Regulation of proximal T cell receptor signaling and tolerance induction by deubiquitinase Usp9X

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The T cell hyperproliferation and autoimmune phenotypes that manifest in mice lacking E3 ubiquitin ligases such as Cbl, ITCH, or GRAIL highlight the importance of ubiquitination for the maintenance of peripheral T cell tolerance. Less is known, however, about the deubiquitinating enzymes that regulate T cell proliferation and effector function. Here, we define a cell intrinsic role for the deubiquitinase Usp9X during proximal TCR signaling. Usp9X-deficient T cells were hypoproliferative, yet mice with T cell–specific Usp9x deletion had elevated numbers of antigen–experienced T cells and expanded PD-1 and OX40–expressing populations consistent with immune hyperactivity. Aged Usp9x KO mice developed lupus–like autoimmunity and lymphoproliferative disease, indicating that ubiquitin ligases and deubiquitinases maintain the delicate balance between effective immunity and self–tolerance.

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MARK4 (Al-Hakim et al., 2008). Here, we show that Usp9X is a positive regulator of proximal TCR signaling in peripheral T cells and also contributes to T cell tolerance established during intrathymic development.

RESULTS AND DISCUSSION

Usp9X protein is expressed in lymphocytes

Western blots of mouse tissues revealed abundant Usp9X in the brain and appreciable expression in the lung, spleen, lymph nodes, and thymus (Fig. 1 A). Usp9x knock-in mice expressing the fusion protein tdTomato-T2A-Usp9X allowed a more detailed analysis of Usp9x mRNA expression in the hematopoietic compartment (Fig. 1 B). The self-cleaving T2A peptide (Ryan et al., 1991) liberated the fluorescent tdTomato protein, which was detected by flow cytometry in hematopoietic stem cells (HSCs) and, to a lesser extent, in differentiated myeloid and lymphoid cells (Fig. 1 C). These data are reminiscent of in situ hybridization analyses where expression of Usp9x during embryogenesis decreased as cell-fate became restricted (Wood et al., 1997). Although tdTomato mRNA was expressed similarly in myeloid and lymphoid cells (Fig. 1 C), T and B cells contained significantly more Usp9X protein than granulocytes and monocytes (Fig. 1 D), which is consistent with posttranslational regulation of Usp9X protein abundance.

Usp9X regulates proximal TCR signaling events

Neither Usp9x−/− nor Usp9x+/− mice survived embryogenesis, so we explored Usp9X function in lymphoid cells from mice bearing Usp9x conditional KO alleles that were deleted in hematopoietic cells with a vavCre transgene or in T cells with a CD4.cre transgene (Fig. 2 A). Naïve Usp9x KO CD4+ T cells exhibited a proliferation defect when cultured with antibodies to CD3 and CD28, incorporating significantly less [3H]-thymidine than their WT counterparts (Fig. 2 B). As Usp9x KO CD4+ T cells proliferated normally in response to PMA and ionomycin (Fig. 2 C), which directly activate PKC and elevate intracellular calcium, it can be concluded that Usp9X regulates proximal TCR signaling events. The TCR-dependent proliferation defect was not secondary to altered development in the absence of Usp9X because wild-type CD4+ T cells exhibited the same defect after siRNA knockdown of Usp9x in culture (Fig. 2 D). It is worth noting that the proliferation defect caused by Usp9X deficiency was observed in CD8+ T cells as well as CD4+ T cells (Fig. 2 E).

Although Usp9x KO T cells expressed normal amounts of TCR/CD3 on their surface (Fig. 2 A) and phosphorylated the proximal kinase ZAP70 normally in response to TCR engagement (Fig. 2 F), phosphorylation of the ZAP70 substrates LAT, Vav, and Slp-76 was markedly reduced when compared with WT control T cells (Fig. 2 F). Therefore, Usp9X is required for transduction of the activation signal from ZAP70 to its substrates. Whether Usp9X targets ZAP70 directly or indirectly remains to be determined. Of note, PKC-dependent phosphorylation of CARMA1, subsequent binding of CARMA1 to Bcl10, and the nuclear translocation of the p65 NF-κB subunit occurred normally in Usp9x KO T cells (Fig. 2, G–I). Consequently, our genetic and biochemical data do not support the reported model wherein the role of Usp9X during T cell activation is to modulate the CARMA1–Bcl10–MALT1 (CBM) complex downstream of PKC (Park et al., 2013).

Figure 1. Usp9X protein is expressed in lymphocytes. (A) Usp9X protein expression in a panel of adult murine tissues. (B) Organization of the tdTomato-Usp9x knock-in allele. (C) Representative histogram and collated MFI (median fluorescence intensity) of tdTomato-Usp9x mRNA. CMP, common myeloid progenitor; CLP, common lymphoid progenitor; Mac, macrophage; Gran, granulocyte; Plat, platelet; Den, dendritic cells; T and B, T and B lymphocytes. (D) Usp9X protein expression in hematopoietic cell subsets. All experiments were conducted a minimum of two independent times with littermate controls on a C57BL/6 background. Each square represents an independent mouse. ****, P ≤ 0.0001 using a two-tailed unpaired Student’s t test.
Figure 2. Usp9X regulates proximal TCR signaling events. (A) Representative histograms of TCR and CD3 levels on naive CD4+ T cells purified from CD4.cre mice. The specificity (T vs. B lymphocytes) and kinetics of deletion using CD4.cre was confirmed by Western blot. (B) Naive CD4+ T cell proliferation in response to CD3/CD28. Data represents mean ± SEM of 5–7 independent mice per genotype/time point. (C) Naive CD4+ T cell proliferation in response to PMA/ionomycin. Data represents mean ± SEM of 7–11 independent mice per genotype/time point. (D) Proliferation of wild-type T cells transfected with a nontargeting control (NTC) or Usp9x siRNAs (siUsp9x) that effectively reduced both Usp9x mRNA and protein levels. Data represent mean ± SEM of three independent transfections. (E) CD8+ (CD4.cre) T cell proliferation. Data represents mean ± SEM of 3 independent mice per genotype. (F and G) Naive CD4+ T cells (CD4.cre) were stimulated for the indicated times and proteins were fractionated and probed with the indicated antibodies. N.S., nonstimulated. The induction of tyrosyl-phosphorylated proteins was monitored with a phospho-tyrosine (p-Tyr)-specific antibody. (H) CBM complex formation in naive CD4+ T cells (CD4.cre) was assessed by co-immunoprecipitation of CARMA1 with Bcl10. (I) Nuclear translocation of the p65 subunit of NF-κB was monitored by subcellular fractionation of naive CD4+ T cells. DP, double-positive; DN, double-negative. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; and ****, P ≤ 0.0001 of wild-type versus Usp9x KO using a two-tailed unpaired Student’s t test. PMA/I, PMA/ionomycin. c.p.m., counts per minute.
Usp9x deficiency expands antigen–experienced, PD-1, and OX40-expressing T cell populations in vivo

Next, we assessed how the reduced signaling capacity of Usp9x KO T cells influenced T cell homeostasis in the whole animal. Unless specified, all subsequent analyses were conducted with the T cell–specific deleter strain CD4.cre. At 12 wk of age, Usp9x KO mice contained approximately threefold more CD4+ CD62L− CD25− CD44hi antigen–experienced (effector–memory) T cells in the spleen and lymph nodes than their WT counterparts (Fig. 3 A), an expansion evident as early as 4 wk of age (Fig. 3 C). The number of naive CD8− T cells were also reduced in Usp9x KO mice (Fig. 3 B). Consequently, the ratio of both CD4+ and CD8+ memory to naive T cells was consistently elevated in Usp9x KO mice (Fig. 3, A and B).

To gain further insight into the defects caused by Usp9X deficiency, WT and Usp9x KO total T cells freshly isolated from 8–12-wk-old CD4.cre mice were analyzed by RNA sequencing. Interestingly, Usp9x KO cells expressed 3.6-fold more Pdcd1 mRNA encoding the inhibitory receptor PD-1 than did WT cells. Consistent with this result, Usp9x KO mice contained ~10-fold more PD-1−/high memory cells in the spleen than WT control mice, and numbers in the lymph nodes were also elevated (Fig. 3 D). Expression of Tnfrsf4 encoding the stimulatory receptor OX40 was also increased in Usp9x KO T cells. By flow cytometry, Usp9x KO spleens and lymph nodes contained more memory CD4+ cells that were OX40+ (Fig. 3 E). OX40 provides co-stimulatory signals for a sustained T cell response (Croft et al., 2009), is up-regulated in several human autoimmune diseases, and therefore is indicative of generalized immune hyperactivity.

Usp9x deficiency in T cells causes spontaneous lupuslike autoimmunity and lymphoproliferative disease

Given the skewing of T cell subsets in the Usp9x KO mice, we monitored a cohort of aging CD4.cre mice for signs of autoimmunity. Unlike control mice, Usp9x KO mice exhibited progressive splenomegaly (Fig. 4 A) due to increased numbers of CD4+ T cells, monocytes, and B cells (Fig. 4 B) and significant thymic hypertrophy due to infiltration of activated T cells and B cells from the perithymic lymph nodes (Fig. 4 A and not depicted). Of note, spleens from mice where Usp9X had been deleted specifically in B cells (using CD19.cre) were not significantly larger than control spleens (Fig. 4 C), revealing that a T cell–intrinsic defect was the primary driver of the pathology observed. Elevation of dsDNA autoantibodies (Fig. 4 D) and the presence of hypergammaglobulinemia, specifically of the IgG class (Fig. 4 E), were consistent with systemic lupus erythematosus (SLE)–like disease. Although SLE is classically viewed as a B cell–driven disease, emergent evidence confirms that T cells are crucial for enhancing autoantibody production by providing the signals that B cells require to differentiate, proliferate, and mature (Shlomchik et al., 2001).

Multiple visceral organs of Usp9x KO mice contained more perivascular and periductular lymphoplasmacytic cuffs in comparison to control mice (Fig. 4 F). Many aggregates contained a high proportion of plasma cells, including Mott cells that contain cytoplasmic immunoglobulin inclusions. Although glomerulonephritis is relatively common in older C57BL/6 mice, 7 out of 7 40–48-wk-old female Usp9x KO mice had severe disease compared with only 1 out of 5 WT mice. By 40 wk of age, Usp9x KO mice exhibited hallmarks
and B cells suggested that this was a hyperplastic rather than neoplastic process. Defective intrathymic T cell development in the absence of Usp9X Defects in T cell development in the thymus may drive autoimmunity in Usp9x KO mice. Normally, a strong TCR–MHC interaction causes deletion of autoreactive T cells, whereas a weaker interaction promotes the maturation of double-positive (DP) thymocytes into either CD4 or CD8 single-positive (SP) thymocytes.
(Jameson et al., 1995; Kisielow and von Boehmer, 1995; Alam et al., 1996). A preTCR complex coupled to an intracellular signaling pathway resembling that present in mature T cells is also required for immature double-negative (DN) thymocytes to transition to the DP stage (Groves et al., 1996; Cheng et al., 1997; Pivniouk et al., 1998; Zhang et al., 1999). Given that TCR-induced activation and phosphorylation of ZAP70 substrates was perturbed in Usp9x KO DP thymocytes (Fig. 5, A and B), we hypothesized that both these processes would be affected.

Deletion of Usp9x at all stages of thymocyte development (with vavCre) resulted in fewer thymocytes at the DN4 stage and an overall reduction in thymic cellularity (Fig. 5 C). Usp9x KO CD69+ DP thymocytes also expressed less Bim than their WT counterparts (Fig. 5 D). Bim is the proapoptotic protein required for the death of autoreactive T cells (Liston et al., 2004; Baldwin and Hogquist, 2007), so these data are consistent with impaired negative selection. Deletion of Usp9x during the DN-to-DP transition with CD4.cre (Fig. 2 A) yielded a greater proportion of CD4 and CD8 SP thymocytes (Fig. 5 E). Consistent with a selection shift model (Sakaguchi et al., 2003; Fig. 5 F), the increase in the proportion of SP cells in these animals is likely to reflect the fact that fewer thymocytes had reached the threshold for negative selection and, as a consequence, fewer T cells bearing autoreactive TCRs were eliminated.

In sum, we show that Usp9X is required for normal T cell development and proliferation. Inherited mutations causing primary human immunodeficiency are rare, so analyses of genetic alterations such as T cell–specific deletion of Usp9x that subtly reduce TCR signal strength may inform our understanding of a greater proportion of human immunopathology.

Figure 5. Defective T cell development in Usp9x KO mice. (A) Thymocyte activation was assessed by the degree of calcium flux elicited by cross-linked anti-CD3 antibody or the calcium ionophore ionomycin. Data are representative of three independent mice per genotype. (B) Western blot analysis confirmed that Usp9x KO thymocytes exhibit similar signaling defects to peripheral T cells. (C) Immature thymic subsets in the Vav.cre strain were enumerated using CD25 and CD44 co-staining to track the transition from DN1 to DN4 before differentiation in to DP thymocytes. (D) Intracellular FACS analysis of the proapoptotic molecule Bim in 4-wk-old Vav.cre mice. (E) Analysis of thymic subsets in 12-wk-old CD4.cre mice. (F) Graphical representation of the selection shift hypothesis. The dashed line denotes a signal strength that in wild-type T cells would result in deletion but in Usp9x KO T cells, results in positive selection. All experiments were conducted a minimum of two independent times with littermate controls on a C57BL/6 background. Each square represents an independent mouse. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; and ****, P ≤ 0.0001 of wild-type versus Usp9x KO using a two-tailed unpaired Student’s t test.
MATERIALS AND METHODS

Mice. The loxP-targeted allele of Usp9X was generated by Lexicon using C57BL/6 embryonic stem cells and standard molecular biology techniques. The loxP sites flank exon 31 of Usp9X. Usp9X+/− mice were crossed to C57BL/6-Gt(ROSA)26Sortm16(Cre)Arte (Taconic), Tg(Vav1-cre)1Cwi (MGI ID: 5527187), or B6.Cg-Tg(CD4cre)1Cwi N9 (Taconic) deleter strains and backcrossed to a C57BL/6 background for >10 generations. The complete open reading frame of the tdTomato fluorescent protein and a 2A sequence was inserted between the 5′ untranslated region (UTR) and the translation initiation codon present in exon 2 of Usp9X. B6. 129S6-Sigd2tm12Vys/J mice were purchased from The Jackson Laboratory. All experiments were conducted with the approval of the Genentech Institutional Animal Care and Use Committee.

Cell surface and intracellular staining and FACS. Fluorescent antibodies to cell surface antigens were diluted 1/50 in 1 mg/ml CD16/CD32 (BD: 553142) to block Fc receptors, 10% FCS/PBS and incubated with cells for 30 min on ice. Before analysis, cells were resuspended in 2 µg/ml propidium iodide (BD: 356663) to exclude dead cells. For quantitation of total cell number, a defined number of unlabeled Calibrite beads (BD; 349502) were added per sample and numbers calculated according to the following equation: (total number, a defined number of gated beads) × no. of gated cells. For absolute number per organ, the total cell number was multiplied by the dilution factor. Data were obtained using an LSR II Flow Cytometer (BD) and all post-acquisition analyses were performed in FlowJo (v8.4.5). The following antibodies were used for multiparameter FACS analysis and cell sorting and were all purchased from BD with the exception of F4/80: B20 (RA3-6B2), CD3 (145-2C11), CD4 (H12-29.1), CD8 (53–6.7), CD11b (M1/70), CD11c (HL3), CD19 (I3D), CD24 (M1/69), CD25 (PC61), CD41 (MWReg30), CD44 (IM7), CD62L (H129-19), and 1% Triton) supplemented with inhibitors and clarified as described above.

Calcium flux. Naive CD4+ T cells were resuspended at 5 × 10^6 cells in 1 ml room temperature loading buffer (0.5%/BSA/HBSS with magnesium and calcium). Indo-1 (Life Technologies; I-1223) was added to a final concentration of 2 µg/ml and samples incubated in a 37°C waterbath for 30 min. Samples were centrifuged for 6 min at 180 g and resuspended at 3 × 10^4 C/ml in loading buffer supplemented with 2 µg/ml propidium iodide. A baseline reading was taken for 30 s before the addition of 10 µg/ml of anti-CD3 anti-body. After a further 60 s of equilibration, 100 µg/ml of goat anti-hamster cross-linking secondary (Vector Laboratories) was added and the degree of calcium flux quan-tified by the change in the ratio of Indo-1/FITC Indo-1/Rhod. Naive T cells were loaded with Indo-1 and a baseline reading obtained for 30 s before the addition of 2 µg/ml of ionomycin. A Fortessa FACS machine (BD) with a UV excitation filter was used to acquire this data.

Biochemical analyses. For signaling studies, purified T cells were rested for 1 h in complete medium at 37°C/5% CO2 before stimulation with CD3/CD28-coated microbeads (Dynabeads Mouse T cell activator). Cells were lysed in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, and 0.1% SDS) supplemented with protease (Roche) and phosphatase inhibitors (Roche). After clarification of the lysates by centrifugation, protein concentrations were determined using the BCA protein quantification method (Thermo Fisher Scientific). Between 15 and 50 µg of total protein and a prestained molecular weight marker (Novex; LC5625) were separated using SDS-PAGE electrophoresis. Western blots were probed with the following antibodies: Usp9X (Genentech; clone 4B3.1.1), phospho-ZAP70 (Cell Signaling Technology), total ZAP70 (Cell Signaling Technology), phospho-PLC-γ1 (Cell Signaling Technology), total PLC-γ1 (Cell Signaling Technology), phospho-CARMA1 (Cell Signaling Technology), phospho-Bcl10 (Cell Signaling Technology), total Bcl10 (Cell Signaling Technology), total p65 (Cell Signaling Technology), total Lamin B1 (Cell Signaling Technology), total Tubulin (Cell Signaling Technology), phospho-teratoma knock-in (KI) genotyping primers 5′-AGGGCAGAGGAAGTC-3′, 5′-CAATGGTATTGCAGCACCATAAGT-3′, and 5′-TGCCCTGTGTCGAGATGATA-3′ amplified 283-bp (WT) and 324-bp (KI) genomic DNA fragments. All experiments were conducted with the approval of the Genentech Institutional Animal Care and Use Committee.

Lymphocyte purification and thymidine proliferation assay. Naive CD4+ T cells were purified from homogenized spleens using magnetic bead selection to deplete non-T and CD8+ T cell populations and CD62L for positive selection of naive cells (Miltenyi Biotec). CD8+ and total T cells were purified by magnetic depletion (Miltenyi Biotec). All procedures were performed in accordance with the manufacturer’s protocols. Purity was typically >90% for both wild-type and Usp9x KO samples. For proliferation assays using CD4+ T cells, T cells were sorted using a FACSAria (BD) to obtain CD4+ CD62L- CD44+ CD25− naive T cells with purity typically >99%. Purified cells were seeded at 10^5 CD4 T cells/ml in a U-bottom 96-well microtiter plate (Costar; 3795). For polyclonal T cell activation using CD3/CD28 antibodies, plates were coated with 5 µg/ml of anti-CD3 (BD: clone 145-2C11) and 10 µg/ml anti-CD28 (BD; clone 37.51) overnight at 4°C and washed three times with PBS before the addition of purified T cells in cell culture medium (10% heat-inactivated FCS, penicillin, streptomycin, nonessential amino acids, l-Glutamine, sodium pyruvate, and 50 µM 2-β-mercaptoethanol) and incubation at 37°C/5% CO2. Cells were treated with 10 ng/ml PMA and 0.5 µM ionomycin and plated as described above. At the indicated times, 0.5 µCi of tritiated thymidine was added to each well and allowed to incorporate for 24 h before harvesting on to a Unifilter-96, GF/C microplate (Perkin Elmer). Counts were obtained by reading scintillation fluid and analysis using a TopCount microplate scintillation reader (Perkin Elmer).

Blood was collected by cardiac puncture and serum obtained using Microtainer Serum Separator Tubes
ELISA kits for autoantibody detection were purchased from Alpha Diagnostics. Serum was diluted 1:100 in low NSB sample diluent for all autoantibody ELISAs. Units per ml were determined with reference to a standard curve. ELISA kits from Abcam were used to assess immunoglobulin levels in serum diluted between 1:20,000 and 1:50,000. All kits were used according to the manufacturer’s instructions. Total levels were determined with reference to a standard curve, whereas absorbance at 450 nM was used to compare the levels of IgA. Absorbance readings at 450 nM were obtained using a SpectraMax 190 microplate reader (Molecular Devices) and absolute concentrations calculated using SoftMaxPro software (Molecular Devices).

**Immunohistochemistry and immunofluorescence.** CD3 and CD20 immunohistochemistry was performed on paraffin-embedded sections of pancreas, thymus, lung, liver, kidney, heart, mesenteric lymph node, spleen, and sternum. The slides were processed by an outpatient (Dako) with manufacturer’s target retrieval before a 60-min incubation with a 1/20 dilution of CD3 (Thermo Fisher Scientific; clone SPT), 2 μg/ml CD20 (Thermo Fisher Scientific), or 2 μg/ml naive rabbit IgG (polyclonal) as a negative control. The ABC-peroxidase Elite kit (Vector Laboratories) with 3, 3′-Diaminobenzidine (DAB) as the chromogen was used as the detection system. Hematoxylin and eosin-stained sections were obtained by standard histochecmical techniques.

**RNAseq.** RNA was extracted from total peripheral T cells using RNeasy Mini kits (Qiagen) with on-column DNase treatment. 5 μg of pooled RNA (n > 15 mice per genotype) was submitted for analysis using the Illumina HiSeq 2000 platform. RNAseq reads were first aligned to ribosomal RNA sequences to remove ribosomal reads. The remaining reads were aligned to the human reference genome (NCBI Build 37) using SNuPAN version 2011-12-28, allowing maximum of 3 mismatches per 100 base sequence parameter: -M -n 10-B 2-I 1-w 20000-E 1-parmax- ma = 200,000). Annotation normalization was noted based on the RefSeq database downloaded on November 30, 2011. To quantify gene expression levels, the number of reads mapped to the exons of each RefSeq gene was calculated. Differential expression analysis on the count data were performed using the R package DESeq, which is based on a negative binomial distribution and uses shrinkage estimation for the variance of each count. A cutoff of 1.5-fold change with a corrected p-value of <0.05 was deemed significant.

**Statistical analysis.** Statistical comparisons were made in Prism (v.5.0d) using a two-tailed unpaired Student’s t test. P-values <0.05 were deemed significant.

We thank Sascha Rutz for advice regarding the use of chemically stabilized siRNAs, Hanne Varn for the calcium flux protocol, Wyne Lee for provision of NZB/W F1 hybrid sera and kidneys, and Felix Chou for CD3 and CD20 immunohistochemistry. We also thank Nobuhiko Kayagaki and Kim Newton for critical discussions, Kim Newton for help with manuscript preparation and Laszlo Komuves for imaging support. We thank the Next-generation sequencing, necropsy, histology and FACS staff at Genentech for their technical support.

The authors declare no additional competing financial interests.

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