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Epigenetic regulation of IL-12-dependent T cell proliferation

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ABSTRACT

It is well established that the cytokine IL-12 and the transcription factor STAT4, an essential part of the IL-12 signaling pathway, are critical components of the Th1 differentiation process in T cells. In response to pathogenic stimuli, this process causes T cells to proliferate rapidly and secrete high amounts of the cytokine IFN-γ, leading to the Th1 proinflammatory phenotype. However, there are still unknown components of this differentiation pathway. We here demonstrated that the expression of the histone methyltransferase *Mll1* is driven by IL-12 signaling through STAT4 in humans and mice and is critical for the proper differentiation of a naïve T cell to a Th1 cell. Once MLL1 is up-regulated by IL-12, it regulates the proliferation of Th1 cells. As evidence of this, we show that Th1 cells from *Mll1*$^{+/-}$ mice are unable to proliferate rapidly in a Th1 environment in vitro and in vivo. Additionally, upon restimulation with cognate antigen *Mll1*$^{+/-}$, T cells do not convert to a Th1 phenotype, as characterized by IFN-γ output. Furthermore, we observed a reduction in IFN-γ production and proliferation in human peripheral blood stimulated with tetanus toxoid by use of a specific inhibitor of the MLL1/menin complex. Together, our results demonstrate that the *MLL1* gene plays a previously unrecognized but essential role in Th1 cell biology and furthermore, describes a novel pathway through which *Mll1* expression is regulated. *J. Leukoc. Biol.* 98: 601–613; 2015.

Introduction

The local host cytokine response to external stimuli dictates the type of innate- and adaptive-immune responses that subsequently occur. Many of the cytokine signals received by cells are transmitted into the nucleus via STAT proteins. As a classic example, production of IL-12 during the innate-immune response causes CD4$^+$ T cells to skew toward a proinflammatory Th1 phenotype, characterized by production of IFN-γ and expression of the transcription factor T-bet. In contrast, exposure of CD4$^+$ T cells to IL-4 skews them toward an alternative Th2 phenotype characterized by the production of the cytokines IL-4, IL-5, and IL-13 and expression of the transcription factor GATA3. In both cases, the particular cytokine milieu is also responsible for developing the T cell recall response. Thus, mice deficient in the STAT6/IL-4 or the STAT4/IL-12 signaling pathway have aberrant T cell memory responses [1–3]. STAT4 phosphorylation by JAK proteins results in its migration to the nucleus, where it functions as a transcription factor for IL-12. Likewise, STAT6 phosphorylation by JAK causes the expression of IL-4. For IL-12 and IL-4, the respective STAT proteins act as transcription factors upon phosphorylation by JAK proteins. Once in the nucleus, STAT promotes the expression of target genes that are important for T cell differentiation and function. In Th1 cells, these genes include *Ifng*, *Il18r1*, *Irf1*, and *Irf4* [4–6]. Some of these STAT target genes are involved in cell proliferation [7], an important feature of effector and memory T cell subsets. For example, STAT5 is known to induce the expression of *Ccnd1* and *Ccnd2*, 2 genes critical for the proliferation of lymphocytes [8, 9]. Although it has been demonstrated that IL-12-driven proliferation through STAT4 is essential to proper Th1 function, the mechanism by which this proliferation occurs is unknown [10].

In addition to STAT4 and IL-12, the transcription factor T-bet has been shown to be an essential regulator of a functional Th1 response [10, 11]. T-bet, a downstream target of STAT4, may function by directly opposing the transcription factor GATA-3 instead of actively differentiating cells toward the Th1 lineage [12]. In agreement with this, it has been demonstrated that there are STAT4-dependent but T-bet-independent genes expressed during Th1 differentiation [13]. Furthermore, T cells deficient in

Abbreviations: $^{-/-}$ = deficient, BCG = *Bacillus* Calmette-Guerin, BMDC = bone marrow-derived dendritic cell, CCND = cyclin D, CD62L = cluster of differentiation 62 ligand, Cdkn = cyclin-dependent kinase inhibitor, ChIP = chromatin immunoprecipitation, DC = dendritic cell, EDU = 5-ethynyl-2′-deoxyuridine, H3K4Me3 = histone H3 lysine 4 methylation, IRF = IFN regulatory factor, MHC II = MHC class II, MLL1 = mixed lineage leukemia 1, PPD = purified protein derivative, qPCR = quantitative PCR, rh = recombinant human, Tbx21 = T-box 21, WT = wild-type

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Tbx21, the gene for T-bet, can differentiate into Th1 cells without difficulty under optimal conditions in vitro [12]. These studies hint that there may be other factors controlling IL-12-induced Th1 development that are independent of T-bet and STAT4 itself, although to date, none have been found [13, 14].

Evidence of epigenetic regulation of T cell phenotype and data supporting that substantial chromatin modification occurs upon differentiation exists for nearly every T cell subset [14–18]. However, in the majority of cases, the chromatin modifiers that regulate these processes have not been identified. It is our hypothesis that proper expression of certain chromatin-modifying enzymes is dictated by exposing T cells to a specific cytokine environment, thus causing T cell differentiation. These enzymes regulate gene expression by altering chromatin structure to a conformation that makes a particular gene accessible or inaccessible to binding of transcription factors. We hypothesize that up-regulation of these chromatin-modifying enzymes in a cytokine-dependent manner is essential to the differentiation and maintenance of the relevant T cell phenotype. Here, we present evidence that the chromatin-modifying histone methyltransferase MLL1 is a previously unknown factor involved in Th1 cell differentiation. Our data indicate that this gene is involved in STAT4-dependent Th1 cell differentiation and also the maintenance of the Th1 cell memory phenotype.

The *Mll1* gene was originally cloned from a subset of acute leukemia patients with cells expressing lymphoid and myeloid markers, indicative of a transformed multipotent progenitor cell [19]. *MLL1* is a histone methyltransferase for H3K4Me, a mark associated with transcription activation, and is cleaved into its active form by the enzyme Taspase1 [20]. The proper functioning of *MLL1* is dependent on interaction with other proteins required for binding to chromatin, including the tumor suppressor menin and the scaffold protein lens epithelium-derived growth factor [21, 22]. Previous studies in mice have demonstrated that the *Mll1* gene is essential for embryonic development [23], as well as differentiation in multiple cell types, including hematopoietic stem cells [24], Th2 memory cells [25, 26], and neural cells [27]. In addition, *Mll1* function has been shown to regulate cellular proliferation by affecting G1/S- and M-phase cell-cycle progression in mouse fibroblasts [20, 28]. These functions are partly achieved through MLL1-dependent regulation of cyclin genes.

Although numerous studies have been done to determine how the MLL1 protein regulates the transcription of target genes—most notably, *homeobox A9* [29]—to date, there is no data on how the *Mll1* gene itself is regulated. Here, we demonstrate that *Mll1* expression is driven by IL-12 signaling, and is a critical factor for Th1 biology that regulates T cell proliferation, an important aspect of the T cell differentiation process [30, 31]. To examine the interplay between *Mll1* expression and the local cytokine milieu, we used an in vivo model of a Th1 response to mycobacterial antigens in the form of PPD. We found that in normal T cells, the up-regulation of *Mll1* by IL-12 is critical to the proper differentiation of the Th1 lineage. Deletion of 1 *Mll1* allele in mice leads to a striking defect in the formation of Th1 cells, characterized by a significant reduction in the proliferation of $Mll1^{+/-}$ CD4 T cells in vitro and in vivo. This reduced proliferative response was correlated with a reduction in expression of *Ccnd3*, a critical gene for T cell proliferation [32]. In addition $Mll1^{+/-}$ CD4 T cells failed to mount an appropriate recall response to the antigen, a finding that mirrors the studies detailing the role of *Mll1* in the Th2 response [25, 33]. To determine if *Mll1* is important in the human-recall response, we used a specific inhibitor of the MLL1/menin complex [34] in human T cell/DC cocultures stimulated with tetanus toxoid. We found that this inhibitor decreased T cell proliferation and cytokine output during the human in vitro-recall response. These latter data represent a novel approach to controlling aberrant T cell-driven inflammatory processes.

MATERIALS AND METHODS

Mice

$Mll1^{+/-}$ mice were obtained from Dr. Yali Dou and bred as heterozygotes at the University of Michigan. For granuloma formation, 1.0×10^5 live *Mycobacterium* (BCG strain) were nonsurgically instilled intratracheally. Two weeks later, 5000 PPD-conjugated, sized sepharose beads [35] were injected intravenously, and mice were analyzed at day 2 or 4 postinjection. $Mll1^{+/-}$ mice were used, as $Mll1^{-/-}$ mice are embryonically lethal.

Cell culture

Murine cell culture. Single-cell suspensions were made from whole spleens that were then centrifuged and incubated with cold RBC lysis buffer for 1 min. CD4 cells were then isolated by use of the CD4 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Isolated cells were checked for purity by use of anti-CD4 PE/Cy7 antibody clone RM4.5 (BioLegend, San Diego, CA, USA). Cells isolated from the draining lymph node for coculture were isolated by the same method. Th1 cells were generated by use of anti-CD3 antibody (eBioscience, San Diego, CA, USA), coated on plates at 3 μg/ml for 2 h at 37°C in PBS. Th0 and Th1 cells received 3 μg/ml anti-CD28 (eBioscience). Th1 cultures also received 10 μg/ml anti-IL-4 (eBioscience) and 10 ng/ml rIL-12 (R&D Systems, Minneapolis, MN, USA) to abrogate IL-4 signaling caused by endogenously produced IL-4 and to promote IL-12 signaling to affect Th1 differentiation. To eliminate effects of endogenously produced IL-12, Th0 cells also received 10 μg/ml anti-IL-12. All cell culture was done in complete RPMI containing 10% heat-inactivated FCS (Atlas Biologicals, Fort Collins, CO, USA) with 1% nonessential amino acids, 1% sodium pyruvate, and 1% penicillin/streptomycin. Except for FCS, all cell-culture reagents were purchased from Lonza (Basel, Switzerland). For ChIP assays, 1.0×10^7 cells were cultured in 6 well plates in 1.5 ml media. For all other assays, 1.0×10^5 cells were plated in anti-CD3 antibody-coated, 96 well, flat-bottom plates in 100 μλ media. For cocultures, BMDCs were derived from naïve animals by culturing bone marrow in 10 ng/ml rGM-CSF for 10 d and harvesting nonadherent cells. T cells were cultured with DCs at a 10:1 ratio in 96 well, flat-bottom plates with 10 μg/ml PPD antigen. For all experiments (see Fig. 6 for exceptions), T cells were analyzed at the indicated time point, postinitial stimulation, with anti-CD3/anti-CD28 in the described culture conditions. No additional stimulation was required to generate the depicted readouts. Cells were stimulated for 5 d, rested for 3 d, and then restimulated with plate-coated anti-CD3 (3 μg/ml) and soluble anti-CD28 (3 μg/ml) for 48 h (see Fig. 6A and B).

Human cell culture. Peripheral blood was isolated from normal, healthy donors (ages 18–65), immediately diluted 1:1 with saline, and overlaid on Ficoll (GE Healthcare, Pittsburgh, PA, USA), according to the manufacturer's instructions. Blood was drawn under Institutional Review Board HUM00075841. $CD4^+$ cells were isolated from peripheral blood by use of a Human CD4 Isolation Kit II from Miltenyi Biotec. For generation of Th1 cells, cells were cultured for 7 d in the presence of 50 ng/ml rhIL-12 (R&D Systems) and 10 μg/ml anti-IL-4 (eBioscience) on plates coated with anti-human CD3/CD28 antibody as above. Complete media were as described above, with the substitution of 10% AB human serum (Sigma-Aldrich,

St. Louis, MO, USA) for FCS. For cocultures with DCs, $CD14^+$ cells were isolated from the blood by use of Miltenyi Biotec CD14 beads before $CD4^+$ T cell isolation. $CD14^+$ cells were grown in 50 ng/ml rhIL-4 and 50 ng/ml rhGM-CSF for 7 d at a concentration of 1.0×10^6 cells/ml in complete RPMI with FCS. Floating and loosely adherent cells were collected by washing flasks several times with media. At this point, T cells cryopreserved on the day of the blood draw in KM Banker II freezing media (CosmoBio, Tokyo, Japan) at 5.0×10^7/ml were thawed and cultured with DCs at a 10:1 ratio in 96 well, flat-bottom plates with 1 μg/ml tetanus toxoid added. Cultures were incubated for 6 d before analysis.

Proliferation

In vitro proliferation. Cells (1.0×10^5) cells were cultured in 100 μλ in a 96 well, flat-bottom plate for the indicated amount of time. [^{3}H]Thymidine was added at 12 h before the time point at a concentration of 0.5 μCi/well. Samples were harvested by use of a Cambridge PHD harvester, and wells were washed 3× with dH20. Counts were measured by use of an LS6500 scintillation counter (Beckman Coulter, Brea, CA, USA).

In vivo proliferation. Mice were injected with 100 μl of a 1 mg/ml solution of EDU Invitrogen, Grand Island, NY, USA), 12 h before analysis. EDU incorporation was assessed by use of the Click-iT kit, per the manufacturer's instructions.

ChIP

ChIP was performed by use of the Chromatin Immunoprecipitation Assay Kit (#17-295) from Millipore (Billerica, MA, USA). Anti-STAT4 (sc-486) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). STAT1, STAT5, and STAT6 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Analysis of the promoters of *Ccnd3*, *Tbx21*, *Ifnγ*, and *Il12rb2* genes was performed by use of a custom EpiTect array purchased from Qiagen (Valencia, CA, USA). The STAT4 binding sites on the *Mll1* promoter were assessed by use of primers (**Table 1**).

Imaging

Fluorescence microscopy was performed with a Zeiss LSM 510 microscope and viewed with a UV and HeNe laser. Pictures for light microscopy were taken with an Olympus BX43 microscope with an Olympus DP73 camera.

Flow cytometry

Flow cytometry was done on an LSR II with 488, 633, and 405 nM lasers. Antibodies to T-bet, IFN-γ, CD4, CD44, CD62L, CD69, CD8, CD11b, CD11c, Gr-1, and MHC II were purchased from BioLegend and used at a 1:200 dilution to stain between 1.0×10^4 and 1.0×10^7 cells in 200 μl FACS buffer (containing 1% FCS and 0.002 M EDTA). Anti-IL-12RB2 antibody was purchased from R&D Systems and used at a dilution of 1:100. For EDU treatment, 100 μl of a 10 mg/ml solution of EDU was injected into mice, 24 h before analysis (Invitrogen). Cells were then labeled according to the manufacturer's instructions. To analyze only viable cells, the LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen) was used, according to the manufacturer's instructions. All cell culture and lung experiments were analyzed by first gating on viable cells and subsequently determining populations of interest. CFSE (Invitrogen) was used to label cells at a concentration of 10 nM in a 5% FCS/PBS solution. Cells were incubated for 5 min at room temperature in the dark and then washed 2× with complete media before plating [36]. For intracellular cytokine staining, cells were incubated with a 0.05 μl GolgiStop and 0.05 μl GolgiPlug (BD Biosciences, San Jose, CA, USA) in 100 μl media for 6 h before surface staining. Cells were then fixed for 15 min with 5% normal-buffered formalin and washed 3× with BD Perm/Wash buffer before staining for intracellular proteins. For lung flow cytometry, minced lungs were digested in 5 ml of a 1 mg/ml solution of Collagenase A (Roche Diagnostics, Indianapolis, IN, USA) and 10 Kunitz units of DNAse I (Sigma-Aldrich) in complete medium for 45 min at 37°C in a shaking incubator. Samples were subsequently passed 15× through a 5 ml syringe with an 18 G needle, filtered through 100 μM Nitex (WildCo, Yulee, FL, USA) to remove debris, and RBCs then lysed by use of buffer purchased from BioLegend. EDU staining was performed by use of the AlexaFlour 488 EDU kit, according to the manufacturer's instructions (Invitrogen), with volumes adjusted for a 96 well plate format (total volume of 100 μl/reaction). In vivo proliferation was measured by injection of 1 mg EDU, dissolved in 100 μl PBS, 24 h before analysis. In vitro EDU proliferation was measured by pulsing a 100 μl culture of cells in a 96 well plate with 1 μl of a 10 mg/ml solution of EDU dissolved in sterile PBS (final concentration of 100 μg/ml).

Flow cytometry for Mll1 was performed by use of Mll1 antibody NB-600-256 at 1:1000 dilution (Novus Biologicals, Littleton, CO, USA). This antibody was conjugated directly to the Pacific Blue fluorochrome by use of an antibody conjugation kit from Invitrogen. Before conjugation, the storage buffer of the antibody was removed and replaced with PBS by use of Amicon Ultracel 100K centrifugal filters (Millipore). Flow cytometry for Ccnd3 was performed by incubating cells with ab52598 at a 1:500 dilution (Abcam, Cambridge, MA, USA) for 20 min, followed by 2 washes with Perm/Wash Buffer. Secondary anti-rabbit allophycocyanin (R&D Systems) was used at a dilution of 1:200 with an incubation time of 20 min.

RNA

RNA was isolated by Trizol (Invitrogen) and reverse transcribed by use of iScript (Bio-Rad Laboratories, Hercules, CA, USA). qPCR was performed on an Applied Biosystems (Carlsbad, CA, USA) 7500 analyzer by use of TaqMan reagents. All genes were normalized to *gapdh* to conduct ΔΔ comparative threshold analysis.

Statistics

Statistical analysis was performed as indicated by use of Prism 6 (GraphPad Software, La Jolla, CA, USA). Peak calling assays were performed by use of Homer [37] and the mm9 build of the *Mus musculus* genome.

TABLE 1. Primers used to assess the STATS binding sites on the Mll1 promoter

Primer	Human	Mouse
STAT4-binding site 1	F: CGGGGCGAATGGCTCTCC	F: AAATCCCTTGGCACAGAC
	R: CCGGCGAGGGGGTCTGTG	R: CCAGGGAAGGAGGCGGAGATG
STAT4-binding site 2	F: CGTTGCCCCTTCCCCATTAGTA	F: TTCAGGTTCAGTCAATGGCTATGC
	R: CGCCACTGGCTGAACCTGAACT	R: CCCCTTCCCCTGCGATTCAAACT
STAT4-binding site 3	F: GAAGAAGGGTTGTGATAGGGAAGA	F: ATATGGAGGGGGATTCA
	R: TCAAAAGGGGAGGGAGAACCAAT	R: CTAAAAGGCCACCAAGAT
Il2ra	F: TGGTACTTCCTAGAAGAAACTGCT	F: AAGAGGAGGCAATCTGGGTCAGATA
	R: GGTCAGAAATTTGTTGTCCGC	R: GGTCAACTGTATAAAGCTGAGTCTC
STAT6 Hs: *Spint1* Mm: *Rad50*	F: GCCTTCGCTCTAGGACTGG	F: CCACACACTGGGATGTGTAGCTCA
	R: AACCACAGGCTTCCAAGAAA	R: AGACCCAGCTCCTCAGAAGGTAGT

F, forward; Hs, *Homo sapiens*; Mm, *Mus musculus*, R, reverse.

RESULTS

Mll1$^{+/-}$ mice display altered granuloma pathology accompanied by differences in cytokine levels in the lung

To determine if *Mll1* was important in a T cell-driven Th1 response, we infected mice with live *Mycobacterium* (BCG strain) and then 14 d later, challenged them with PPD-coated beads via intravenous tail-vein injection. Injected beads lodge in the lungs of the mice and cause the formation of granulomas. Previous research has indicated that 4 d postinjection is the peak inflammatory response in WT mice. We found that 4 d was also the peak of granuloma formation in *Mll1*$^{+/-}$ mice; however, the pathology associated with granulomas in *Mll1*$^{+/-}$ was altered significantly compared with WT mice.

The dominant response to challenge with PPD beads is IFN-γ production by CD4$^+$ T cells [35]. Histologic examination of granulomas revealed that WT granulomas were compact and consisted mainly of mononuclear cells, whereas *Mll1*$^{+/-}$ granulomas consisted of large numbers of granulocytic polymorphonuclear cells (**Fig. 1A** and **B**). We also observed a reduction in IFN-γ levels in the lungs of *Mll1*$^{+/-}$ mice at the RNA and protein level (Fig. 1C and D). The reduction in IFN-γ levels in the lung correlated with a reduction in effector CD4$^+$ T cells, as determined by a decrease in the number of CD4$^+$ cells bearing the signature of CD44^{hi}CD62l^{lo} expression. There was no difference in the total number of CD4$^+$ lung or lymph node nor in the number of CD4$^+$ memory cells (CD44^{hi}CD62l^{lo}CCR7$^+$; Fig. 1F) at these sites. We also observed no difference in CD8$^+$ T cells or CD19$^+$ cells in the lung or lymph node. The predominance of granulocytes at the site of the granuloma was correlated with an increase in lung neutrophils (Gr1^{hi}MHC II$^-$) in *Mll1*$^{+/-}$ mice, as determined by flow cytometry (Fig. 1E). There was no change in the number of macrophages or DCs. Immunofluorescent staining for the H3K4Me3 modification mediated by MLL1 revealed a paucity of this mark in *Mll1*$^{+/-}$ granulomas (Fig. 1G), thus confirming that MLL1 was nonfunctional in these mice. We did not detect an increase in IL-17 at the protein or mRNA level in *Mll1*$^{+/-}$ mice nor was there increased production of IL-17 from cultures of draining lymph nodes restimulated with PPD antigen (data not shown). To determine how *Mll1* expression is altered during T cell differentiation, we FACS-sorted naïve, effector, and memory cells from the spleens of WT mice immunized with PPD/CFA for 14 d. We found that *Mll1* expression is highest in naïve and memory cells and reduced in effector cells (Fig. 1H). To determine the level of *Mll1* expression in *Mll1*$^{+/-}$ mice, we then sorted naïve and effector cells from the lungs and lymph nodes of PPD immunized and challenged mice. We found that *Mll1* expression was not altered in naïve cells in the lung or lymph node. However, effector cells isolated from the lungs of *Mll1*$^{+/-}$ mice had 50% reduced *Mll1* expression compared with WT controls (Fig. 1H).

Transferred *Mll1*$^{+/-}$ CD4$^+$ T cells recapitulate the phenotype of *Mll1*$^{+/-}$ mice

We next tested if CD4$^+$ T cells are responsible for the observed changes in granuloma pathology in *Mll1*$^{+/-}$ mice by transferring splenic CD4$^+$ T cells from *Mll1*$^{+/-}$ mice or littermate controls into *Rag2*$^{-/-}$ mice. Recipients were then sensitized and challenged as described above. The transfer of CD4$^+$ T cells from *Mll1*$^{+/-}$ mice into *Rag2*$^{-/-}$ mice reproduced all of the features of the granuloma, initially observed in *Mll1*$^{+/-}$ mice. These included reduced IFN-γ in the lung (**Fig. 2A**), a decrease in *Mll1* mRNA expression (Fig. 2B), and an increase in granuloma size (Fig. 2C and D). Histologic examination again revealed granulomas with increased numbers of polymorphonuclear cells in mice receiving *Mll1*$^{+/-}$ T cells (Fig. 2D). Additionally, we observed an increase in neutrophils in the lung and a decrease in total CD4 cells in the lung (Fig. 2E and F) by flow cytometry. The decrease in total CD4 cells was likely observable because of the decreased amount of CD4 cells present in *Rag2*$^{-/-}$ mice, which in turn amplifies the contribution of the effector population to the total pool of T cells. Thus, these experiments confirmed the results seen above in *Mll1*$^{+/-}$ mice, showing the importance of the *Mll1*$^{+/+}$ gene to the expression of a proper Th1 phenotype.

Th1 cells undergo *Mll1*-dependent proliferation

The MLL1 protein has been implicated previously in the regulation of the cell cycle and the proliferation of fibroblasts [28]. As our data indicated a reduced number of CD4$^+$ effector cells in vivo after PPD immunization and challenge, we next determined the ability of *Mll1*$^{+/-}$ T cells to proliferate under Th1 conditions in vitro. For these experiments, we assessed the ability of WT and *Mll1*$^{+/-}$ CD4 T cells to incorporate [^{3}H]thymidine under Th0 (anti-CD3/anti-CD28 + anti-IL-12) and Th1 (anti-CD3/anti-CD28 + anti-IL-4 + rIL-12) conditions. We found that *Mll1*$^{+/-}$ T cells under Th0 conditions were able to proliferate as well as WT Th0 cells at all time points (48–120 h; data not shown). However, under Th1 conditions, *Mll1*$^{+/-}$ cells had a reduced, proliferative capacity, as measured by [^{3}H]thymidine uptake (**Fig. 3A**). By calculating the ratio of Th1 proliferation compared with Th0 proliferation, we found that IL-12-dependent proliferation started at 72 h post-CD3 stimulation and was greatest in WT cells at 120 h poststimulation (Fig. 3B). In *Mll1*$^{+/-}$ Th1 cells, the ratio of proliferation fell below 1 at 72 h, indicating that *Mll1*$^{+/-}$ Th1 cells proliferated less than *Mll1*$^{+/-}$ Th0 cells at this time. The proliferative ratio between *Mll1*$^{+/-}$ Th1 and Th0 cells continued to decrease through 120 h poststimulation. With the use of the nucleotide analog EDU, we tested whether *Mll1*$^{+/-}$ cells had reduced proliferation in vivo by pulsing PPD-sensitized and -challenged *Rag2*$^{-/-}$ mice on day 3 postbead injection and assessed the incorporation of this analog by flow cytometry, 24 h later. As described previously, these mice received WT or *Mll1*$^{+/-}$ T cells, 2 wk before analysis. We found a decrease in proliferation of total CD4 T cells from *Mll1*$^{+/-}$ mice in the lung and draining lymph nodes (Fig. 3C).

Cells deficient in functional MLL1 protein have been shown to have reduced expression of cell-cycle genes and an increase in expression of select tumor-suppressor genes [20]. We next examined the expression of a panel of genes involved in cell-cycle progression, including family members from the A-, B-, D-, and E-type cyclins. We observed decreased expression of *Ccnd3* in *Mll1*$^{+/-}$ Th1 cells (Fig. 3D). There was no decrease in the expression of other cell-cycle genes (*Ccna2, -b1, -b2, -d1, -e1, -e2*) or increase in expression of tumor-suppressor genes (*Cdkn1a, Cdkn1b, Cdkn2a, Cdkn2c*), as measured by qPCR (data not

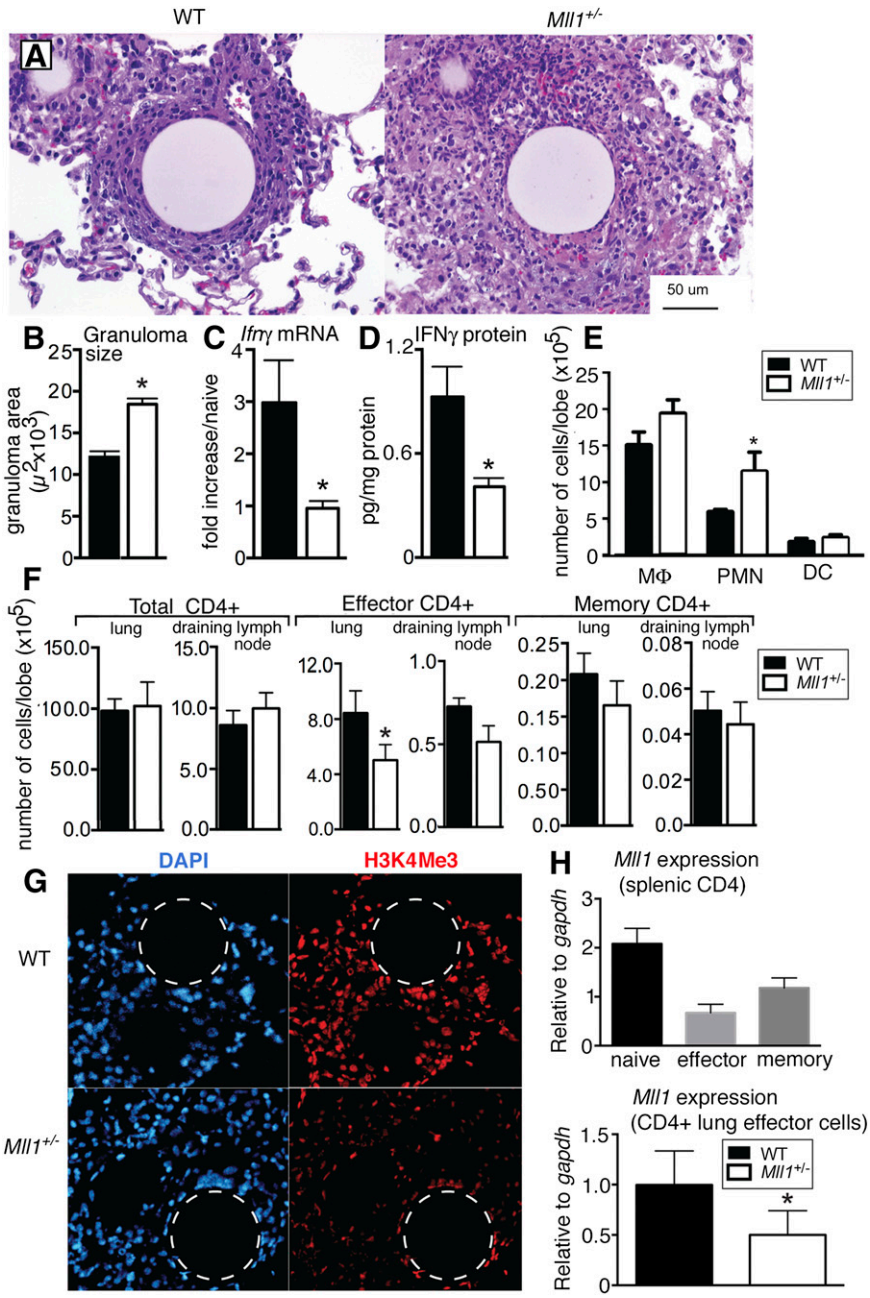

Figure 1. *Mll1*$^{+/-}$ mice have an altered response to PPD antigen challenge at 4 d post-PPD bead challenge. (A and B) H&E staining of lungs from WT and *Mll1*$^{+/-}$ mice at 4 d postbead injection and a graph quantifying significant differences in granuloma size. *$P < 0.03$, as determined by Student's *t* test. (C and D) Quantification of IFN-γ at protein and mRNA levels. (E) Analysis of infiltrating myeloid cell populations in the lung. Results are representative of 3 separate experiments. *$P < 0.007$, as determined by Student's *t* test. Neutrophils [polymorphonuclear neutrophils (PMN)] were defined as autofluorescent-negative CD11b$^+$CD11c$^-$Ly6G$^+$, DCs as autofluorescent-negative CD11c$^+$CD11b$^+$Ly6C$^+$MHC II$^+$, and macrophages (MΦ) as autofluorescent-negative CD11b$^+$F/480$^+$MHC II^{midhigh}. (F) Analysis of total CD4$^+$ T cells, as well as T cell subsets in the lung and lymph node. Effector CD4 cells were defined as CD44^{hi}CD62L^{lo}, and memory cells were defined as CD44^{hi}CD62L$^+$CCR7$^+$. *$P < 0.05$, as determined by Student's *t* test. (G) Staining of lung sections with antibodies to H3K4Me3 in WT and *Mll1*$^{+/-}$ mice. The beads at the center of the granulomas are represented by the dashed circles. (H) *Mll1* expression in T cell subsets isolated from the spleen by FACS at 14 d post-PPD immunization. Effector and memory cells were isolated, as described in F. Naïve cells were defined as CD44^{lo}CD62L$^+$. Expression of *Mll1* in CD4$^+$ effector cells isolated from the lungs of PPD-immunized and -challenged mice at 4 d postinjection. For all data, $n = 3$–5 animals/experiment. *$P < 0.05$, as determined by Student's *t* test. Results are representative of 3 independent experiments.

shown). We also found a reduction in *Ccnd3* expression in effector (CD44^{hi}CD62L^{lo}) T cells isolated from the lungs of *Mll1*$^{+/-}$ mice at 4 d postbead injection (Fig. 3E). To determine whether *Mll1*$^{+/-}$ cells also contained less Ccnd3 protein, we performed flow cytometry for Ccnd3 on WT and *Mll1*$^{+/-}$ Th1 cultures (Supplemental Fig. 1A). We found a reduced amount of Ccnd3$^+$ *Mll1*$^{+/-}$ cells at 96 and 120 h poststimulation with anti-CD3, confirming that *Mll1*$^{+/-}$ cells have reduced Ccnd3 at the RNA and protein level at later time points post-CD3 activation in the presence of IL-12 (Fig. 3D–F).

Because of the critical role of IL-2 and the IL-2R CD25 in T cell proliferation, we assessed in vitro-activated T cells for the presence of the CD25 molecule by flow cytometry and found no differences between *Mll1*$^{+/-}$ cells and littermate controls in Th0 or Th1 conditions at any time point (data not shown). As an additional test to determine if IL-2 caused the observed reduced proliferation, we added rIL-2 to in vitro cultures and assessed [^{3}H]thymidine uptake at 96 h postactivation (Supplemental Fig. 1B). We observed an increase in proliferation in Th0 and Th1 cultures of both genotypes when IL-2 was added compared with no IL-2 controls. However, there was a significant decrease in [^{3}H]thymidine uptake in *Mll1*$^{+/-}$ Th1 cells compared with Th1 cells from WT littermate controls in the presence of rIL-2 (Supplemental Fig. 1B). These data suggest that the reduced proliferation in *Mll1*$^{+/-}$ Th1 is at least partially dependent on IL-2.

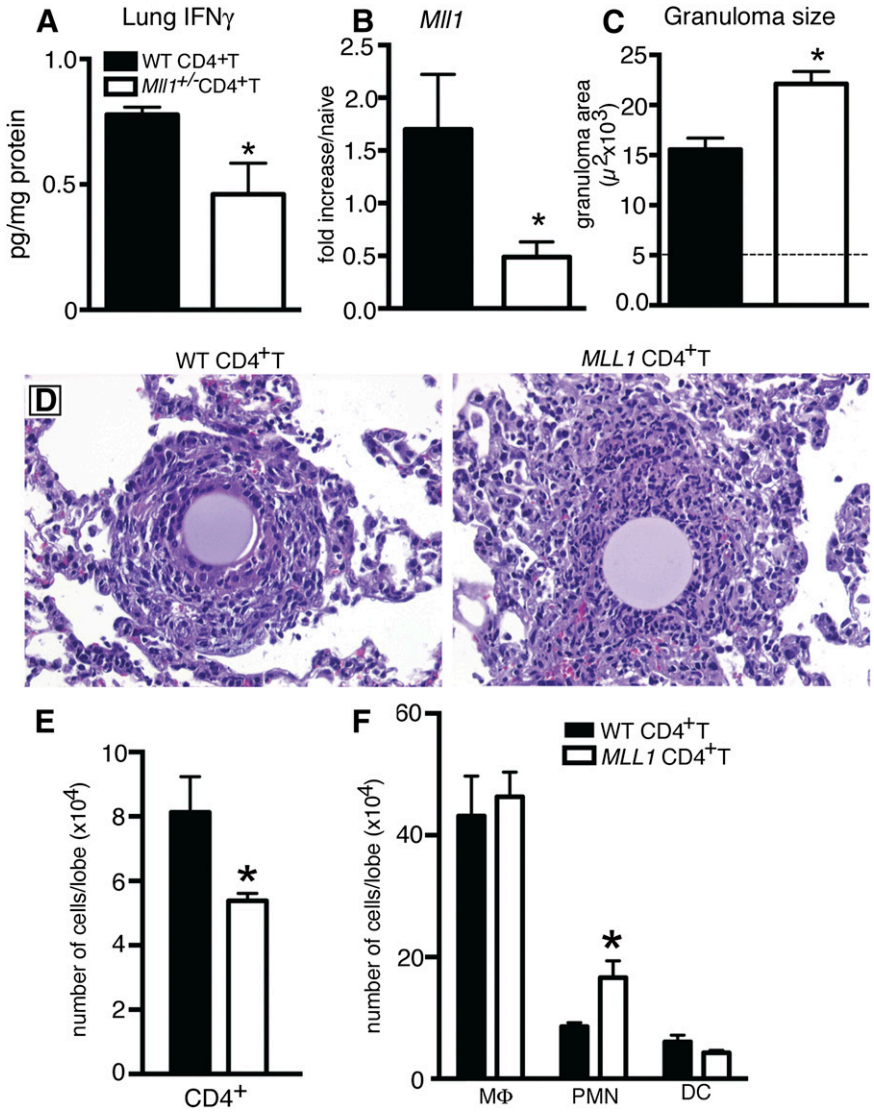

Figure 2. *Mll1*$^{+/-}$ CD4$^+$ T cells cause altered granuloma pathology in *Rag2*$^{-/-}$ mice. Quantification of IFN-γ protein (A) and *Mll1* mRNA expression (B) in the lungs of *Rag2*$^{-/-}$ mice receiving *Mll1*$^{+/-}$ T cells after PPD bead challenge. (C and D) Average granuloma size in Rag2$^{-/-}$ mice receiving a transfer of *Mll1*$^{+/-}$ CD4$^+$ T cells following sensitization with CFA/PPD and subsequent challenge with PPD-coated beads. (E and F) Quantification of the number of total CD4$^+$ T cells and myeloid cells in the lung. Myeloid cell populations were defined as in Fig. 1F. *$P \leq 0.0,1$ as determined by Student's t test; $n = 3$–5 animals/experiment. Results are representative of 3 independent experiments.

We also assessed cell death during and after in vitro stimulation with anti-CD3/anti-CD28 antibody by propidium iodide staining (Supplemental Fig. 1C). We found no change in cell death during primary stimulation (up to 120 h post-anti-CD3/anti-CD28 stimulation). However, we did find an increase in propidium iodide$^+$ cells in cultures containing *Mll1*$^{+/-}$ Th1 cells during the resting phase of the culture. These data eliminate the possibility that the reduction in proliferation that we observed at 120 h postactivation in Th1 cultures was a result of an excess of cell death at late time points of activation.

To correlate proliferation with cytokine production, we labeled CD4$^+$ T cells with CFSE and cultured them in Th1 conditions. At 96 h postactivation, we stained these samples with anti-IFN-γ and anti-IL-12RB2. Analysis indicated that there were significantly less *Mll1*$^{+/-}$ IFN-γ$^+$ cells that had undergone 4 cell divisions. In contrast, IFN-γ$^-$ *Mll1*$^{+/-}$ T cells were more proliferative than IFN-γ$^-$ WT cells, suggesting that only those cells producing IFN-γ cytokine were reduced in proliferative capacity (Fig. 3G). This observation is important, as it will drive experiments (seen in Fig. 4B). For all experiments in which we measured IFN-γ, production was measured during the normal course of T cell differentiation, driven by activation with anti-CD3/anti-CD28 in the presence or absence of IL-12 and anti-IL-4.

As IFN-γ was found at reduced levels in the lungs of *Mll1*$^{+/-}$ mice and in *Mll1*$^{+/-}$ T cell cultures, we assessed expression of several factors associated with Th1 cell biology, including the transcription factor T-bet and the cytokine receptor IL-12RB2, a critical receptor for Th1 cell proliferation and function [38]. In vitro, we observed a significant reduction in *Tbx21* mRNA expression at 96 h postactivation in Th1 cultures (data not shown). We also observed a reduction in T-bet$^+$ effector cells in *Mll1*$^{+/-}$ mice sensitized and challenged with PPD antigen (Fig. 3H). In addition, there was a reduction of IL-12RB2$^+$ effector cells in these mice (Fig. 3I). Taken together, these data suggest that *Mll1* is an important IL-12-driven Th1 proliferation that occurs as a result of T cell receptor activation.

IL-12 causes STAT4-dependent *Mll1* expression

It is well established that the IL-12/STAT4 pathway is critical to the differentiation of Th1 cells [4, 10, 13, 39, 40]. To determine

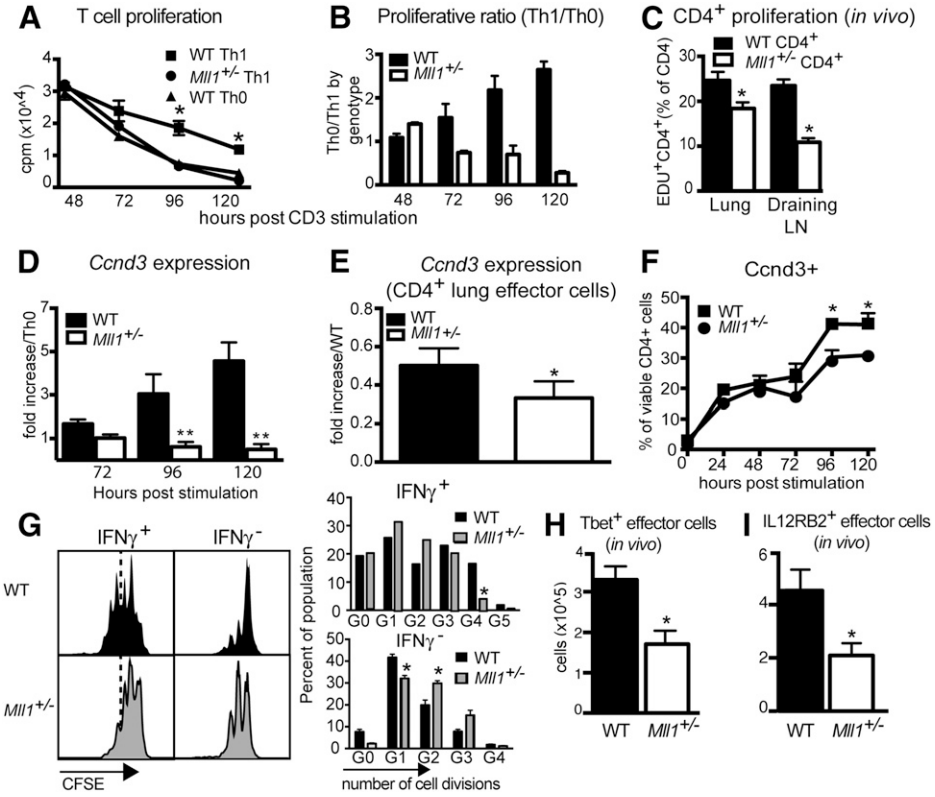

Figure 3. *Mll1*$^{+/-}$ Th1 cells have reduced proliferative capacity and have reduced expression of *CCND3*. (A) Assessment of proliferation of WT and *Mll1*$^{+/-}$ T cells under Th0 and Th1 conditions by [^{3}H]thymidine uptake. *$P \leq 0.01$, as determined by 1-way ANOVA. (B) Depiction of the ratio of T cells proliferating in the presence (Th1) vs. the absence (Th0) of IL-12. This ratio is shown for WT and *Mll1*$^{+/-}$ cells at the indicated time points. (C) Proliferation of CD4$^+$ T cells in vivo after transfer into *Rag2*$^{-/-}$ mice during PPD challenge, as determined by EDU incorporation and surface staining for total CD4$^+$ T cells. *$P \leq 0.03$, as determined by Student's *t* test. (D) Quantification of expression of *Ccnd3*, as determined by qPCR after in vitro activation. **$P \leq 0.0001$, as determined by 1-way ANOVA. (E) Expression of *Ccnd3* in lung-effector CD4 cells from PPD-immunized mice isolated as described in Fig. 1G. *$P < 0.05$, as determined by Student's *t* test. (F) Determination of the number of Ccnd3$^+$ cells in cultures of WT and *Mll1*$^{+/-}$ T cells cultured under Th1 conditions, as measured by flow cytometry. *$P \leq 0.01$, as determined by 1-way ANOVA. (G) Intracellular staining for IFN-γ in CFSE-labeled cultures of WT and *Mll1*$^{+/-}$ cells activated under Th1 conditions. We observed a significant decrease in CD4 IFN-γ$^+$ cells at 96 h postactivation. The data also depict the percent of IFN-γ$^+$ and IFN-γ$^-$ cell populations undergoing multiple cell divisions in WT and *Mll1*$^{+/-}$ cells. *$P \leq 0.0001$, as determined by 2-way ANOVA. (H and I) Flow cytometric assessment of T-bet and IL-12RB2 protein levels on CD4$^+$ effector cells in vivo, 4 d post-PPD bead challenge. Effector cells were analyzed as CD44hi CD62Llo. *$P < 0.03$, as determined by Student's *t* test. In vitro experiments were performed 3 times with the pooled cells of 2–3 animals for each replicate. In vivo experiments were done with n = 3–5 animals/group and repeated twice.

whether *Mll1* is a target of the STAT4 signal transduction pathway, we assessed the ability of IL-12-dependent STAT4 to alter *Mll1* expression in vitro. We initially observed an increase in *Mll1* gene expression at 48–72 h postactivation by use of anti-CD3 antibody. This increase in expression only occurred in cells cultured in the presence of IL-12 and was reduced significantly when Th1 cells were incubated with the JAK inhibitor tofacitinib [41] (**Fig. 4A**). To characterize further the role of IL-12 in upregulation of *Mll1*, we developed a flow cytometry assay to assess Mll1 protein levels by use of cells deficient in *Mll1* (Supplemental Fig. 2A). We used this assay to assess the amount of Mll1 protein induced in response to IL-12 (Fig. 4B and Supplemental Fig. 2B). Consistent with the reduced expression of *Mll1* that we observed in effector cells (Fig. 1H), we found that the number of Mll1$^+$ cells was highest at 24 h postactivation (Supplemental Fig. 2B) and subsequently decreased over time, correlating with an increase in cellular activation. As we had observed a decrease in IFN-γ$^+$ cells in *Mll1*$^{+/-}$ mice, we also assessed Mll1 expression in conjunction with expression of IFN-γ. We found that the peak of Mll1 expression occurred in IFN-γ$^+$ cells at 72 h postactivation with the addition of 10–25 ng/ml IL-12 (Fig. 4B). As IL-12 increased Mll1 expression in IFN-γ$^+$ T cells, we hypothesized that the *Mll1* gene was a target of the JAK/STAT pathway. To determine if the IL-12 signaling pathway was directly responsible for increasing *Mll1* transcription, we activated T cells from *Stat4*$^{-/-}$ mice under Th1 conditions and assessed expression of Mll1 at 72 h postactivation by EDU incorporation and expression of IFN-γ. Although the total number of cells incorporating EDU was not different between *Stat4*$^{-/-}$ cells and controls (Supplemental Fig. 2C), we found a significant reduction in Mll1$^+$ EDU$^+$ cells in *Stat4*$^{-/-}$ mice and cells treated with tofacitinib (Fig. 4C–E). Figure 4D demonstrates that Mll1 expression in T cells is associated with those cells that incorporated high levels of EDU, indicating rapid proliferation. We also observed a reduction in Mll1$^+$ IFN-γ$^+$ cells in these same cultures (Fig. 4F and G). Figure 4F demonstrates that Mll1 expression is associated with IFN-γ production. For these experiments, cells were analyzed at 96 h postactivation with anti-CD3/anti-CD28 in the presence of rIL-12 and anti-IL-4.

We then investigated the relationship between *Mll1* expression and STAT4 by use of a previously published data set, in which ChIP-seq was performed on WT and *Stat4*$^{-/-}$ Th1 cells by use of an antibody against STAT4 [42], again with use of a 96 h time point. We identified 2 peaks within the *Mll1* gene with significant fold enrichment over that observed in *Stat4*$^{-/-}$ cells (Fig. 4H). We searched within these peaks for potential STAT4-binding sites [4] and found 3 separate sites within the large peak and a single, conserved binding site within the small, intergenic peak. With the focus on these potential binding sites, we found that STAT4 was bound to the promoter of the *Mll1* gene at significantly higher levels than found in Th0 cells (Fig. 4I), at 96 h post-CD3 stimulation under Th1 conditions. These results were significant

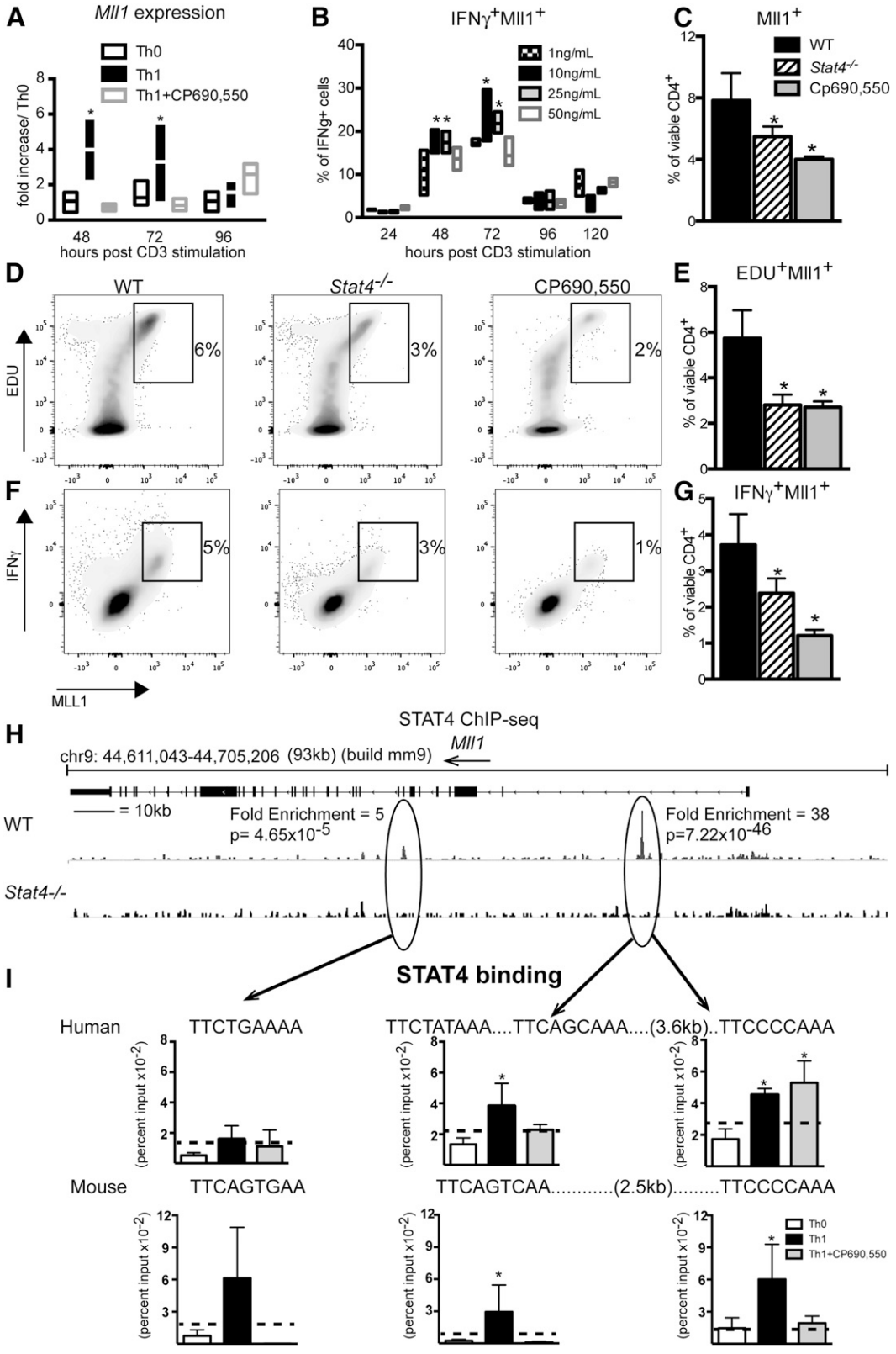

Figure 4. STAT4 binds to the *Mll1* promoter. (A) PCR data assessing the levels of *Mll1* in T cells cultured under normal Th0 or Th1 conditions or Th1 conditions with the addition of 1 μM of the JAK inhibitor tofacitinab (CP690,550). $*P \leq 0.05$, as determined by 1-way ANOVA. (B) Assessment of $Mll1^+$ expression in IFN-γ^+ cells in response to increasing doses of rIL-12. $*P \leq 0.01$, as determined by 2-way ANOVA. (C) The number of $Mll1^+$ cells in WT and $Stat4^{-/-}$ cells and WT cells treated with the JAK inhibitor CP690,550. (D) Flow diagrams depicting the relationship between Mll1 expression and

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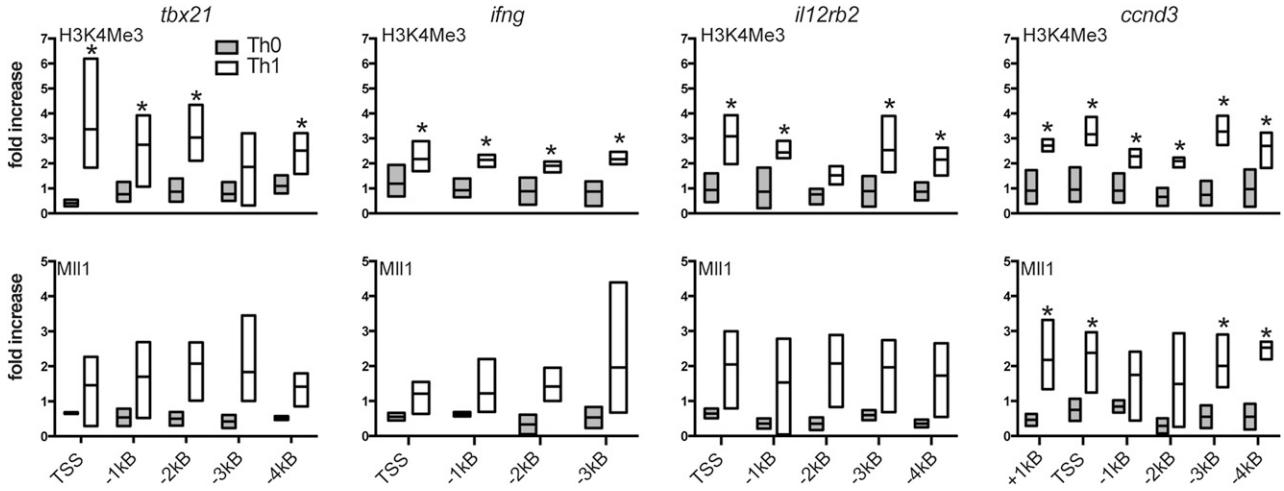

Figure 5. MLL1 binds to genes critical to Th1 cell differentiation. ChIP assay to quantify H3K4Me3 modifications and MLL1 binding to the promoters of *Tbx21*, *Ifng*, *IL12RB2*, and *Ccnd3* in murine Th0 and Th1 cells. TSS, Transcription start site. Negative numbers on the abscissa represent kilobases upstream of the TSS. Data are pooled from 3 independent experiments performed with 1.0×10^7 pooled cells from 3 to 5 animals for each replicate. $^*P \leq 0.05$ by 1-way ANOVA.

within the large peak at the beginning of the gene. In mice, all STAT4 binding was reduced when Th1 cells were exposed to tofacitinab. In humans, ⅓ binding sites displayed reduced STAT4 binding upon exposure to this drug. These sites were specific to STAT4, as performing ChIP with antibodies to STAT1, STAT3, and STAT5 demonstrated no significant change in binding to the promoter of *Mll1* in Th1 cells compared with Th0 controls (data not shown). To ascertain the specificity of our antibody, we assessed binding in a known STAT4-binding site within the promoter of the *Il2* locus [43] and found amplification similar to that observed in the Mll1 gene (Supplemental Fig. 2D and E). We did not observe significant binding to confirmed STAT6-binding sites in humans [44] or mice [45] (Supplemental Fig. 2D and E). These data indicate a conserved up-regulation of *Mll1* in T cells that is mediated by IL-12 and STAT4 signaling. Taken together, these data present evidence that STAT4 regulates the transcription of *Mll1* in human and mouse Th1 cells.

Increased H3K4Me3 and Mll1 binding within the *Ccnd3* promoter under Th1 conditions

To determine if the genes we had found to be down-regulated in $Mll1^{+/-}$ Th1 cells were regulated directly by MLL1 binding to the promoter region, we performed ChIP for the presence of the H3K4Me3 mark and the C-terminal end of MLL1 on the promoter of *Il12rb2*, *Ccnd3*, *Tbx21*, and *Ifng* at 96 h postactivation. In all cases, we found an increase in H3K4Me3 at the promoters of these genes in Th1 cells compared with Th0 cells, indicating that these genes were activated. However, we only observed a significant increase in MLL1 protein binding to the *Ccnd3* promoter under Th1 conditions. Within the *Ccnd3* promoter, MLL1 binding correlated with H3K4Me3, indicating that this gene is a direct target of MLL1 (**Fig. 5**). Although there was a trend toward increased binding of MLL1 on other genes, this trend did not reach significance for any of the indicated genes. These data confirm the importance of MLL1 protein in regulating the Th1 response by demonstrating direct and consistent binding of MLL1 to *Ccnd3*, a target gene with reduced expression in $Mll1^{+/-}$ Th1 cells.

Mll1 is essential for the Th1 recall response

Previous research has demonstrated that $Mll1^{+/-}$ T cells are unable to convert to memory cells during a Th2 response [25]. As we observed increased cell death during the resting phase of our Th1 cultures (Supplemental Fig. 1C and E) and as our in vivo model demonstrated a deficiency in generating Th1 memory in $Mll1^{+/-}$ mice (as demonstrated by a reduced PPD recall response after sensitization), we further characterized the role of the *Mll1* gene and protein in human and mouse recall responses. We observed a reduced amount of IFN-γ produced by $Mll1^{+/-}$ Th1 cells after a period of rest and restimulation, as detailed in Materials and Methods (**Fig. 6A**). In addition, $Mll1^{+/-}$ Th1 cells that had been rested and restimulated did not proliferate as well as WT cells (Fig. 6B). As we had already observed decreased proliferation of $CD4^+$ T cells in response to PPD in vivo (Fig. 3D), we also wanted to determine if these $CD4^+$ T cells were the source of reduced IFN-γ production

proliferation, as determined by incorporation of the nucleotide analog EDU. (E) Analysis of data in (D). (F) Flow diagrams depicting the relationship between Mll1 expression and IFN-γ. (G) Analysis of data in (F). (C, E, and G) $^*P \leq 0.01$, as determined by 1-way ANOVA. (H) A depiction of the ChIP-seq data generated by Wei et al. [42], focused on the *Mll1* gene with regions of interest circled. (I) ChIP assay to determine if STAT4 binds to the *Mll1* promoter in human and mouse Th1 cells. This assay was also done in the presence of 1 μM of the JAK inhibitor CP690,550. The dashed lines represent the background level of detection in tubes immunoprecipitated with control Ig and are averaged among the 3 different conditions (Th0, Th1, and Th1 + CP690,550) for each primer set. $^*P < 0.05$ by 1-way ANOVA. Experiments that use murine cells were performed 3 times with the pooled cells of 3–5 animals for each replicate. Human data were generated by pooling experiments from 2 normal, healthy donors. Mouse cells were analyzed at 96 h postactivation, and human cells were analyzed at 144 h postactivation.

during PPD challenge in our mouse model. Thus, we isolated $CD4^+$ T cells from the draining lymph nodes of $Mll1^{+/-}$ -sensitized and -challenged mice and cocultured these cells with WT BMDCs in the presence of PPD antigen. Analysis of these samples revealed significantly reduced IFN-γ protein production in cultures containing $Mll1^{+/-}$ T cells (Fig. 6C).

To test the relevance of MLL1 in the human immune system, we measured T cell proliferation and cytokine secretion induced by tetanus toxoid in donors that had received a tetanus vaccine. In humans, vaccination with tetanus toxoid produces a robust Th1 memory response. To determine the importance of the MLL1 protein in this memory response, we used a specific inhibitor of the menin/MLL1 complex (MI-2-2) [34]. We found that this inhibitor had no effect on the viability of $CD4^+$ T cells up to a 15 μM dose. We first observed a significant increase in proliferation and IFN-γ production in cultures pulsed with tetanus toxoid compared with cultures containing no antigen. In addition, increasing doses of MI-2-2 caused a reduction in T cell proliferation and IFN-γ production (Fig. 6D). These data indicate that the interaction of menin with Mll family members is important in driving the proliferation and cytokine production associated with the human Th1 memory response.

DISCUSSION

We have demonstrated that the *Mll1* gene is critical to Th1 differentiation in humans and mice. In mice, $Mll1^{+/-}$ T cells have a significant reduction in several factors associated with Th1 biology, including IFN-γ production, expression of T-bet and IL-12RB2, and a decrease in the proliferative capacity of Th1 cells. Our data show that an intact IL-12 signaling pathway via STAT4 is important for proper *Mll1* expression in Th1 cells in humans and mice. Analysis of a dose-response experiment revealed that although Mll1 decreases in all $CD4^+$ cells activated with anti-CD3, the presence of IL-12 increases Mll1 levels in IFN-γ^+ cells. This specific up-regulation may allow for a rapid proliferation of IFN-γ^+ effector cells to mediate an immune response. Given that *Mll1* is up-regulated by the STAT4 pathway, it is likely that other cytokines that signal through STAT4, including IL-23, also up-regulate the expression of *Mll1*. However, this possibility has not been formally tested.

Our results are in contrast to a previous report that $Mll1^{+/-}$ T cells exhibited no change in Th1 skewing characterized by early IFN-γ production in vitro [25]. Our discovery of the crucial role of *Mll1* in the Th1 response occurred because of the significant changes in pathology observed in our in vivo experiments. The previous cited study examined the in vitro Th1 response by use of PMA and ionomycin to elicit IFN-γ production. We believe that studying the antigen-driven Th1 response was critical to our discovery of the crucial role of *Mll1* in this response, as PMA/ionomycin treatment of $Mll1^{+/-}$ Th1 cells also resulted in no difference in IFN-γ production in our system (data not shown).

The transition of naïve T cells to memory cells requires an intermediate effector phase, in which high-affinity T cell clones rapidly expand in response to antigenic stimulation. As the response resolves, the majority of these effector cells dies off,

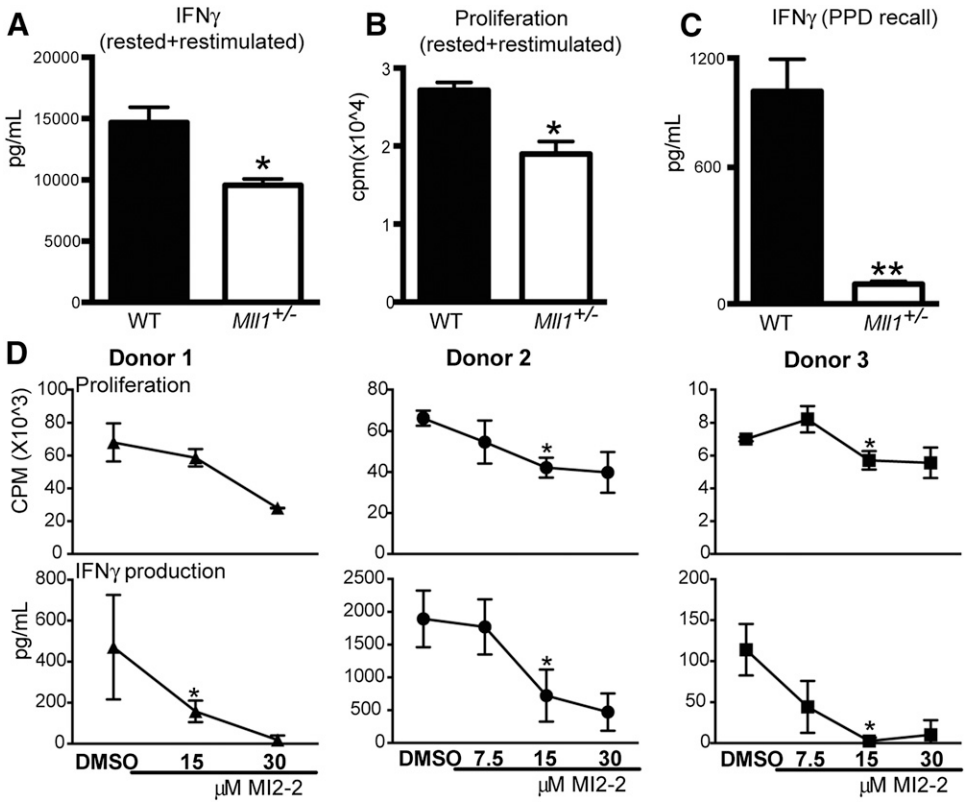

Figure 6. *Mll1* is essential for the T cell recall response. (A) IFN-γ production by $Mll1^{+/-}$ $CD4^+$ T cells in vitro after 5 d of activation and 3 d of rest, followed by an additional 48 h of stimulation with anti-CD3/anti-CD28. $^*P \leq 0.03$ by Student's *t* test. (B) Proliferation of $Mll1^{+/-}$ T cells in the same conditions as in (A). $^*P \leq 0.03$ by Student's *t* test. (C) IFN-γ production from $CD4^+$ T cells isolated from the lungs of PPD-sensitized and -challenged mice that were then cocultured with BMDCs pulsed with PPD for 48 h. $^{**}P \leq 0.001$ by Student's *t* test. (D) Assessment of proliferation and IFN-γ production during the tetanus toxoid recall response when cocultures of T cells and monocyte-derived DCs from the same donor were incubated with the Mll1/menin inhibitor MI-2-2 for 5 d in the presence of tetanus toxoid. All comparisons in (D) are made by use of the DMSO control. $^*P < 0.05$ by Student's *t* test compared with the 15 μM dose of inhibitor. Culture supernatants were analyzed by bioplex (A and C) or standard sandwich ELISA (D). Proliferation was measured by pulsing with [^{3}H]thymidine on day 4 of culture and measuring uptake over an 18 h period. (A–C) Data are pooled from 3 independent experiments with the pooled cells of 2–3 animals for each replicate.

leaving a small pool of memory cells to respond the next time the antigen is encountered [46]. Much of our data indicate that *Mll1* is essential for proper Th1 cell-effector function. These data include a reduction of effector CD4 T cells in $Mll1^{+/-}$ mice in response to PPD immunization and challenge, a reduction in proliferation of $Mll1^{+/-}$ effector cells in vitro, and an increase in $Mll1^{+}IFN\text{-}\gamma^{+}$ cells starting at 72 h postactivation during in vitro differentiation. However, our data also indicate that as T cells become activated, Mll1 expression is reduced (Fig. 1H and Supplemental Fig. 2B). We conclude from these data that although *Mll1* expression is reduced during T cell activation within the total population, it is increased in those cells that produce IFN-γ, thus providing these cytokine-producing cells with a proliferative advantage, perhaps by increased expression of *Ccnd3*. As previous studies and our own data in Fig. 6 demonstrate that *Mll1* is involved in the memory response, it is possible that the expression of *Mll1* during the effector phase is a requirement for a specific T cell to differentiate fully into a memory cell.

Although a number of cyclin genes and cyclin-dependent kinase inhibitors are expressed in T cells activated via anti-CD3/anti-CD28 antibody stimulation, we specifically identified that increased *Mll1* expression is linked to proper expression of *Ccnd3* expression in CD4 T cells. *Ccnd3* is a particularly important gene for T cells, as $Ccnd3^{-/-}$ mice have normal development except for a decrease in thymocyte and peripheral T cell numbers [47]. Under normal conditions, T cells in the periphery up-regulate *Ccnd3* expression in response to IL-12 and IL-2 [48–50]. In $Mll1^{+/-}$ T cells, proliferation was only affected under Th1 conditions, in which IL-12 was present (Fig. 3A and B). Addition of exogenous IL-2 only partially corrected this defect in proliferation (Fig. 3C). Therefore, we hypothesized that the IL-12/STAT4/MLL1 pathway plays a unique role in T cell proliferation. As evidence of this, we have demonstrated that IL-12 signaling caused significant and unique H3K4Me3 modifications in the promoter region of the *Ccnd3* locus that are not present in cells activated with anti-CD3/anti-CD28 antibody alone (Fig. 5). These modifications correlate with the binding of MLL1 to the same promoter regions as those with H3K4Me3 modifications. Out of the genes that we examined that are essential for the Th1 response, this correlation between MLL1 binding and H3K4Me3 modifications was significant only for *Ccnd3*. However, we did observe a trend of MLL1 binding to the promoter of *Tbx21* and *IL12RB2*, 2 other genes associated with the Th1 response. Consistent with previous research regarding the regulation of gene transcription, we observed MLL1 binding and H3K4Me3 modifications at sites 3–4 kb distal to the transcription start site of the *Ccnd3* gene. It has been demonstrated that chromatin modifications at enhancer regions, distal to transcription start sites or gene promoters, can control cell-specific gene expression [51], a result consistent with our observations on the *Ccnd3* gene.

Our data demonstrate that removing the JAK component of the JAK/STAT pathway results in reduced STAT4 binding to the *Mll1* promoter. These results suggest that inhibiting a JAK/STAT pathway in T cells can alter the epigenetic process that determines T cell phenotype. These data were confirmed by the reduction of proliferating and cytokine-producing cells expressing MLL1 in $Stat4^{-/-}$ mice. Furthermore, our data demonstrate, for the first time, that a specific cytokine pathway regulates *MLL1* transcription in T cells in humans and mice. The presence of a STAT-binding site in the intragenic region of *Mll1* is consistent with previous findings that binding sites for these transcription factors can occur in places other than the promoter of the gene [52]. Although the exact role of intergenic transcription factor-binding sites is unknown, it is associated with a number of processes, including T cell differentiation and proliferation [53, 54]. A recent study has suggested that STAT proteins can alter enhancer regions by increasing H3K4Me1 modifications within the chromatin [15]. The STAT4-binding site within the *Mll1* gene may be one of these regions. It is likely that STAT4 and other factors regulate the expression of *Mll1* in instances where rapid, nonhomeostatic cell proliferation is required. As evidence of this, $Mll1^{+/-}$ mice do not have any alterations in hematopoiesis under normal circumstances [55]. Furthermore, this same study demonstrated that hematopoietic cells with no functional copies of the *Mll1* gene were able to reconstitute an irradiated mouse when WT cells were absent. These data demonstrate that homeostatic proliferation is unaltered when *Mll1* is nonfunctional. These findings further suggest that the MLL1 protein complex may be a novel target for immune disorders mediated by T cell responses.

The end result of *Mll1* haploinsufficiency was altered pathology in an antigen-driven, granulomatous response. We observed a significant increase in granuloma size within the lungs of $Mll1^{+/-}$ mice. The observed pathology was accompanied by an increase in neutrophils within the granuloma, without an increase in IL-17 production. As this phenotype was recapitulated in $Rag2^{-/-}$ mice receiving $Mll1^{+/-}$ $CD4^{+}$ T cell transfer (Fig. 2), the altered phenotype is a result of defects in the $Mll1^{+/-}$ $CD4^{+}$ T cells. It has previously been reported that IFN-γ signaling can decrease the number of neutrophils generated from the bone marrow via a suppressor of cytokine signaling 3-dependent mechanism [56]. We also observed increases in IL-1, CXCL1, and CXCL2 in the in the lungs of $Mll1^{+/-}$ mice and $RAG2^{-/-}$ mice receiving $Mll1^{+/-}$ T cells (data not shown). We believe these increases are responsible for neutrophil recruitment. In a model of experimental autoimmune encephalomyelitis, IFN-γ-deficient mice had increased levels of the chemokine CXCL2 [57], thus suggesting that reduction in IFN-γ during an immune response can cause aberrant expression of chemokines. Taken together with our data, we believe that the change in pathology observed in $Mll1^{+/-}$ mice or $Rag2^{-/-}$ mice receiving $Mll1^{+/-}$ T cells is solely a result of the reduced production of IFN-γ by these $Mll1^{+/-}$ T cells.

AUTHORSHIP

M.S. wrote the manuscript, designed and performed experiments, and interpreted data. T.I. designed and performed experiments and interpreted data. R.M.A., D.K., N.K., and C.P. performed experiments contributing to the manuscript. K.C., W.F.C., and N.G. contributed to experimental design. J.G. and T.C. provided compound MI-2-2. Y.D. and S.L.K. interpreted data and contributed to the experimental design. S.L.K. was the principal investigator.

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DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

1. Pakpour, N., Zaph, C., Scott, P. (2008) The central memory CD4+ T cell population generated during *Leishmania major* infection requires IL-12 to produce IFN-gamma. *J. Immunol.* **180,** 8299–8305.
2. Li, Q., Eppolito, C., Odunsi, K., Shrikant, P. A. (2006) IL-12-programmed long-term CD8+ T cell responses require STAT4. *J. Immunol.* **177,** 7618–7625.
3. Finkelman, F. D., Morris, S. C., Orekhova, T., Mori, M., Donaldson, D., Reiner, S. L., Reilly, N. L., Schopf, L., Urban, Jr., J. F. (2000) Stat6 regulation of in vivo IL-4 responses. *J. Immunol.* **164,** 2303–2310.
4. Good, S. R., Thieu, V. T., Mathur, A. N., Yu, Q., Stritesky, G. L., Yeh, N., O'Malley, J. T., Perumal, N. B., Kaplan, M. H. (2009) Temporal induction pattern of STAT4 target genes defines potential for Th1 lineage-specific programming. *J. Immunol.* **183,** 3839–3847.
5. Lund, R. J., Chen, Z., Scheinin, J., Lahesmaa, R. (2004) Early target genes of IL-12 and STAT4 signaling in Th cells. *J. Immunol.* **172,** 6775–6782.
6. O'Shea, J. J., Plenge, R. (2012) JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. *Immunity* **36,** 542–550.
7. Calò, V., Migliavacca, M., Bazan, V., Macaluso, M., Buscemi, M., Gebbia, N., Russo, A. (2003) STAT proteins: from normal control of cellular events to tumorigenesis. *J. Cell. Physiol.* **197,** 157–168.
8. Martino, A., Holmes IV, J. H., Lord, J. D., Moon, J. J., Nelson, B. H. (2001) Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-2. *J. Immunol.* **166,** 1723–1729.
9. Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C., Downward, J., Pestell, R. G., Kanakura, Y. (1999) Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J.* **18,** 1367–1377.
10. Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., Kung, A. L., Cereb, N., Yao, T. P., Yang, S. Y., Reiner, S. L. (2001) Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* **292,** 1907–1910.
11. Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., Glimcher, L. H. (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100,** 655–669.
12. Usui, T., Preiss, J. C., Kanno, Y., Yao, Z. J., Bream, J. H., O'Shea, J. J., Strober, W. (2006) T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *J. Exp. Med.* **203,** 755–766.
13. Thieu, V. T., Yu, Q., Chang, H. C., Yeh, N., Nguyen, E. T., Sehra, S., Kaplan, M. H. (2008) Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. *Immunity* **29,** 679–690.
14. Kanno, Y., Vahedi, G., Hirahara, K., Singleton, K., O'Shea, J. J. (2012) Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annu. Rev. Immunol.* **30,** 707–731.
15. Vahedi, G., Takahashi, H., Nakayamada, S., Sun, H. W., Sartorelli, V., Kanno, Y., O'Shea, J. J. (2012) STATs shape the active enhancer landscape of T cell populations. *Cell* **151,** 981–993.
16. Allan, R. S., Zueva, E., Cammas, F., Schreiber, H. A., Masson, V., Belz, G. T., Roche, D., Maison, C., Quivy, J. P., Almouzni, G., Amigorena, S. (2012) An epigenetic silencing pathway controlling T helper 2 cell lineage commitment. *Nature* **487,** 249–253.
17. Mukasa, R., Balasubramani, A., Lee, Y. K., Whitley, S. K., Weaver, B. T., Shibata, Y., Crawford, G. E., Hatton, R. D., Weaver, C. T. (2010) Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity* **32,** 616–627.
18. Agarwal, S., Rao, A. (1998) Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* **9,** 765–775.
19. Domer, P. H., Fakharzadeh, S. S., Chen, C. S., Jockel, J., Johansen, L., Silverman, G. A., Kersey, J. H., Korsmeyer, S. J. (1993) Acute mixed-lineage leukemia t(4;11)(q21;q23) generates an MLL-AF4 fusion product. *Proc. Natl. Acad. Sci. USA* **90,** 7884–7888.
20. Takeda, S., Chen, D. Y., Westergard, T. D., Fisher, J. K., Rubens, J. A., Sasagawa, S., Kan, J. T., Korsmeyer, S. J., Cheng, E. H., Hsieh, J. J. (2006) Proteolysis of MLL family proteins is essential for Taspase1-orchestrated cell cycle progression. *Genes Dev.* **20,** 2397–2409.
21. Schuettengruber, B., Martinez, A. M., Iovino, N., Cavalli, G. (2011) Trithorax group proteins: switching genes on and keeping them active. *Nat. Rev. Mol. Cell Biol.* **12,** 799–814.
22. Yokoyama, A., Cleary, M. L. (2008) Menin critically links MLL proteins with LEDGF on cancer-associated target genes. *Cancer Cell* **14,** 36–46.
23. Hess, J. L., Yu, B. D., Li, B., Hanson, R., Korsmeyer, S. J. (1997) Defects in yolk sac hematopoiesis in Mll-null embryos. *Blood* **90,** 1799–1806.
24. Yagi, H., Deguchi, K., Aono, A., Tani, Y., Kishimoto, T., Komori, T. (1998) Growth disturbance in fetal liver hematopoiesis of Mll-mutant mice. *Blood* **92,** 108–117.
25. Yamashita, M., Hirahara, K., Shinnakasu, R., Hosokawa, H., Norikane, S., Kimura, M. Y., Hasegawa, A., Nakayama, T. (2006) Crucial role of MLL for the maintenance of memory T helper type 2 cell responses. *Immunity* **24,** 611–622.
26. Onodera, A., Yamashita, M., Endo, Y., Kuwahara, M., Tofukuji, S., Hosokawa, H., Kanai, A., Suzuki, Y., Nakayama, T. (2010) STAT6-mediated displacement of polycomb by trithorax complex establishes long-term maintenance of GATA3 expression in T helper type 2 cells. *J. Exp. Med.* **207,** 2493–2506.
27. Lim, D. A., Huang, Y. C., Swigut, T., Mirick, A. L., Garcia-Verdugo, J. M., Wysocka, J., Ernst, P., Alvarez-Buylla, A. (2009) Chromatin remodelling factor Mll1 is essential for neurogenesis from postnatal neural stem cells. *Nature* **458,** 529–533.
28. Liu, H., Cheng, E. H., Hsieh, J. J. (2007) Bimodal degradation of MLL by SCFSkp2 and APCCdc20 assures cell cycle execution: a critical regulatory circuit lost in leukemogenic MLL fusions. *Genes Dev.* **21,** 2385–2398.
29. Muntean, A. G., Hess, J. L. (2012) The pathogenesis of mixed-lineage leukemia. *Annu. Rev. Pathol.* **7,** 283–301.
30. Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H., Mahowald, M. A., Sider, J. R., Gajewski, T. F., Wang, C. R., Reiner, S. L. (1998) Helper T cell differentiation is controlled by the cell cycle. *Immunity* **9,** 229–237.
31. Kaech, S. M., Ahmed, R. (2001) Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nat. Immunol.* **2,** 415–422.
32. Weng, A. P., Aster, J. C. (2003) No T without D3: a critical role for cyclin D3 in normal and malignant precursor T cells. *Cancer Cell* **4,** 417–418.
33. Nakayama, T., Yamashita, M. (2009) Critical role of the Polycomb and Trithorax complexes in the maintenance of CD4 T cell memory. *Semin. Immunol.* **21,** 78–83.
34. Shi, A., Murai, M. J., He, S., Lund, G., Hartley, T., Purohit, T., Reddy, G., Chruszcz, M., Grembecka, J., Cierpicki, T. (2012) Structural insights into inhibition of the bivalent menin-MLL interaction by small molecules in leukemia. *Blood* **120,** 4461–4469.
35. Chensue, S. W., Warmington, K., Ruth, J., Lincoln, P., Kuo, M. C., Kunkel, S. L. (1994) Cytokine responses during mycobacterial and schistosomal antigen-induced pulmonary granuloma formation. Production of Th1 and Th2 cytokines and relative contribution of tumor necrosis factor. *Am. J. Pathol.* **145,** 1105–1113.
36. Quah, B. J., Warren, H. S., Parish, C. R. (2007) Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat. Protoc.* **2,** 2049–2056.
37. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., Cheng, J. X., Murre, C., Singh, H., Glass, C. K. (2010) Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* **38,** 576–589.
38. Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L., Murphy, K. M. (2002) T-bet is a STAT1-induced regulator of IL-12R expression in naïve CD4+ T cells. *Nat. Immunol.* **3,** 549–557.
39. Kaplan, M. H., Sun, Y. L., Hoey, T., Grusby, M. J. (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* **382,** 174–177.
40. Nishikomori, R., Usui, T., Wu, C. Y., Morinobu, A., O'Shea, J. J., Strober, W. (2002) Activated STAT4 has an essential role in Th1 differentiation and proliferation that is independent of its role in the maintenance of IL-12R beta 2 chain expression and signaling. *J. Immunol.* **169,** 4388–4398.
41. Ghoreschi, K., Jesson, M. I., Li, X., Lee, J. L., Ghosh, S., Alsup, J. W., Warner, J. D., Tanaka, M., Steward-Tharp, S. M., Gadina, M., Thomas, C. J., Minnerly, J. C., Storer, C. E., LaBranche, T. P., Radi, Z. A., Dowty, M. E., Head, R. D., Meyer, D. M., Kishore, N., O'Shea, J. J. (2011) Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550). *J. Immunol.* **186,** 4234–4243.
42. Wei, L., Vahedi, G., Sun, H. W., Watford, W. T., Takatori, H., Ramos, H. L., Takahashi, H., Liang, J., Gutierrez-Cruz, G., Zang, C., Peng, W.,

O'Shea, J. J., Kanno, Y. (2010) Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* **32,** 840–851.
43. O'Sullivan, A., Chang, H. C., Yu, Q., Kaplan, M. H. (2004) STAT4 is required for interleukin-12-induced chromatin remodeling of the CD25 locus. *J. Biol. Chem.* **279,** 7339–7345.
44. Elo, L. L., Järvenpää, H., Tuomela, S., Raghav, S., Ahlfors, H., Laurila, K., Gupta, B., Lund, R. J., Tahvanainen, J., Hawkins, R. D., Oresic, M., Lähdesmäki, H., Rasool, O., Rao, K. V., Aittokallio, T., Lahesmaa, R. (2010) Genome-wide profiling of interleukin-4 and STAT6 transcription factor regulation of human Th2 cell programming. *Immunity* **32,** 852–862.
45. Köck, J., Kreher, S., Lehmann, K., Riedel, R., Bardua, M., Lischke, T., Jargosch, M., Haftmann, C., Bendfeldt, H., Hatam, F., Mashreghi, M. F., Baumgrass, R., Radbruch, A., Chang, H. D. (2014) Nuclear factor of activated T cells regulates the expression of interleukin-4 in Th2 cells in an all-or-none fashion. *J. Biol. Chem.* **289,** 26752–26761.
46. Pepper, M., Jenkins, M. K. (2011) Origins of CD4(+) effector and central memory T cells. *Nat. Immunol.* **12,** 467–471.
47. Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowski, C., Yu, Q., Ferrando, A. A., Levin, S. D., Geng, Y., von Boehmer, H., Sicinski, P. (2003) Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* **4,** 451–461.
48. Yoo, J. K., Cho, J. H., Lee, S. W., Sung, Y. C. (2002) IL-12 provides proliferation and survival signals to murine CD4+ T cells through phosphatidylinositol 3-kinase/Akt signaling pathway. *J. Immunol.* **169,** 3637–3643.
49. Miyatake, S., Sakuma, M., Saito, T. (1997) Induction of interleukin-2 unresponsiveness and down-regulation of the JAK-STAT system upon activation through the T cell receptor. *Eur. J. Immunol.* **27,** 1816–1823.
50. Ajchenbaum, F., Ando, K., DeCaprio, J. A., Griffin, J. D. (1993) Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T lymphocytes. *J. Biol. Chem.* **268,** 4113–4119.
51. Heintzman, N. D., Hon, G. C., Hawkins, R. D., Kheradpour, P., Stark, A., Harp, L. F., Ye, Z., Lee, L. K., Stuart, R. K., Ching, C. W., Ching, K. A., Antosiewicz-Bourget, J. E., Liu, H., Zhang, X., Green, R. D., Lobanenkov, V. V., Stewart, R., Thomson, J. A., Crawford, G. E., Kellis, M., Ren, B. (2009) Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* **459,** 108–112.
52. Robertson, G., Hirst, M., Bainbridge, M., Bilenky, M., Zhao, Y., Zeng, T., Euskirchen, G., Bernier, B., Varhol, R., Delaney, A., Thiessen, N., Griffith, O. L., He, A., Marra, M., Snyder, M., Jones, S. (2007) Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nat. Methods* **4,** 651–657.
53. Durant, L., Watford, W. T., Ramos, H. L., Laurence, A., Vahedi, G., Wei, L., Takahashi, H., Sun, H. W., Kanno, Y., Powrie, F., O'Shea, J. J. (2010) Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* **32,** 605–615.
54. Schraml, B. U., Hildner, K., Ise, W., Lee, W. L., Smith, W. A., Solomon, B., Sahota, G., Sim, J., Mukasa, R., Cemerski, S., Hatton, R. D., Stormo, G. D., Weaver, C. T., Russell, J. H., Murphy, T. L., Murphy, K. M. (2009) The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* **460,** 405–409.
55. McMahon, K. A., Hiew, S. Y., Hadjur, S., Veiga-Fernandes, H., Menzel, U., Price, A. J., Kioussis, D., Williams, O., Brady, H. J. (2007) Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. *Cell Stem Cell* **1,** 338–345.
56. De Bruin, A. M., Libregts, S. F., Valkhof, M., Boon, L., Touw, I. P., Nolte, M. A. (2012) IFNγ induces monopoiesis and inhibits neutrophil development during inflammation. *Blood* **119,** 1543–1554.
57. Savarin, C., Stohlman, S. A., Hinton, D. R., Ransohoff, R. M., Cua, D. J., Bergmann, C. C. (2012) IFN-γ protects from lethal IL-17 mediated viral encephalomyelitis independent of neutrophils. *J. Neuroinflammation* **9,** 104.

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