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*J Immunol* 2009; 183:1110-1119; Prepublished online 24 June 2009;
doi: 10.4049/jimmunol.0803671
http://www.jimmunol.org/content/183/2/1110

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/06/25/jimmunol.0803671.DC1

References

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SUMO Conjugation Contributes to Immune Deviation in Nonobese Diabetic Mice by Suppressing c-Maf Transactivation of IL-4

Jianmei W. Leavenworth,* Xiaojing Ma, Yin-yuan Mo,* and Mary E. Pauza2*†

It is not clear why the development of protective Th2 cells is poor in type 1 diabetes (T1D). c-Maf transactivates the IL-4 gene promoting Th2 cell development; therefore, abnormalities in c-Maf may contribute to reduced IL-4 production by CD4 cells from nonobese diabetic (NOD) mice. In this study we demonstrate that despite normal expression, c-Maf binds poorly to the IL-4 promoter (IL-4p) in NOD CD4 cells. Immunoblotting demonstrates that c-Maf can be modified at lysine 33 by SUMO-1 (small ubiquitin-like modifier 1). Sumoylation is facilitated by direct interaction with the E2-conjugating enzyme Ubc9 and increases following T cell stimulation. In transfected cells, sumoylation decreases c-Maf transactivation of IL-4p-driven luciferase reporter activity, reduces c-Maf binding to the IL-4p in chromatin immunoprecipitation assays, and enhances c-Maf localization into promyelocytic leukemia nuclear bodies. Sumoylation of c-Maf is increased in NOD CD4 cells as compared with CD4 cells from diabetes-resistant B10.D2 mice, suggesting that increased c-Maf sumoylation contributes to immune deviation in T1D by reducing c-Maf access to and transactivation of the IL-4 gene. The Journal of Immunology, 2009, 183: 1110–1119.

Received for publication November 3, 2008. Accepted for publication May 18, 2009.

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Many studies point to a correlation between the development of type 1 diabetes (T1D)3 and decreased Th2 responses (1–4). Poor IL-4 production may contribute to the disease process by limiting protective Th2 responses (5), restricting the activation of nonpathogenic T cells (6), and reducing B7.2 expression on dendritic cells that would otherwise curb islet-specific CTL maturation (7). Although reduced IL-4 production is a well-established observation in T1D, the mechanisms contributing to its dysregulation are not clear.

Cellular muscular aponeurotic fibrosarcoma (c-Maf) is a lineage-specific transcription factor that promotes Th2 cell development through direct transactivation of the IL-4 gene (8). An association between T1D and abnormal c-Maf function is suggested by previous studies performed with c-Maf transgenic, nonobese diabetic (NOD) mice where constitutive overexpression of c-Maf in T cells did not effectively increase IL-4 production nor protect NOD mice from T1D (9). These results were unique to NOD mice, because transgenic c-Maf production significantly increased T cell IL-4 production and reduced T1D onset in other transgenic, adoptive transfer, and virally inducible mouse models of TID on B10.D2 genetic backgrounds (9). Abnormal c-Maf function in NOD mice is further suggested by an inability to detect c-Maf binding to the IL-4 promoter (IL-4p) in Tc2 cells (10). Together, these existing data suggest a link between poor IL-4 production and the failure of c-Maf to bind the IL-4p in T cells from diabetes-prone NOD mice.

Several mechanisms can be envisioned as potentially responsible for reduced c-Maf binding to the IL-4p in NOD T cells, including genetic polymorphisms in c-Maf coding or IL-4p sequences, reduced c-Maf expression, or altered posttranslational modification of c-Maf. Morel and colleagues previously reported that sequences surrounding and including the Maf response element (MARE) within the IL-4p in NOD mice are identical with those of C57BL/6 mice (10). More complete data now available in the Mouse Genome Informatics single nucleotide polymorphism (SNP) database (dbSNP) (National Center for Biotechnology Information build 37; dbSNP build 128) confirm and extend this observation. No SNPs are found within the c-Maf coding sequence when comparing the NOD sequence with those of diabetes-resistant C57BL/6, B10.D2, and BALB/c strains. Furthermore, no polymorphisms are observed within the established MARE site of the IL-4p. However, four SNPs are found further upstream (SNP identification nos. rs26969121 (located at −391 relative to the transcriptional start site), rs26969120 (−461), rs13495225 (−512), and rs26969118 (−597)). None of these involve potential MARE sites as determined by the AliBaba2.1 and TFSEARCH in silico transcription factor binding site prediction programs (11). Furthermore, c-Maf mRNA expression in NOD T cells is similar to that in BALB/c T cells (10). Because neither polymorphisms in c-Maf or IL-4p nor changes in c-Maf expression levels are likely responsible for reduced c-Maf binding to the IL-4p in NOD T cells, we explored the possibility that posttranslational sumoylation affects c-Maf binding to the IL-4p.

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Received for publication November 3, 2008. Accepted for publication May 18, 2009.

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1 This work was supported by grants from the Juvenile Diabetes Research Foundation and the Southern Illinois University Central Research Committee (to M.E.P.).

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3 Abbreviations used in this paper: T1D, type 1 diabetes; CHIP, chromatin immunoprecipitation; c-Maf, cellular muscular aponeurotic fibrosarcoma; DN, dominant negative; HEK, human embryonic kidney 293; IL-4p, IL-4 promoter; IP, immunoprecipitation; luc, luciferase; L2D, leucine zipper domain; MARE, Maf response element; NEM, N-ethylmaleimide; NOD, nonobese diabetic; NOR, nonobese diabetes resistant; PML, promyelocytic leukemia; PML-NB, PML nuclear body; RIPA, radioimmunoprecipitation assay; SNP, single nucleotide polymorphism; dbSNP, SNP database; SUMO, small ubiquitin-like modifier; TAD, transactivation domain; WB, Western blotting.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803671
Sumoylation influences a diverse array of cellular functions and appears particularly important for transcription (12–15). Sumoylation can enhance, but more commonly represses, transcription (12). Although no SNPs have been identified in the coding sequence of murine small ubiquitin-like modifier (SUMO)-1 (Mouse Genome Informatics dbSNP query comparing NOD, C57BL/6, B10.D2, and BALB/c), a polymorphism in the coding sequence of human SUMO-4 was recently identified and associated with T1D (16, 17). However, the validity of this association has been questioned (18–20). Furthermore, the expression of SUMO-4 in human cells is likely to be very limited (21), and no murine ortholog has been identified. Despite the controversial linkage with T1D, the established polymorphisms clearly influence the ability of SUMO-4 to stabilize IkB and enhance heat shock transcription factors, demonstrating that changes in sumoylation can have measurable effects on specific cellular functions (16, 17).

In this study, we demonstrate that in CD4 cells c-Maf is subject to conjugation by SUMO-1 and that levels of SUMO-c-Maf are enhanced following CD4 cell activation. Sumoylation of c-Maf at lysine 33 reduces its transactivational capacity by impairing c-Maf binding to the IL-4p. Sumoylation correlates with c-Maf trafficking into promyelocytic leukemia (PML) nuclear bodies (PML-NBs), suggesting that subnuclear sequestration contributes to reduced promoter binding and to transcriptional repression. Moreover, the proportion of sumoylated c-Maf is increased in activated NOD CD4 cells, providing new insights into the defective binding of c-Maf to the IL-4p and the poor IL-4 production characteristic of CD4 cells from diabetes-prone NOD mice.

Materials and Methods

Mice

Six- to 9-week-old age-matched female NOD, B10.D2, or nonobese diabetes resistant (NOR) mice were used as sources of CD4 cells (The Jackson Laboratory). Mice were bred and maintained under specific pathogen-free conditions at Southern Illinois University School of Medicine (Springfield, IL) in accordance with National Institutes of Health and institutional guidelines.

In vitro purification and activation of CD4 cells

CD4 cells were purified from spleen and lymph nodes pooled from 3–5 mice per strain as described previously (22). Purified CD4 cells were used immediately or activated with 5–10 μg/ml plate-bound anti-CD3 (145-2C11; BD Pharmingen) and 1 μg/ml soluble anti-CD28 (37.51; BD Pharmingen) for 8–36 h in RPMI 1640 (Invitrogen) plus 10% FBS (Atlanta Biologicals), 10 mM HEPES (Invitrogen), 50 μM 2-ME, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete RPMI medium) before activation. In some cases, 20 ng/ml IL-6 (PeproTech) was added.

Plasmid construction

Plasmids for the expression of full-length c-Maf (FlagMafCD3S) or mutants lacking the transactivation domain (TAD) (FlagMafTAD) or leucine zipper domain (LZD) (FlagMafLALZD) were constructed using standard molecular cloning techniques as described in the supplemental Extended Methods (8). Lyxine to arginine point mutants were constructed from FlagMafCDS using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Murine pIL-4 (–157/+68)-luc (H4p-luc; where luc stands for luciferase) reporter and pCMV-Myc-SUMO-1 (MySUMO-1), pCMV-Myc-Ubc9DN (MycUbc9DN; where DN is dominant negative), or pSG9mGam1wt-Myc (MycGam1) expression plasmids and their corresponding parental vectors (pCMV-Myc and pSG9mMyc) were previously described (23–25). Primer pairs used for cloning are listed in supplemental Table I. The integrity of all plasmids was confirmed by DNA sequencing (University of Illinois, Urbana-Champaign, IL).

Transient transfections and luciferase reporter assays

Human embryonic kidney 293 (HEK) and murine thymoma (EL4) cell lines were obtained from American Type Culture Collection and maintained in DMEM (Irvine Scientific) containing 10% FBS, 1 mM sodium pyruvate (Sigma-Aldrich), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete DMEM). Cells were cultured for 24 h and then transfected using ExGen500 (Fermentas) according to the manufacturer’s instructions. In all cases, the total amount of DNA transfected was kept constant by the addition of a control plasmid. EL4 cells were cultured in complete DMEM for 18–24 h after transfection and then treated with 1 μM ionomycin for 24 h before downstream analyses. For luciferase assays, EL4 cells were cotransfected with H4p-luc, pMuc2-Renilla (Novagen), and full-length or mutant c-Maf plasmids. Firefly (Renilla) luciferase enzymatic activities were assessed as described (22).

Immunoprecipitation (IP) and Western blotting (WB)

Cells were lysed in radioimmune precipitation assay (RIPA) buffer (25 mM Tris (pH 8.2), 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 0.1% azide) plus 0.1 mM PMSF and a protease inhibitor cocktail (Sigma-Aldrich). For detection of SUMO-conjugated proteins, transfected HEK cells or CD4 cells were washed twice with ice-cold PBS supplemented with 20 mM N-ethylmaleimide (NEM; Sigma-Aldrich) and lysed in SDS lysis buffer (5% SDS, 0.15 M Tris (pH 6.7), and 30% glycerol) diluted 1/2 in RIPA buffer (25 mM Tris (pH 8.2), 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 0.1% azide) containing 0.1 mM PMSF, protease inhibitor cocktail, and 20 mM NEM (26). In some assays, cytoplasmic and nuclear fractions were prepared separately using a nuclear extract kit (Active Motif) according to the manufacturer’s protocol. Cytoplasmic and nuclear fractions were collected in 1× hypotonic buffer (kit component) containing 20 mM NEM. Nuclear pellets were resuspended in SDS/ RIPA buffer, sonicated briefly, and centrifuged. Supernatants were collected as nuclear fractions. Protein concentrations were determined using the DC reagent kit (Bio-Rad) (22). For immunoprecipitations, 500 μg of total cell lysates were suspended in 1 ml of ice-cold PBS and 0.5% Nonidet P-40 and incubated with 2 μg of mouse anti-Flag (M2, Sigma-Aldrich), 2 μg of rabbit anti-c-Maf (M-153, Santa Cruz Biotechnology), or 5 μg of mouse anti-SUMO-1 (21C7; Invitrogen) overnight at 4°C before precipitation with 50 μl of protein G-Sepharose (GE Healthcare) for 1 h at 4°C. Immunoprecipitates were washed three times with PBS with 0.5% Nonidet P-40. Immunoprecipitates or 50 μg of lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and subjected to Western transfer (22). To detect exogenous (plasmid-derived) or endogenous (native) c-Maf proteins, membranes were incubated overnight at 4°C with anti-Flag or anti-c-Maf, respectively. Sumoylated c-Maf was detected using either anti-c-Myc (9E10; Clontech) or anti-SUMO-1 (D-11; Santa Cruz Biotechnology) Ab. Primary Abs were detected by incubation with goat anti-mouse IgG-HRP (Calbiochem) or goat anti-rabbit IgG HRP (Santa Cruz Biotechnology) for 1 h at room temperature. In some cases, blots were incubated with Clean-Blot IP detection reagent (Pierce) or mouse IgG F(ab)’2. Cytoplasmic and nuclear fractions were treated with the nuclear topoisomerase I, cytoplasmic β-tubulin, or β-actin, membranes were stripped with 64 mM Tris (pH 6.8), 2% SDS, and 0.1 M 2-ME at 50°C for 30 min, rebloked, and probed with goat anti-topoisomerase I (C-15; Santa Cruz Biotechnology), mouse anti-β-tubulin (AA2; Millipore), or mouse anti-β-actin (AC-15; Sigma-Aldrich), respectively. Donkey anti-goat IgG HRP (Santa Cruz Biotechnology) or goat anti-mouse HRP were used as secondary Abs. Protein signals were detected using SuperSignal West Pico chemiluminescence kit (Pierce) and visualized on a LAS-3000 imaging system (Fujiﬁlm). Data were quantitated by densitometry using Multi-Gauge software (Fujiﬁlm).

Immunocytochemistry and confocal microscopy

Triple immunofluorescence staining was performed as previously described (27). Briefly, 16 h post-transfection 1.5 × 10^6 HEK cells were split into a two-well Lab-Tek chamber slide (Nalge Nunc) coated with poly-lysine and cultured for another 16 h. Cells were washed once with PBS, fixed for 30 min in 1:1 methanol/aceton at −20°C, and then rinsed three times in PBS. After blocking with PBS and 0.05% Tween 20 (PBST) plus 1% BSA for 1 h at room temperature, slides were incubated with rabbit anti-PML (H238; Santa Cruz Biotechnology) in PBST plus 0.5% BSA overnight at 4°C. Slides were washed four times in PBST plus 0.5% BSA and incubated with goat anti-rabbit IgG-Cy3 (Invitrogen) for 2 h at room temperature. Cells were then stained with mouse anti-c-Maf and then goat anti-mouse IgG-Alexa Fluor 568 (Invitrogen) to visualize SUMO-1. Finally, slides were incubated with anti-Flag-FITC (1/100; Sigma-Aldrich) overnight at 4°C to detect c-Maf protein. Fluorescence was detected using an

* The online version of this article contains supplemental material.
Olympus confocal microscope (×100 original magnification) and analyzed using Fluoview software (Olympus).

**Chromatin immunoprecipitation (ChIP) assays**

ChIP assays were performed as previously described (22). DNA-protein complexes were immunoprecipitated with 2 μg of anti-c-Maf, anti-Flag, anti-H3 (Millipore), anti-acetylated H3 (Millipore), preimmune rabbit IgG (Jackson ImmunoResearch Laboratories), or without any Ab. PCR was performed using primers (supplemental Table I) specific for the native mouse IL-4p (murine IL-4 ChIP primers) or the IL-4p found within the IL-4p-luc reporter construct (IL-4p-luc primers).

**Real-time reverse transcriptase PCR**

Total RNA was isolated from ionomycin-treated EL4 transfectants using RNeasy kits (Qiagen) according to the manufacturer's instructions. Genomic DNA was removed using Turbo DNase (Ambion) and cdNA was generated using SuperScript III reverse transcriptase (Invitrogen) according to each manufacturer's instructions. Real-time PCR were performed using SYBR Green master mix (New England Biolabs) and analyzed on an IQ5 thermal cycler (Bio-Rad). Primer sequences are listed in supplemental Table I. The following cycle conditions were used: 95°C for 15 min and then 40 cycles at 95°C for 15 s, 58°C for 25 s, and 72°C for 30 s, followed by melt curve analysis. Data were analyzed using the Pfaffl method to account for differences in primer efficiencies (28). Levels of IL-4 transcripts found in SUMO-1 transfectants were expressed relative to control vector transfectants using the following formula: fold change = (E_{(p/ΔCt)}^{input}/H1001) / (E_{(p/ΔCt)}^{input}/H1001).

### Results

**IL-4 production and c-Maf binding to the IL-4p are reduced in NOD CD4 cells despite normal c-Maf expression**

To test whether IL-4 production differs for NOD CD4 cells in our system where diabetes-resistant B10.D2 mice were used as controls, we compared intracellular IL-4 production profiles by flow cytometry. The percentage of IL-4-producing cells was reduced by >2-fold for NOD CD4 cells cultured using standard Th2 conditions compared with parallel cultures of B10.D2 CD4 cells (supplemental Fig. 1, A and B). The level of IL-4 protein produced on a per cell basis was also decreased in NOD CD4 cells as revealed by the mean fluorescence intensity of IL-4+ cells (supplemental Fig. 1, A and C). These results are consistent with previous reports showing poor IL-4 production and Th2 development among CD4 cells from NOD mice (1, 4, 29).

Because c-Maf transactivation of IL-4 is dependent on its direct interaction with the IL-4p, we examined c-Maf binding to the IL-4p half-MARE site after 36 h of stimulation with anti-CD3 plus anti-CD28 using ChIP assays. c-Maf binding was detected in B10.D2 CD4 cells consistent with their ability to produce IL-4 (Fig. 1). However, c-Maf association with the IL-4p was nearly undetectable in NOD CD4 cells. DNA that was not subject to IP yielded similar results for both NOD and B10.D2 samples (input), indicating that DNA integrity was similar for both samples. DNA integrity was also maintained following the IP procedure, because similar results were obtained from both NOD and B10.D2 cells with Ab specific for total histone H3. Despite the near absence of c-Maf at the IL-4p half-MARE site in NOD CD4 cells, acetylation of histone H3 remained similar to that observed with B10.D2 cells, indicating an open local chromatin structure (Fig. 1). These results are not unique to comparisons made with the control strain B10.D2. Similar findings were observed when comparing stimulated CD4 cells from NOD mice with those from the closely related but diabetes-resistant NOR mice (supplemental Fig. 2A). Together, these data suggest that although an open chromatin structure is present within the IL-4p, c-Maf binding is greatly reduced among stimulated CD4 cells from young female NOD mice, consistent with poor IL-4 production.

To determine whether reduced c-Maf binding to the IL-4p in NOD CD4 cells is due to decreased c-Maf expression, we performed a series of WBs to measure c-Maf protein levels in NOD compared with those in B10.D2 CD4 cells. Similar c-Maf protein levels were detected in CD4 cells from both strains (Fig. 2A). The kinetics of c-Maf expression following stimulation with anti-CD3 plus anti-CD28 was also similar among the strains (Fig. 2B). IL-6 was previously reported to increase c-Maf expression during early stages of T cell activation (30). c-Maf expression was nearly doubled when IL-6 was added to B10.D2 CD4 cell cultures (Fig. 2A). Similar results were obtained with NOD CD4 cells. Thus, c-Maf protein expression appears normal in NOD CD4 cells.

**c-Maf is sumoylated in primary CD4 cells**

Sumoylation influences transcription factor activity by disrupting DNA recognition in some cases (31–33); we therefore investigated the possibility that c-Maf is a SUMO-1 target protein. Using the sumoylation predication program SUMOplot (Abgent), we found five putative SUMO binding motifs conserved within mouse and human c-Maf proteins, three within the TAD and another two in the LZD (Fig. 3A). To begin examining the possibility that c-Maf is subject to sumoylation, we performed IP assays using B10.D2 CD4 cell lysates prepared in the presence of the cysteine peptidase inhibitor NEM, which blocks desumoylation. In addition to the typical ~50 kDa c-Maf species, a second higher molecular mass species (~72 kDa) was detected (Fig. 3B). The ~20-kDa shift in molecular mass is consistent with monosumoylation (34) and was most evident in CD4 cells following TCR/CD28 engagement (Fig. 3B). This band was not observed in samples immunoprecipitated with rabbit IgG (IgG) or without Ab (No Ab) as negative controls. PCR was conducted on the eluted DNA using primer pairs specific for the MARE site within the mouse IL-4p. Cell lysates (10%) were also assessed without prior IP (Input). B. Results from ChIP assays performed as described in A were quantitated using densitometry of ethidium-stained gels. Results are graphed as fold change relative to total H3 (set as 1) with SD values indicated by error bars. Statistical comparisons of NOD and B10.D2 results were performed using a paired Student’s t test (*, p < 0.05; n = 3).
with the IgG control Ab, indicating that the 72-kDa band is specific for c-Maf. Together, these results suggest that c-Maf is sumoylated following stimulation of CD4 cells.

c-Maf is directly conjugated to SUMO-1 and Ubc9 in vivo

To confirm and extend these findings, we performed IP and WB analyses using HEK cells transiently transfected with c-Maf (Flag-MafCDS) and SUMO-1 (MycSUMO-1) expression plasmids simultaneously. A 72-kDa band was observed when c-Maf was immunoprecipitated using anti-Flag and plots probed with anti-SUMO-1 (Fig. 3C). Likewise, IP with anti-SUMO-1 followed by anti-Flag WBs revealed a band of the same molecular mass (Fig. 3C). A 72-kDa band was also evident in parallel anti-Flag WBs (Fig. 3C; Input). Sumoylated c-Maf was also readily detected when anti-Flag immunoprecipitates were probed with anti-Myc (Fig. 3D). The specificity of these observations was demonstrated by the absence of a 72-kDa band in cells transfected with Flag-MafCDS or MycSUMO-1 alone (Fig. 3D). Together, these assays confirm that SUMO-1 can conjugate c-Maf.

Ubc9 is an E2 conjugation enzyme that is essential for sumoylation of target proteins (35). DN Ubc9 is capable of blocking sumoylation (24). Sumoylation of c-Maf was greatly diminished in HEK transfectants coexpressing MycUbc9DN (Fig. 3D, left). Reduction in band intensity was not due to unequal expression of c-Maf, because a similar amount of c-Maf was detected in input WBs (Fig. 3D, right). When Ubc9-specific Ab was used to probe blots containing anti-Flag (c-Maf) immunoprecipitates, endogenous Ubc9 was detected indicating that direct interaction of Ubc9 with c-Maf occurs and likely promotes SUMO-1 conjugation. c-Maf appeared to preferentially bind to Ubc9DN, because endogenous Ubc9 was not readily detected in immunoprecipitates of
transfectants expressing Ubc9DN (Fig. 3D, left) despite the presence of both forms of Ubc9 in these cells (Fig. 3D, right). Together, these results indicate that SUMO-1 can be conjugated to c-Maf, most likely through the enzymatic activity of Ubc9.

**Sumoylation of c-Maf occurs at lysine 33**

To define the site(s) of SUMO-1 conjugation in c-Maf, two deletion mutants were constructed: one without the TAD domain (FlagMafΔTAD) and the other lacking the LZD (FlagMafΔLZD). SUMO-conjugated c-Maf was detected in HEK cells transfected with MycSUMO-1 plus FlagMafCDS or FlagMafΔLZD, but not FlagMafΔTAD (Fig. 4A). Thus, sumoylation likely occurs within the TAD. To determine whether one or more of the predicted sumoylation sites within this domain are used, we used site-directed mutagenesis to convert lysine at position 29, 33, or 34 to arginine. Sumoylation still occurred with the K29R and K34R mutants, but not the K33R mutant (Fig. 4B). Parallel detection of sumoylated DNA topoisomerase I suggests that the absence of SUMO-c-MafK33R was not due to failure of SUMO-1 expression. These results demonstrate that SUMO-1 conjugation occurs at lysine 33 within the TAD of c-Maf.

**Sumoylated c-Maf accumulates within PML-NBs**

Although c-Maf acts as a transcription factor predominantly in the nucleus, its entry into and localization within the nucleus are poorly understood. Given the ability of sumoylation to influence protein trafficking (36, 37), we tested the effect of sumoylation on c-Maf nuclear localization. As anticipated, WB analyses of HEK transfectants expressing the K33R c-Maf mutant, which sumoylation (23). Expression of Gam1 had no effect on nuclear localization of c-Maf (Fig. 5A). Similar results were obtained with HEK transfectants expressing the K33R c-Maf mutant, which...
cannot be sumoylated (Fig. 5A). Identical findings were observed by immunofluorescence confocal microscopy (supplemental Fig. 3). Together, these results clearly demonstrate that sumoylation does not change the typical nuclear localization pattern of c-Maf.

Sumoylation can also modify transcription factor activity by influencing subnuclear localization (39). Therefore, we examined HEK transfectants expressing c-Maf alone or together with SUMO-1 by immunofluorescence confocal microscopy. In the absence of exogenous SUMO-1, c-Maf was detected primarily in a diffuse pattern throughout the nucleus (supplemental Fig. 3 and Fig. 5B). In the nuclei of transfectants expressing both c-Maf and SUMO-1, c-Maf was not only found in a diffuse pattern but also in discrete punctuate dots or speckles reminiscent of nuclear bodies (supplemental Fig. 3 and Fig. 5B). Merged images of dual-stained transfectants demonstrate that c-Maf colocalized with SUMO-1 within these dots. Simultaneous staining with Ab specific for PML protein demonstrated that these structures are PML-NBs (Fig. 5B). In contrast, c-Maf was not found in PML-NBs in HEK cells transfected simultaneously with the c-Maf K33R mutant and SUMO-1 expression vectors (supplemental Fig. 3 and Fig. 5B). The presence of mutant c-Maf did not itself influence PML-NB formation, because nuclear speckles were still visible at sites of SUMO-1 and PML protein colocalization (Fig. 5B). Together, these results suggest that sumoylation targets c-Maf to PML-NBs without altering its general nuclear translocation property.

Sumoylation represses c-Maf transcriptional capacity

Spatial sequestration of sumoylated c-Maf into PML-NB may limit c-Maf access to its target genes; we therefore examined the influence of sumoylation on c-Maf transcriptional activity using dual luciferase reporter assays. EL4 murine thymoma cells were transfected with an IL4p-luc reporter construct plus c-Maf and/or SUMO-1 expression plasmids (Fig. 6A). Transfectants were treated with 1 μM ionomycin 24 h posttransfection to provide optimal conditions for IL-4p activation (40). Strong luciferase activity was evident in EL4 cells expressing c-Maf relative to control transfectants (pCMV-Flag + pCMV-Myc or pCMV-Flag + MycSUMO-1), demonstrating that c-Maf effectively transactivates the IL-4p-driven luciferase reporter gene (Fig. 6A). In contrast, luciferase activity was reduced by 40% in EL4 cells transfected with FlagMafCDS plus MycSUMO-1, suggesting that sumoylation inhibits c-Maf transcriptional activity. The inclusion of plasmids expressing the SUMO inhibitors Ubc9DN or Gam1 reversed the SUMO-mediated inhibition of c-Maf transcriptional activity (Fig. 6A). The ability of Ubc9DN or Gam1 to restore luciferase activity beyond that achieved by c-Maf alone suggests that EL4 cells express endogenous SUMO-1 capable of influencing c-Maf activity. Together, these data demonstrate that sumoylation inhibits c-Maf transcriptional activation of the IL-4p.

Although these reporter assays provide useful information, they cannot determine whether transcriptional repression of the IL-4p luciferase reporter is due primarily to sumoylation of c-Maf itself or is secondary to the sumoylation of another protein(s). Therefore, reporter assays were performed using K29R, K33R, or K34R c-Maf mutants. Consistent with our previous assays, luciferase activity was markedly reduced in EL4 transfectants expressing sumoylation-competent forms of c-Maf (CDS, K29R, or K34R) plus MycSUMO-1 as compared with parallel transfectants that did not express exogenous SUMO-1 (Fig. 6B). In contrast, luciferase activity among EL4 cells expressing sumoylation-incompetent c-Maf (FlagMafK33R) remained unaffected by exogenous SUMO-1, demonstrating that sumoylation of c-Maf itself reduces its transactivation potential. Similar results were obtained with EL4 cells transfected with a triple mutant (3KR). The 3KR mutant had a nearly identical stimulatory effect on IL-4p-driven reporter activity as the K33R c-Maf mutant (data not shown).

An unusual characteristic of sumoylation is that a significant change in cellular function can be attributed to the conjugation of a relatively small fraction of the target protein (13, 41). Also, we found that a relatively small percentage of c-Maf was sumoylated in EL4 transfectants expressing full-length c-Maf plus SUMO-1 (supplemental Fig. 4). Because these transfection conditions are identical with those in Fig. 6B, these data demonstrate that a small fraction of detectable sumoylated c-Maf can result in a measurable physiologic effect.

Because the IL-4p luciferase construct does not contain distant regulatory elements, we also examined endogenous IL-4 gene expression in EL4 transfectants (Fig. 6C). EL4 cells transfected with
full-length c-Maf together with SUMO-1 expression plasmids had a >2-fold decrease in endogenous IL-4 mRNA levels compared with cells transfected with c-Maf alone. Taken together, these data provide strong support for the hypothesis that sumoylation at lysine 33 represses c-Maf transactivation of the IL-4p in T lineage cells.

**Overexpression of SUMO-1 impairs c-Maf binding to the IL-4p**

The repressive effect of SUMO-1 on c-Maf transactivation of the IL-4p may be the result of reduced c-Maf binding to its target MARE site. Alternatively, sumoylation may alter c-Maf activity without affecting its affinity for the IL-4p. To distinguish between these two possibilities, we performed ChIP assays on EL4 transfectants to examine c-Maf binding to both the exogenous and endogenous IL-4 promoters (Fig. 7, A and B). Following IP with anti-Flag Ab, only a very weak signal was detected by PCR, with EL4 cells expressing both c-Maf and SUMO-1 (Fig. 7A). In contrast, a product was readily detected with EL4 cells transfected with FlagMafCDS alone. Input and anti-H3 immunoprecipitates served as positive controls, demonstrating that DNA quantity and integrity were appropriate. Results obtained with anti-acetylated-H3 immunoprecipitates demonstrated an open local chromatin structure; thus, failure to detect c-Maf at the IL-4p was not due to chromatin inaccessibility. Similar results were observed with primers specific for the endogenous IL-4p, demonstrating that enhanced sumoylation of c-Maf inhibits transactivation of not only the exogenous IL-4p but also of native IL-4p (Fig. 7B). Therefore, impaired c-Maf transactivation of the IL-4p by SUMO conjugation is mainly due to reduced c-Maf binding to the IL-4p. This is also consistent with SUMO-dependent compartmentalization of c-Maf into PML bodies where c-Maf access to the IL-4p half-MARE site is presumably limited.

**FIGURE 7.** Overexpression of SUMO-1 impairs c-Maf binding to the IL-4p. A. ChIP assays were performed using lysates obtained from EL4 cells 2 days posttransfection with FlagMafCDS and IL-4p-luc together with pCMV-Myc control (without SUMO-1) or MycSUMO-1 vectors (with SUMO-1) as in Fig. 6A. Abs specific for histone 3 (H3), acetylated histone 3 (Ac-H3), or Flag (c-Maf) were used for IP of cross-linked lysates as described in Fig. 1A. PCR was conducted on eluted DNA or 10% “Input” DNA using primer pairs specific for the IL-4p-luc construct as diagramed and shown in supplemental Table I (IL4plus). Data are representative of two independent experiments. B. ChIP assays were performed as in A, except that the PCRs were specific for the endogenous IL-4p as diagramed and shown in supplemental Table I (mIL-4 ChIP). Data are from one of three independent experiments.

**Elevated SUMO-c-Maf is found in NOD CD4 cells**

Given that c-Maf binding to the IL-4p is reduced in NOD CD4 cells and that sumoylation can reduce c-Maf engagement with the IL-4p, we examined the possibility that reduced binding of c-Maf to the IL-4p in NOD CD4 cells is the result of increased sumoylation of c-Maf. In reciprocal IP assays using anti-c-Maf and anti-SUMO-1 Abs and subsequent WBs were probed with mouse anti-SUMO-1 or rabbit anti-c-Maf, respectively. Mouse IgG TrueBlot or Clean-Blot IP detection reagent was used respectively. Blots are representative of two independent assays. B. WBs of whole cell lysates from A without prior IP were probed with rabbit anti-c-Maf. Membranes were stripped and reprobed with anti-SUMO-1 and then anti-β-tubulin Abs. Asterisks indicate SUMO-c-Maf and the arrowhead indicates unSUMO-c-Maf. The triangle marks protein consistent in molecular mass with sumoylated RanGAP. Expression of unSUMO-c-Maf was quantitated using densitometry and is expressed as a ratio to β-tubulin. Ratios are given below each lane. C. WBs were performed as described in B, and sumoylated c-Maf protein expression was determined by densitometry. Mean (±SEM) sumoylated c-Maf protein expression was normalized to β-tubulin. Results obtained from activated NOD CD4 cells were compared with those obtained from resting NOD CD4 cells or activated B10.D2 CD4 cells using paired Student’s t test (*, p < 0.05, n = 6).

**FIGURE 8.** Sumoylated c-Maf is increased in NOD compared with B10.D2 CD4 cells. Purified CD4 cells from 8-wk-old NOD or B10.D2 mice were left untreated or treated with 5 μg/ml plate-bound anti-CD3 plus 1 μg/ml soluble anti-CD28 for 36 h. A. Total cell lysates were immunoprecipitated with rabbit anti-c-Maf (top) or mouse anti-SUMO-1 (bottom) Abs and subsequent WBs were probed with mouse anti-SUMO-1 or rabbit anti-c-Maf, respectively. Mouse IgG TrueBlot or Clean-Blot IP detection reagent was used respectively. Blots are representative of two independent experiments. B. WBs of whole cell lysates from A without prior IP were probed with rabbit anti-c-Maf. Membranes were stripped and reprobed with anti-SUMO-1 and then anti-β-tubulin Abs. Asterisks indicate SUMO-c-Maf and the arrowhead indicates unSUMO-c-Maf. The triangle marks protein consistent in molecular mass with sumoylated RanGAP. Expression of unSUMO-c-Maf was quantitated using densitometry and is expressed as a ratio to β-tubulin. Ratios are given below each lane. C. WBs were performed as described in B, and sumoylated c-Maf protein expression was determined by densitometry. Mean (±SEM) sumoylated c-Maf protein expression was normalized to β-tubulin. Results obtained from activated NOD CD4 cells were compared with those obtained from resting NOD CD4 cells or activated B10.D2 CD4 cells using paired Student’s t test (*, p < 0.05, n = 6).
anti-SUMO-1 Abs, a 72-kDa band consistent with sumoylated c-Maf was observed in both NOD and B10.D2 CD4 cells (Fig. 8A). The intensity of this band was stronger for lysates obtained from CD4 cells 36 h after stimulation with anti-CD3 plus anti-CD28 compared with freshly isolated CD4 cells. Band intensity was also greater for NOD compared with B10.D2 CD4 cells. Differences in the amount of SUMO-c-Maf detected for NOD vs B10.D2 CD4 cells was not due to unequal expression of c-Maf, because a similar amount of unsumoylated c-Maf was detected in parallel input WBs (Fig. 8B). A 72-kDa band consistent with sumoylated c-Maf was also evident in input WBs probed with anti-SUMO-1 (Fig. 8B). The apparent sumoylated c-Maf band was more readily visible in NOD CD4 cells following TCR/CD28 stimulation than in resting NOD or B10.D2 CD4 cells (Fig. 8B). To compare sumoylation quantitatively, we performed a series of similar WBs and normalized the 72-kDa band to β-tubulin using densitometry. After stimulation, sumoylated c-Maf levels increased in both NOD and B10.D2 CD4 cells. Of note, the magnitude of increase was ~2-fold for NOD CD4 cells. In contrast, SUMO-c-Maf in activated B10.D2 CD4 cells only reached a level similar to that of freshly isolated NOD CD4 cells. Although sumoylated c-Maf levels are relatively low in freshly isolated CD4 cells, a significant difference was still observed when comparing ex vivo NOD to B10.D2 CD4 cells (Fig. 8C). Similar results were observed when comparing CD4 cells from NOD mice to those of NOR mice (supplemental Fig. 2, B and C). Together, these data demonstrate that the increase in SUMO-1 conjugation of c-Maf that typically occurs as CD4 cells become activated is accentuated in NOD CD4 cells.

Discussion
Prior to this study it was unclear what factors contributed to the impaired Th2 immune responses in humans and animals with T1D (2, 3, 10, 42). The studies presented here demonstrated that enhanced sumoylation of c-Maf limits its access to the IL-4p, thereby reducing IL-4 production. Although sumoylation did not disrupt the strong nuclear localization of c-Maf, it did result in an accumulation of c-Maf within PML-NBs. Importantly, the relative amount of sumoylated c-Maf was increased among activated CD4 cells from NOD mice compared with diabetes-resistant B10.D2 mice. Based on these results, we propose that increases in c-Maf sumoylation contribute to limited IL-4 production by CD4 cells from diabetes-prone NOD mice, creating an environment averse to Th2 development.

Subnuclear reorganization of SUMO-conjugated c-Maf into PML-NBs, as shown by our confocal microscopy studies, is consistent with poor association with the IL-4p. PML-NBs are recognized as dynamic structures capable of sequestering proteins within the nuclear environment (43–46). Thus, sumoylation may disrupt c-Maf transactivation of IL-4, at least in part, by redirecting c-Maf subnuclear localization into PML-NBs thereby limiting its access to the IL-4p. However, we cannot exclude the possibility that additional mechanisms operate simultaneously to deter c-Maf interaction with the IL-4p.

Sumoylation may alter c-Maf interactions with other proteins, thereby disrupting DNA recognition. NFIAT1 is known to synergize with c-Maf forming a unique complex at the IL-4p (8). If sumoylation disrupts c-Maf interaction with NFIAT1, a reduction in both c-Maf and NFIAT1 binding to the IL-4p would be expected. In contrast, sumoylation may enhance c-Maf interactions with other proteins that hinder c-Maf recognition of the half-MARE site within the IL-4p. The NF-κB family member RelA/p65 may be such a protein. Previous studies suggest that RelA interferes with c-Maf binding to the human IL-4p in T cells from minimal change nephrotic syndrome patients (47). Presently, it is not clear what impact sumoylation has on c-Maf interactions with NFIAT1 or RelA, but the ability of sumoylation to alter protein-protein interactions has been observed for other sumoylated factors, including RanGAP and Sp3 (48–50). In some cases, initiation of a protein interaction is dependent on sumoylation, but maintenance of that interaction is not. For example, Smad4 associates with Daxx in a sumoylation-dependent manner, but upon desumoylation Smad4 remains bound to Daxx (51). Similar observations have been made with the CREB-binding protein (52). Such observations may help to explain how small changes in the percentage of protein that is sumoylated can mediate physiologically meaningful effects.

Similarly as in our study, Morel and colleagues previously attributed poor IL-4 production by NOD Tc2 cells to reduced c-Maf binding to the IL-4p (10). However, in their study c-Maf binding appeared normal in committed NOD Th2 cells (10). The reason for the discrepancy between Th2 cells in the Morel study and CD4 cells in our study is not clear. The most notable difference between these studies is the stage of development assessed, i.e., reinforced vs initial Th2 development. The generation of committed Th2 cells from naive CD4 cells requires repeated stimulation in an IL-4-rich, IFN-γ-deficient environment that induces change in transcription factors and chromatin structure, resulting in faithful type 2 cytokine production (53, 54). As Th2 cell commitment is reinforced, the overall level of c-Maf increases, transcription factors capable of synergizing with c-Maf become available and epigenetic changes stabilize the IL-4 locus in an active configuration. Although additional studies are needed, at present we propose that one or more of these events allow normal levels of c-Maf binding to the IL-4p of committed NOD Th2 cells despite an increased proportion of sumoylated c-Maf in established Th2 cells. In contrast, commitment to reliable IL-4 production is tenuous for CD4 cells that have just received their first activation stimulus under nonpolarizing conditions. A Th2 fate is not sealed; expression of type 1 cytokines can occur with subsequent changes in the cytokine environment, Ag, or costimulatory signals (55). Initial TCR and CD28 engagement activates transient global chromatin derepression, allowing universal transcription factors such as NFAT to gain access to the regulatory regions of IL-4 and drive early transcription (56). Although c-Maf is induced following TCR/CD28 engagement even in the absence of exogenous IL-4, the level of c-Maf production is less than that observed with reinforced Th2 cells (30). Thus, we propose that during the initiation stage of CD4 cell activation, IL-4 production is sensitive to c-Maf sumoylation. In cases where the proportion of SUMO-c-Maf is significant, as with NOD CD4 cells, the Th1/Th2 balance is tipped against Th2 cell development.

A few transcription factors known to regulate IL-4, other than c-Maf, are also sumoylated, but the downstream effect differs. The basic leucine zipper transcription factor C/EBPβ promotes IL-4 and represses c-Myc expression (26, 57). Although sumoylation impairs C/EBPβ suppression of c-Myc, transactivation of the IL-4 gene is unaffected (26). JunB is also modified by sumoylation in T cells (58). Its sumoylation appears to promote transactivation of the IL-4 and IL-2 promoters, because SUMO-deficient mutants have reduced transactivation ability. The mechanisms underlying enhanced JunB activity by sumoylation are not clear but may be indirect, requiring other transcriptional regulators induced by T cell activation (58). Unlike C/EBPβ and JunB, sumoylation of c-Maf reduces IL-4 expression by limiting c-Maf interaction with the IL-4p.

GATA-3 is also a critical transcription factor for Th2 cytokine production, but its sumoylation status in T cells is unclear. GATA-3 associates with Ubc9 and the SUMO-E3 ligase PIAS1 in
yeast two-hybrid assays, suggesting that sumoylation is possible; but studies focusing on PIAS1 suggest this may have the opposite effect as that seen with c-Maf (59). Overexpression of PIAS1 in DO11.10.Ca2+/– splenocytes enhances GATA-3 binding to the IL-13 promoter and increases IL-13 production. Surprisingly, PIAS1 does not enhance GATA-3 binding to the IL-4 promoter and has no effect on IL-4 production. On the surface this suggests that sumoylation of GATA-3, if it occurs, is not likely to decrease IL-4 production. However, it is not clear whether PIAS1 facilitates GATA-3 sumoylation. Furthermore, we cannot exclude the possibility that sumoylation regulates IL-4 production by influencing GATA-3 interactions at sites distant to the IL-4 promoter. Interestingly, PIASy enhances sumoylation of GATA-2 and inhibits GATA-2 transcriptional activity, but the PIASy-mediated repression of GATA-2 activity appears to be independent of sumoylation (60). Hence, the contribution of sumoylation to GATA-3-mediated transcription of IL-4 awaits further investigation.

Regulation of SUMO conjugation varies depending on the sub-strate; there is no universal mechanism or pathway currently known to control the enzymatic processes necessary for SUMO conjugation. Oxidative stress, heat shock, osmotic changes, and genotoxic stimuli are commonly known to induce sumoylation, most likely through stress signals associated with the unfolded protein response that occurs as damaged proteins accumulate within the cell (61–63). In the case of T cells, sumoylation of C/EBPβ and JunB increase following stimulation with PMA and ionomycin or through the TCR and CD28 (26, 58). Consistent with these studies, we also found that TCR and CD28 engagement enhances the SUMO conjugation of c-Maf. However, the overall expression of c-Maf also increases, so the ratio of SUMO-c-Maf to unsumoylated c-Maf in acutely activated B10.D2 CD4 cells remains similar to that found among resting CD4 cells (Fig. 8C). Similar results were reported for JunB, suggesting that upon T cell activation the sumoylation machinery is sufficient to maintain SUMO conjugation in the face of increasing transcription factor levels (58). As with B10.D2 CD4 cells, c-Maf levels also increase following stimulation of NOD CD4 cells. However, there is a disproportionate increase in SUMO-c-Maf. Interestingly, the stress response system of NOD T cells is reportedly dysregulated (64). It is tempting to speculate that the sumoylation machinery is inherently more active in NOD CD4 cells and may be central to the increased SUMO-c-Maf to unsumoylated c-Maf ratio observed in NOD CD4 cells following TCR/CD28 engagement.

Although several studies associate dysregulated sumoylation with disease (65–67), our report is the first to link c-Maf sumoylation in CD4 cells with autoimmune diabetes. In normal CD4 cells, SUMO conjugation of c-Maf appears to be a cell-intrinsic mechanism that regulates IL-4 production. Under conditions where the proportion of SUMO-c-Maf is increased, such as in NOD CD4 cells, IL-4 transactivation is reduced even when overall c-Maf expression is increased to the same level as that observed for other normal T cells. In the case of NOD mice, this contributes to the reduced IL-4 production and poor Th2 development characteristic of T1D. At present, it is not clear whether SUMO-c-Maf contrib-utes to the development of diabetes only by inhibiting IL-4 expression or if sumoylation also influences other c-Maf functions, such as T cell sensitivity to apoptotic stimuli (22). Given the polygenic nature of T1D and the complex regulation of IL-4 production, it is likely that multiple factors contribute to the unbalanced immune response observed. Although the data presented in this study demonstrate that enhanced sumoylation of c-Maf contributes to depressed Th2 responses in NOD mice, we cannot completely rule out the possibility that other transcriptional activators of Th2 development are subject to similar modification by sumoylation. Nevertheless, manipulation of SUMO-c-Maf levels may represent a novel target for the development of therapeutic tools for treatment of T1D.

Acknowledgments

We thank Anna Travelstead for acquisition of flow cytometry data and Dr. William Halford and Brandon Rakowski for help with animal husbandry.

Disclosures

The authors have no financial conflict of interest.

References
