^+$ T cell tolerance. To support this, immunostaining experiments revealed a brighter Sirt1 expression in anergic T cells (Supplemental Methods), and Sirt1 was well colocalized with c-Jun in the nuclei (Supplemental Figure 14). We determined that the C terminus of Sirt1 was responsible for its interaction with c-Jun because deletion of the C terminus completely abolished Sirt1/c-Jun interaction as well as its ability to inhibit c-Jun acetylation,

Table 1

Clinical observation of immunized *Sirt1*$^{-/-}$ and *Sirt1*$^{+/-}$ mice

Mouse	Incidence of EAE	Clinical score				Mean maximal disease severity[A]	Day of disease onset	Day of recovery
Sirt1$^{+/-}$	55% (11 of 20)	9	5	3	3	1.12 ± 1.17	12.7 ± 0.97	35
Sirt1$^{-/-}$	87.5% (14 of 16)	2	1	6	7	2.43 ± 1.06	10.5 ± 1.16	53

[A]Disease severity was scored on a scale of 0 to 5: 0, no illness; 1, limp tail; 2, limp tail and hindlimb weakness; 3, hindlimb paralysis; 4, forelimb and hindlimb paralysis; 5 moribund.

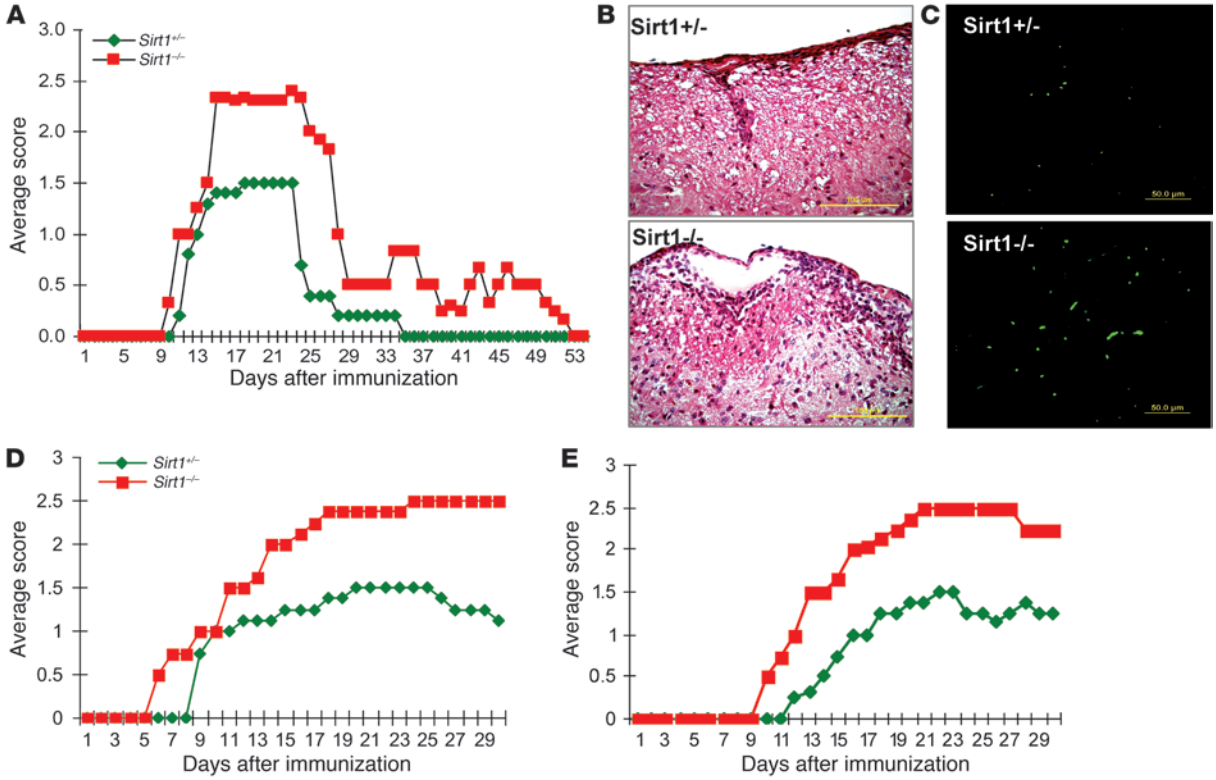

Figure 7
EAE development in *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice. EAE was induced by immunization of Sirt1$^{+/-}$ and Sirt1$^{-/-}$ mice with MOG peptide. (**A**) Disease scores were obtained daily for 55 days after immunization, and the average score is shown. (**B** and **C**) Spinal cord sections of sick mice were analyzed by H&E staining (**B**), or infiltrated cells were detected by Alexa Fluor 488–conjugated anti-CD4 antibody (**C**). Representative images are shown. (**D**) *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice were immunized with MOG peptide emulsified with CFA. CD4$^+$ T cells were isolated from the immunized mice 7 days after immunization. CD4$^+$ cells (5×10^6) were adoptively transferred into T cell–null mice, followed by pertussis toxin injection 1 and 2 days after transfer. EAE was analyzed as described in **A**. (**E**) Naive CD4$^+$ T cells were isolated from *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice and adoptively transferred into T cell–null mice at 5×10^6 cells per mouse. EAE induction and analysis in the recipient mice was performed as described in **A**.

while deletion of the N terminus did not affect their interaction (Figure 6, A, E, and F), and the C terminus alone was sufficient for its interaction with c-Jun (data not shown).

Since activation signals of T cells provided by TCR/CD28 promote c-Jun phosphorylation by JNK (29, 30), we then tested whether this phosphorylation event was responsible for c-Jun interaction with Sirt1. Treatment of cells with a JNK-specific inhibitor, SP600125, which dramatically inhibited c-Jun phosphorylation, did not affect Sirt1/c-Jun interaction (Supplemental Figure 12A). In addition, deletion of the JNK-docking site or the δ domain of c-Jun, or mutation of the predominant phosphorylation serine and threonine within c-Jun, did not affect its interaction with Sirt1 (Supplemental Figure 12B). Immunostaining experiments revealed that c-Jun is mainly distributed in the cytoplasm of naive T cells and that TCR/CD28 stimulation relocated c-Jun into the nucleus (Supplemental Figure 13). In contrast, Sirt1 remained in both cytoplasm and nucleus in naive unstimulated T cells. Therefore, TCR/CD28 stimulation might enhance c-Jun/Sirt1 interaction by promoting c-Jun nuclear translocation. Interestingly, consistent with our finding of increased Sirt1 expression in anergic T cells, a brighter Sirt1 staining that was well colocalized with c-Jun was observed in anergic T cells (Supplemental Figure 14). Based on these findings, we conclude that Sirt1 is a deacetylase of c-Jun, and Sirt1-mediated deacetylation of c-Jun inhibits AP-1 transcriptional activation.

Sirt1 deficiency results in autoimmunity in mice. Breakdown of self-tolerance in CD4$^+$ T cells plays a major role in the development of EAE in mice as well as MS in humans (31). To further investigate the role of Sirt1 in T cell tolerance, *Sirt1*$^{-/-}$ and *Sirt1*$^{+/-}$ mice were immunized with myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) peptide to induce EAE and the animals were monitored for signs of paralysis. The findings indicated that *Sirt1*$^{-/-}$ mice were more susceptible to EAE, as 87.5% (14 of 16) manifested clinical signs of disease after immunization (Table 1). In contrast, 55% (11 of 20) of the *Sirt1*$^{+/-}$ mice showed signs of EAE. In addition, the onset of EAE in the *Sirt1*$^{-/-}$ mice was 2 days earlier relative to the onset in *Sirt1*$^{+/-}$ mice, and the average clinical score was significantly higher than in control mice ($P < 0.005$) (Figure 7A and Table 1). Histological analysis of the spinal cord sections revealed more lymphocyte infiltration in *Sirt1*$^{-/-}$ mice (Figure 7B). Most of the infiltrating cells were T cells, as confirmed by immunostaining (Figure 7C). Thus, we concluded that Sirt1 deficiency promotes susceptibility to EAE.

Next, we determined whether Sirt1-deficient CD4$^+$ T cells are able to transfer EAE to normal animals. *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice were immunized with MOG peptide in CFA. CD4$^+$ T cells were isolated

and adoptively transferred into T cell–deficient mice. As shown in Figure 7D, mice recipients of *Sirt1*$^{-/-}$ CD4$^+$ T cells showed signs of EAE as early as 5 days after transfer. In contrast, disease development in mice that received *Sirt1*$^{+/-}$ T cells was delayed by 3–4 days. In addition, a significantly increased average clinical score was observed in recipients of *Sirt1*$^{-/-}$ versus *Sirt1*$^{+/-}$ T cells. Furthermore, experiments using naive *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ T cells for the adoptive transfer demonstrated that the clinical severity of EAE was also increased in mice that received *Sirt1*$^{-/-}$ T cells (Figure 7E). Therefore, increased T cell activation and breakdown of T cell tolerance appear to be critical for the development of autoimmunity in *Sirt1*$^{-/-}$ mice.

We then asked whether aging *Sirt1*$^{-/-}$ mice (11 months old or older) develop spontaneous autoimmune responses (Supplemental Methods). Sera from *Sirt1*$^{-/-}$ mice had higher amounts of anti-nuclear antibodies than did sera from *Sirt1*$^{+/-}$ (Supplemental Figure 15A). This was supported by the stronger fluorescence in the nuclei of NIH3T3 cells when the staining was made with the sera from *Sirt1*$^{-/-}$ versus *Sirt1*$^{+/-}$ mice (Supplemental Figure 15B), which is similar to findings from a recent study of *Sirt1*$^{-/-}$ mice on a mixed genetic background (32). Next, we tested whether self-reactive antibodies deposit in the kidney glomerules. Kidney tissue sections were prepared, frozen with OCT, fixed, saturated with normal goat IgM and IgG, and stained with fluorescence-labeled goat anti-mouse IgM or IgG. As shown in Supplemental Figure 17C, deposition of both IgM and IgG was evident in the kidney glomerules of *Sirt1*$^{-/-}$ mice, while only background staining was observed in kidney sections of *Sirt1*$^{+/-}$ mice. Lymphocyte infiltration was observed in the liver, lung, and kidney of all 5 *Sirt1*$^{-/-}$ mice examined. In contrast, no obvious lymphocyte infiltration was observed in tissues from any *Sirt1*$^{+/-}$ mice (Supplemental Figure 15D). These results indicate that Sirt1 deficiency results in the development of an autoimmune syndrome in mice.

The CD4$^+$CD25$^+$FoxP3$^+$ Tregs that suppress autoreactive T cells are critical for autoimmune suppression (33, 34), and in some instances TGF-β is essential for Treg function (35). Interestingly, Sirt1 has been shown to destabilize Smad7, a suppressive transcription factor in the TGF-β pathway (36). Smad7 expression strongly affects in vitro Treg differentiation induced by TGF-β (37, 38). Therefore, loss of Sirt1 function might impair the development and/or function of Tregs and consequently contribute to the autoimmune phenotype in *Sirt1*$^{-/-}$ mice. To test this hypothesis, we compared the percentages of Tregs between *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice. The percentages of CD4$^+$FoxP3$^+$ populations, as well as their suppressive functions, were indistinguishable between the 2 strains (Supplemental Figure 16, A and B), indicating that Sirt1 deficiency does not affect Treg development and function.

An alternative for *Sirt1*$^{-/-}$ involvement in autoimmunity may be increased Th17 differentiation. To test this premise, we compared the percentages of IL-17$^+$ populations between heterozygous and *Sirt1*$^{-/-}$ CD4$^+$ T cells upon polarization with TGF-β and IL-6 (39). CD4$^+$ T cells were purified from *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice and cultured with anti-CD3, anti-CD28, TGF-β, and IL-6 for 5 days. The production of IL-17 and IFN-γ was detected by intracellular staining with anti–IL-17–PE and anti–IFN-γ–FITC, respectively. As shown in Supplemental Figure 18C, the percentages of IL-17$^+$ cells were indistinguishable between *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ T cells, indicating that loss of Sirt1 function does not affect Th17 polarization in vitro. Collectively, our findings suggest that breakdown of CD4$^+$ T cell tolerance due to the lack of Sirt1 functions is related to uncontrolled activation of autoreactive T cells.

Discussion

The findings presented in this report suggest that anergic signals induce upregulation of Sirt1 expression, which suppresses AP-1 transcriptional activity, leading to inhibition of T cell activation and maintenance of peripheral T cell tolerance. A lack of Sirt1 causes a breakdown of peripheral tolerance, and *Sirt1*$^{-/-}$ mice are more susceptible to autoimmune diseases.

Autoreactive T cells are generally eliminated by negative selection during thymic development (central tolerance). Self-reactive thymic escapees remain harmless due to the lack of costimulation when they detect antigen, a phenomenon known as peripheral tolerance (1–3). Although the molecular mechanisms underlying peripheral tolerance remain largely unknown, progress has been made that sheds light on how TCR stimulation without CD28 signaling induces unresponsiveness of autoimmune T cells (1, 2). This imbalanced stimulation of autoreactive T cells activates the transcription factor NFAT, possibly together with other unknown transcription factors, for the transcription of genes to induce and maintain peripheral T cell tolerance (19, 40). Recent studies reported that anergy induction is a process that upregulates expression of a cascade of inhibitory proteins including the E3 ubiquitin ligases Cbl-b, Itch, and Grail (20, 41). These upregulated E3 ubiquitin ligases selectively target T cell activators for ubiquitination-mediated degradation and/or functional suppression. Our study here defines what we believe is a new anergic gene, *Sirt1*, for peripheral T cell tolerance, because TCR-mediated signaling alone was sufficient for its transcription and Sirt1 suppressed T cell responses to TCR/CD28 stimuli. One interesting observation was that Sirt1 transcription was significantly higher in T cells with TCR stimulation alone than with both TCR and CD28 together, suggesting that CD28 stimulation may suppress Sirt1 expression to allow T cells to be activated. This CD28-mediated Sirt1 downregulation seems to depend on TCR signaling, because Sirt1 was not transcribed when naive T cells were stimulated with anti-CD28 in the absence of TCR stimulation (data not shown). Thus, it is likely that binding of the MHC/peptide complex to TCRs without costimulation induces Sirt1 expression and the consequent T cell anergy, while ligation of the TCR and CD28 costimulatory molecule leads to downregulation of Sirt1, allowing for T cell activation. How the CD28-mediated signal blocks Sirt1 transcription remains to be defined.

One signature of *Sirt1*$^{-/-}$ T cells was that full-scale activation did not require costimulation, suggesting that a "short-cut" signal transduction pathway that links TCR to CD28 elements is put in place by the lack of Sirt1. This short-cut pathway likely plays a major role in the breakdown of T cell tolerance. Similar results have been observed in *Cbl-b*$^{-/-}$ T cells showing a vigorous T cell activation independent of CD28 stimulation (17). However, in *Sirt1*$^{-/-}$ T cells, unlike *Cbl-b*$^{-/-}$ T cells, CD28 stimulation further enhances activation, suggesting that Sirt1 inhibits the signal transduction mediated by both TCR and CD28 in T cells. Signal transduction was inhibited at multiple stages when anergic T cells were stimulated by self-antigen and costimulators. For instance, altered expression of Fyn and Lck protein tyrosine kinases and altered patterns of early tyrosine phosphorylation have been correlated with deficient IL-2 production in anergic T cells (42–47). Initial interpretations suggested that the defect in IL-2 production of anergic T cells emanates from translational regulation because stimulation of anergic cells failed to induce *Il2* mRNA (48). A milestone in the understanding of how IL-2 transcription is silenced in

anergic T cells transpired from the finding that anergic T cells display selective inhibition of AP-1 transcriptional activity (4). This is consistent with our recent report showing that AP-1 is a molecular target for FoxP3 to maintain the unresponsiveness of Tregs (49). AP-1 transcriptional activation, which is crucial for IL-2 transcription, is triggered by both TCR and CD28 stimulation in T cells (50, 51). When self-reactive T cells see antigen in the absence of CD28 stimulation, Sirt1 expression is upregulated, which inhibits the TCR-mediated AP-1 transcriptional activity. However, costimulation through CD28 counters Sirt1 activity, leading to T cell activation. The study in this report elucidates the mechanism underlying AP-1 inhibition and indicates that Sirt1-mediated c-Jun acetylation leads to AP-1 inhibition in anergic T cells.

Acetylation and deacetylation of transcription factors represent critical processes that dynamically regulate gene transcription (52). In the case of c-Jun, a recent study found that acetylation at lysines 268, 271, and 273 is required for c-Jun transcriptional activation, and mutations of these 3 lysines to arginine completely abolished c-Jun transcriptional activity (53). The finding that c-Jun acetylation is diminished in anergic T cells indicates that AP-1 transcriptional activity is suppressed by c-Jun deacetylation. Sirt1 is solely responsible for the suppression of c-Jun acetylation during induction of T cell anergy because the lack of Sirt1 resulted in hyperacetylation of c-Jun and the breakdown of T cell tolerance. Also, it should be noted that TCR stimulation alone could not induce c-Jun acetylation in naive CD4$^+$ T cells, possibly because TCR-mediated signaling upregulates Sirt1 transcription. Indeed, when *Sirt1*$^{-/-}$ T cells were stimulated with anti-CD3 antibody alone, c-Jun acetylation was highly detected. Given that TCR stimulation in the absence of costimulation induces T cell tolerance (2), the finding of Sirt1-mediated AP-1 deacetylation defines what we believe is a novel molecular mechanism underlying T cell tolerance.

Recent studies have found that Sirt1 interacts with AP-1 in fibroblasts and epithelial cells (54, 55). We demonstrate here that Sirt1 interacts with c-Jun to form a protein complex that catalyzes c-Jun deacetylation in anergic T cells. Protein-protein interactions are specifically regulated by extracellular stimuli. Indeed, Sirt1/c-Jun interaction requires TCR/CD28 stimulation because their interaction is not detectable in naive T cells. It is surprising that Sirt1 interacts with c-Jun independent of JNK activation, because treatment with a JNK-specific inhibitor or mutation of the phosphorylation sites within c-Jun did not affect the interaction, suggesting that TCR/CD28 signaling regulates Sirt1/c-Jun interaction by other mechanisms, which is interesting to further characterize. Therefore, TCR/CD28 signaling regulates T cell activation and tolerance not only by altering Sirt1 transcription but also by controlling its access to substrate proteins such as c-Jun. Our laboratory is currently investigating the precise mechanisms underlying the regulation of Sirt1/c-Jun interaction by activation and/or anergic signals in T cells.

Overall, the findings that Sirt1 inhibits T cell activation and is required for T cell tolerance imply that activators of Sirt1 might be useful as therapeutic reagents for the treatment/prevention of autoimmune diseases such as MS, rheumatoid arthritis, and type 1 diabetes. Indeed, a Chinese herbal medicine, Huzhang (*polygonum cuspidatum*), which is one of the richest known sources of Sirt1 activator, resveratrol, has been widely used for the treatment of autoimmune diseases, particularly rheumatoid arthritis, in China. Resveratrol has been found to attenuate EAE development by suppressing T cell activation in mice (56). Also, Sirt1 activators have been successfully used for treatment of type 2 diabetes (57). Finally, the findings that Sirt1 inhibits T cell activation and is required for T cell tolerance suggest that a Sirt1 activator may help in the treatment of both autoimmune diseases and type 2 diabetes.

Methods

Mice. *Sirt1*$^{+/-}$ mice (9) were backcrossed for 5 or 6 generations onto the C57BL/6 genetic background. Consistent with previous reports (10, 58), further backcrossing reduced the survival of *Sirt1*$^{-/-}$ mice. OT-II transgenic mice on the C57BL/6 background (DO11.10 TCR transgenic mice) were purchased from The Jackson Laboratory. Some *Sirt1*$^{+/-}$ mice were bred with OT-II transgenic mice to generate *Sirt1*$^{+/-}$OTII and *Sirt1*$^{-/-}$OTII mice. AP-1 luciferase transgenic mice were purchased from The Jackson Laboratory and bred with *Sirt1*$^{+/-}$ animals. T cell–null mice (both αβ and γδ) (59) were purchased from the Jackson Laboratory. All mice used in this study were maintained and used at the University of Missouri mouse facility under pathogen-free conditions according to institutional guidelines. All animal study protocols were approved by the University of Missouri Institutional Animal Care and Use Committee.

T cell proliferation and cytokine production. In vitro T cell proliferation and stimulation were performed as previously described (60). Briefly, purified CD4$^+$ T cells were cultured with or without anti-CD3 or anti-CD3 plus anti-CD28 for 3 days. Cells treated with PMA (20 ng/ml) plus ionomycin (0.5 μM) were used for control experiments. For proliferation analysis, cells were chased with ^{3}H-thymidine (0.5 μCi/well) for 16 hours, and ^{3}H-thymidine incorporation was measured. For cytokine production, supernatants of stimulated cells were collected, and concentrations of IL-2, IFN-γ, IL-4, and IL-5 were analyzed by ELISA.

Animal immunization and analysis of T cell–mediated immune responses. *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice (8–10 weeks old) were immunized subcutaneously at the base of the tail with OVA protein (200 μg/mouse) emulsified in 100 μl CFA (Sigma-Aldrich). Total cells from draining lymph nodes were isolated 7 days later and cultured with different doses of OVA protein. For the proliferation assay, cells were cultured for 3 days and then chased with ^{3}H-thymidine (0.5 μCi/well) for additional 16 hours, and ^{3}H-thymidine incorporation was analyzed.

For T cell–mediated humoral immune responses, mice were immunized with OVA/CFA and boosted with OVA/IFA 10 days after the first immunization. Sera were collected 4 days after the first immunization and 5 days after the second immunization. The concentrations of OVA-specific immunoglobulins including IgG1, IgG2a, IgG3, and IgM were measured by ELISA.

Induction of T cell anergy in mice and in vitro. For in vivo CD4 T cell anergy induction, *Sirt1*$^{+/-}$OTII or *Sirt1*$^{-/-}$OTII mice were treated with a single dose of $OVA_{323–339}$ peptide (200 μg in 100 μl PBS per mouse) by intravenous injection. Mice injected with 100 μl of PBS were used as controls. Ten days after tolerization, total cells from draining lymph nodes were isolated and cultured with different amounts of $OVA_{323–339}$ peptide. CD4$^+$ T cell proliferation to $OVA_{323–339}$ peptide was analyzed by ^{3}H-thymidine incorporation. The in vitro CD4$^+$ T cell anergy induction was performed as previously described (19). Briefly, CD4$^+$ cells were purified using anti-CD4–coated magnetic beads (Miltenyi Biotec) and then stimulated with plate-bound anti-CD3 and anti-CD28 (0.5 μg/ml) in the presence of IL-12 (10 ng/ml) and anti–IL-4 (10 μg/ml). IL-2 (10 U/ml) was added at days 3 and 7, respectively. Cells were washed with PBS and treated with 0.5 mM ionomycin for 16 hours. Cells were subsequently washed with PBS 3 times, rested for 2–4 hours, and restimulated with plate-bound anti-CD3 plus anti-CD28 or IL-2 to evaluate proliferation.

Anti-nuclear antibody analysis. Sera from *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice were collected. Anti-nuclear antibody (ANA) concentrations were measured by ELISA using a commercial kit (Alpha Diagnostic International Inc.). To

confirm the presence of ANA, pre-fixed NIH3T3 cells were stained with diluted (1:100) sera from these mice, followed by immunostaining with Alexa Fluor 488–conjugated anti-mouse Ig antibody. Cells were visualized under a fluorescence microscope.

Induction and clinical assessment of EAE. EAE was induced as previously reported (61). Six- to 8-week-old *Sirt1*$^{+/-}$ and *Sirt1*$^{-/-}$ mice were immunized with MOG peptide (amino acid 35–55, MEVGWYRSPFSRVVHLYRNGK) emulsified with CFA (200 μg per mouse). Mice were also given pertussis toxin (200 ng per mouse) on day 0 and day 2 via tail vein injection. All mice were weighed and examined for clinical symptoms and assigned scores on a scale of 0–5 as follows: 0, no overt signs of disease; 1, limp tail; 2, limp tail and partial hindlimb paralysis; 3, complete hindlimb paralysis; 4, complete hindlimb and partial forelimb paralysis; 5, moribund state or death. Some mice were euthanized at day 17 to 18, and brains and spinal cords were collected for histological analysis.

Dual luciferase assay. HEK-293 cells in 12-well plates were transfected with pRL-TK (Promega) and pAP-1 luciferase plasmids, along with various expression plasmids, using the Lipofectamine transfection reagent (Invitrogen). The pRL-TK plasmid contains the *Renilla reniformis* (sea pansy) luciferase gene under the transcriptional control of the herpes virus thymidine kinase promoter and constitutively expresses low levels of renillar luciferase. Therefore, it can be used as a control. Transfected cells were lysed, and the luciferase activity in cell lysates was analyzed using a Dual Luciferase Reporter assay kit (Promega). Luciferase activity was measured as relative light units using a luminometer (Turner BioSystems Inc.).

Immunoprecipitation and Western blotting. Transiently transfected HEK-293 and mouse primary T cells were washed with ice-cold PBS, resuspended NP-40 lysis buffer with protease inhibitor, and incubated on ice for 15 minutes. Insoluble fractions were removed by centrifugation (15,000 *g* for 15 minutes). Supernatants were pre-cleaned with protein G–Sepharose at 4°C for 15 minutes and then incubated with antibody (1 μg/ml) for 1 hour, followed by incubation with protein G–Sepharose beads for additional 2 hours. The protein G–Sepharose beads were washed 4 times with lysis buffer, dissolved with 4× Laemmli's buffer and boiled for 5 minutes. Supernatants were subjected to SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% (wt/vol) skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membrane was incubated overnight at 4°C with the indicated primary antibodies followed by HRP-conjugated secondary antibody. Membranes were then washed and visualized with ECL. When necessary, membranes were stripped using stripping buffer (Bio-Rad) and reprobed with various antibodies.

EMSA. Nuclear protein extracts from unstimulated or stimulated CD4 cells with anti-CD3 or anti-CD3 and anti-CD28 were prepared using a nuclear extraction kit (Active Motif Inc.) according to the manufacturer's instructions. Protein concentration was determined using the Bradford protein assay (Bio-Rad). Oligonucleotides for AP-1 were labeled with biotin-11 UTP using a Biotin 3′ end DNA labeling kit (Pierce). Binding reactions were performed in a 25-μl volume. Each reaction contained 2 μg nuclear extract, 2 μg polydeoxinosinic-deoxycytidylic acid (dI:dC; Sigma-Aldrich), 50 mM NaCl, 10 mM Tris-HCl, 4% (vol/vol) glycerol, 0.5 mM DTT, 0.5 mM EDTA, 5 mM $MgCl_2$, and 20 fmol biotin-labeled oligonucleotides. Reactions were incubated for 20 minutes at room temperature, then electrophoresed through a 5% or 7% polyacrylamide gel with 0.5× TBE running buffer. Gels were transferred onto Hybond N^+ membrane, followed by cross-linking at 120 mJ/cm^2 using a commercial UV light cross-linker Spectrolinker XL-1500UV cross-linker (Spectronics Inc.). Biotin-labeled AP-1 was detected by chemiluminescence using Phototope-Star detection kit (New England Biolab).

Statistics. All data are expressed as mean ± SD. All in vitro experiments were performed in triplicate in at least 3 independent experiments. In vivo analyses were performed using 5 mice per group, unless otherwise specified. The Student's unpaired 2-tailed *t* test was used to calculate statistical significance for differences between 2 groups. A *P* value less than 0.05 was considered significant.

Note added in proof. Sirt1 may regulate T cell activation and anergy by targeting other molecules besides c-Jun. Indeed, a recent study suggests that Sirt1 inhibits NF-κB transcriptional activation in T cells (62).

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1. Anasetti, C., Tan, P., Hansen, J.A., and Martin, P.J. 1990. Induction of specific nonresponsiveness in unprimed human T cells by anti-CD3 antibody and alloantigen. *J. Exp. Med.* **172**:1691–1700.
2. Jenkins, M.K., and Schwartz, R.H. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* **165**:302–319.
3. LaSalle, J.M., Tolentino, P.J., Freeman, G.J., Nadler, L.M., and Hafler, D.A. 1992. Early signaling defects in human T cells anergized by T cell presentation of autoantigen. *J. Exp. Med.* **176**:177–186.
4. Kang, S.M., et al. 1992. Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science.* **257**:1134–1138.
5. Haigis, M.C., and Guarente, L.P. 2006. Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. *Genes Dev.* **20**:2913–2921.
6. Afshar, G., and Murnane, J.P. 1999. Characterization of a human gene with sequence homology to Saccharomyces cerevisiae SIR2. *Gene.* **234**:161–168.
7. Vaquero, A., et al. 2004. Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Mol. Cell.* **16**:93–105.
8. Bordone, L., and Guarente, L. 2005. Calorie restriction, SIRT1 and metabolism: understanding longevity. *Nat. Rev. Mol. Cell Biol.* **6**:298–305.
9. McBurney, M.W., et al. 2003. The mammalian SIR-2alpha protein has a role in embryogenesis and gametogenesis. *Mol. Cell. Biol.* **23**:38–54.
10. Cheng, H.L., et al. 2003. Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **100**:10794–10799.
11. Gao, X., Xu, Y.X., Janakiraman, N., Chapman, R.A., and Gautam, S.C. 2001. Immunomodulatory activity of resveratrol: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production. *Biochem. Pharmacol.*

62:1299–1308.
12. Wu, S.L., et al. 2006. Apoptosis of lymphocytes in allograft in a rat liver transplantation model induced by resveratrol. *Pharmacol. Res.* **54**:19–23.
13. Falchetti, R., Fuggetta, M.P., Lanzilli, G., Tricarico, M., and Ravagnan, G. 2001. Effects of resveratrol on human immune cell function. *Life Sci.* **70**:81–96.
14. Feng, Y.H., et al. 2004. Differential regulation of resveratrol on lipopolysacchride-stimulated human macrophages with or without IFN-gamma pre-priming. *Int. Immunopharmacol.* **4**:713–720.
15. Kraft, A.S., and Anderson, W.B. 1983. Phorbol esters increase the amount of Ca2+, phospholipid-dependent protein kinase associated with plasma membrane. *Nature.* **301**:621–623.
16. Bennett, J.P., Cockcroft, S., and Gomperts, B.D. 1979. Ionomycin stimulates mast cell histamine secretion by forming a lipid-soluble calcium complex. *Nature.* **282**:851–853.
17. Chiang, Y.J., et al. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature.* **403**:216–220.
18. Murphy, K.M., Heimberger, A.B., and Loh, D.Y. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science.* **250**:1720–1723.
19. Macian, F., et al. 2002. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell.* **109**:719–731.
20. Heissmeyer, V., et al. 2004. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol.* **5**:255–265.
21. Anandasabapathy, N., et al. 2003. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity.* **18**:535–547.
22. Safford, M., et al. 2005. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat. Immunol.* **6**:472–480.
23. Olenchock, B.A., et al. 2006. Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol.* **7**:1174–1181.
24. Zha, Y., et al. 2006. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. *Nat. Immunol.* **7**:1166–1173.
25. Bandyopadhyay, S., et al. 2007. Interleukin 2 gene transcription is regulated by Ikaros-induced changes in histone acetylation in anergic T cells. *Blood.* **109**:2878–2886.
26. Thomas, R.M., et al. 2007. Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in CD4+ T lymphocytes. *J. Immunol.* **179**:7305–7315.
27. Wojcik, H., Griffiths, E., Staggs, S., Hagman, J., and Winandy, S. 2007. Expression of a non-DNA-binding Ikaros isoform exclusively in B cells leads to autoimmunity but not leukemogenesis. *Eur. J. Immunol.* **37**:1022–1032.
28. Rincon, M., and Flavell, R.A. 1994. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J.* **13**:4370–4381.
29. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* **7**:2135–2148.
30. Derijard, B., et al. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell.* **76**:1025–1037.
31. Lassmann, H., Brunner, C., Bradl, M., and Linington, C. 1988. Experimental allergic encephalomyelitis: the balance between encephalitogenic T lymphocytes and demyelinating antibodies determines size and structure of demyelinated lesions. *Acta Neuropathol.* **75**:566–576.
32. Sequeira, J., et al. 2008. sirt1-null mice develop an autoimmune-like condition. *Exp. Cell Res.* **314**:3069–3074.
33. Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* **4**:330–336.
34. Khattri, R., Cox, T., Yasayko, S.A., and Ramsdell, F. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat. Immunol.* **4**:337–342.
35. Chen, W., et al. 2003. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* **198**:1875–1886.
36. Kume, S., et al. 2007. SIRT1 inhibits transforming growth factor beta-induced apoptosis in glomerular mesangial cells via Smad7 deacetylation. *J. Biol. Chem.* **282**:151–158.
37. Fantini, M.C., et al. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25– T cells through Foxp3 induction and down-regulation of Smad7. *J. Immunol.* **172**:5149–5153.
38. Dominitzki, S., et al. 2007. Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells. *J. Immunol.* **179**:2041–2045.
39. Jain, R., et al. 2008. Innocuous IFNgamma induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. *J. Exp. Med.* **205**:207–218.
40. Macian, F., Garcia-Rodriguez, C., and Rao, A. 2000. Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. *EMBO J.* **19**:4783–4795.
41. Liu, Y.C., Penninger, J., and Karin, M. 2005. Immunity by ubiquitylation: a reversible process of modification. *Nat. Rev. Immunol.* **5**:941–952.
42. Gajewski, T.F., Qian, D., Fields, P., and Fitch, F.W. 1994. Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. *Proc. Natl. Acad. Sci. U. S. A.* **91**:38–42.
43. Baier-Bitterlich, G., Baier, G., Gulbins, E., Coggeshall, K.M., and Altman, A. 1995. The role of p56lck in CD4-mediated suppression of CD3-induced early signaling events in T lymphocytes. *Life Sci.* **56**:1287–1297.
44. Deckert, M., Tartare-Deckert, S., Hernandez, J., Rottapel, R., and Altman, A. 1998. Adaptor function for the Syk kinases-interacting protein 3BP2 in IL-2 gene activation. *Immunity.* **9**:595–605.
45. Boussiotis, V.A., Freeman, G.J., Berezovskaya, A., Barber, D.L., and Nadler, L.M. 1997. Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science.* **278**:124–128.
46. Boussiotis, V.A., et al. 2000. p27kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nat. Med.* **6**:290–297.
47. Boussiotis, V.A., Lee, B.J., Freeman, G.J., Gribben, J.G., and Nadler, L.M. 1997. Induction of T cell clonal anergy results in resistance, whereas CD28-mediated costimulation primes for susceptibility to Fas- and Bax-mediated programmed cell death. *J. Immunol.* **159**:3156–3167.
48. Go, C., and Miller, J. 1992. Differential induction of transcription factors that regulate the interleukin 2 gene during anergy induction and restimulation. *J. Exp. Med.* **175**:1327–1336.
49. Lee, S., Gao, B., and Fang, D. 2008. FoxP3 maintains Treg unresponsiveness by selectively inhibiting the promoter DNA-binding activity of AP-1. *Blood.* **111**:3599–3606.
50. Muegge, K., et al. 1989. Interleukin-1 costimulatory activity on the interleukin-2 promoter via AP-1. *Science.* **246**:249–251.
51. Serfling, E., et al. 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. *EMBO J.* **8**:465–473.
52. Glozak, M.A., Sengupta, N., Zhang, X., and Seto, E. 2005. Acetylation and deacetylation of non-histone proteins. *Gene.* **363**:15–23.
53. Wang, Y.N., Chen, Y.J., and Chang, W.C. 2006. Activation of extracellular signal-regulated kinase signaling by epidermal growth factor mediates c-Jun activation and p300 recruitment in keratin 16 gene expression. *Mol. Pharmacol.* **69**:85–98.
54. Dey, S., et al. 2008. Interactions between SIRT1 and AP-1 reveal a mechanistic insight into the growth promoting properties of alumina (Al2O3) nanoparticles in mouse skin epithelial cells. *Carcinogenesis.* **29**:1920–1929.
55. Gao, Z., and Ye, J. 2008. Inhibition of transcriptional activity of c-JUN by SIRT1. *Biochem. Biophys. Res. Commun.* **376**:793–796.
56. Singh, N.P., Hegde, V.L., Hofseth, L.J., Nagarkatti, M., and Nagarkatti, P. 2007. Resveratrol (trans-3,5,4′-trihydroxystilbene) ameliorates experimental allergic encephalomyelitis, primarily via induction of apoptosis in T cells involving activation of aryl hydrocarbon receptor and estrogen receptor. *Mol. Pharmacol.* **72**:1508–1521.
57. Milne, J.C., et al. 2007. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature.* **450**:712–716.
58. Lee, I.H., et al. 2008. A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. *Proc. Natl. Acad. Sci. U. S. A.* **105**:3374–3379.
59. Mombaerts, P., et al. 1992. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature.* **360**:225–231.
60. Gao, B., Lee, S.M., and Fang, D. 2006. The tyrosine kinase c-Abl protects c-Jun from ubiquitination-mediated degradation in T cells. *J. Biol. Chem.* **281**:29711–29718.
61. Legge, K.L., et al. 2002. On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. *J. Exp. Med.* **196**:217–227.
62. Kwon, H.S., et al. 2008. Human immunodeficiency virus type 1 Tat protein inhibits the SIRT1 deacetylase and induces T cell hyperactivation. *Cell Host Microbe.* **3**:158–167.