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Critical Role for the Transcription Regulator CCCTC-Binding Factor in the Control of Th2 Cytokine Expression

Claudia Ribeiro de Almeida,†† Helen Heath,‡ Sanja Krpic,♦ Gemma M. Dingjan,* Jan Piet van Hamburg,* Ingrid Bergen,‡ Suzanne van de Nobelen,‡ Frank Sleutels,‡ Frank Grosveld,♦ Niels Galjart,§ and Rudi W. Hendriks§

Differentiation of naive CD4+ cells into Th2 cells is accompanied by chromatin remodeling at the Th2 cytokine locus allowing the expression of the IL-4, IL-5, and IL-13 genes. In this report, we investigated the role in Th2 differentiation of the transcription regulator CCCTC-binding factor (CTCF). Chromatin immunoprecipitation analysis revealed multiple CTCF binding sites in the Th2 cytokine locus. Conditional deletion of the CTCF gene in double-positive thymocytes allowed development of peripheral T cells, but their activation and proliferation upon anti-CD3/anti-CD28 stimulation in vitro was severely impaired. Nevertheless, when TCR signaling was circumvented with phorbol ester and ionomycin, we observed proliferation of CTCF-deficient T cells, enabling the analysis of Th2 differentiation in vitro. We found that in CTCF-deficient Th2 polarization cultures, transcription of IL-4, IL-5, and IL-13 was strongly reduced. By contrast, CTCF deficiency had a moderate effect on IFN-γ production in Th1 cultures and IL-17 production in Th17 cultures was unaffected. Consistent with a Th2 cytokine defect, CTCF-deficient mice had very low levels of IgG1 and IgE in their serum, but IgG2c was close to normal. In CTCF-deficient Th2 cultures, cells were polarized toward the Th2 lineage, as substantiated by induction of the key transcriptional regulators GATA3 and special AT-rich binding protein 1 (SATB1) and down-regulation of T-bet. Also, STAT4 expression was low, indicating that in the absence of CTCF, GATA3 still operated as a negative regulator of STAT4. Taken together, these findings show that CTCF is essential for GATA3- and SATB1-dependent regulation of Th2 cytokine gene expression. The Journal of Immunology, 2009, 182: 999–1010.

Protection against pathogens relies on the ability of T cells to give rise to various effector cell fates upon activation. Classically, naive CD4+ T cells are thought to undergo programmed differentiation into mainly two functionally distinct subsets, termed Th1 and Th2 (for review, see: Refs. 1 and 2). Th1 cells, which produce IFN-γ, are predominantly involved in cellular immunity against intracellular pathogens. Two major signaling pathways facilitate Th1 development, one involving IL-12/STAT4 and the other involving IFN-γ/STAT1/T-bet (3–5). Th2 cells, producing IL-4, IL-5, and IL-13, mediate humoral immunity and are essential for the eradication of parasitic worms, but also mediate allergic responses. Th2 cytokine production is dependent on the transcription factor GATA3, which is rapidly induced by IL-4 through STAT6 (6–10). Recently, a distinct effector T cell subset has been described, termed Th17. These cells produce IL-17 and control a wide range of infections at mucosal surfaces and are implicated in the pathogenesis of several autoimmune diseases first thought to be caused by deregulated Th1 function (reviewed in Ref. 2). In mice, both TGF-β and IL-6 are required to drive Th17 differentiation through activation of the orphan nuclear receptors RORγt and RORα (11, 12). Differentiation of pathogenic Th17 cells is developmentally related to anti-inflammatory Foxp3+ regulatory T (Treg) cells, which can be generated in vitro by stimulation with TGF-β in the absence of IL-6 (13).

Subset-specific expression of cytokine genes in T cells involves unique transcriptional, epigenetic, and structural mechanisms. When naive T cells are stimulated with Ag, they show low transcription of both IFN-γ and Th2 cytokines (14, 15). Th2 cytokine gene promoters and the Th2 locus control region come into close spatial proximity to form a higher-order chromatin structure, suggesting that early expression of the Th2 cytokines in naive T cells is supported by an initial “poised” chromatin configuration (16). Upon Th2 differentiation, a substantial increase in the transcriptional activity of IL-4, IL-5, and IL-13 and concomitant silencing of IFN-γ are observed. The converse pattern of gene activation and silencing is present in differentiating Th1 cells. Such polarized patterns of cytokine gene expression are achieved through the activation of cell type-specific transcription factors and chromatin remodeling proteins which bind to cis-regulatory elements of cytokine genes, thus initiating substantial and reciprocal alterations in the chromatin structure of the IFN-γ and Th2 cytokine loci (reviewed in Refs. 17 and 18). Indeed, both STAT6 and GATA3 are responsible for the establishment and/or maintenance of the

Abbreviations used in this paper: Treg, regulatory T; SATB1, special AT-rich binding factor 1; CTCF, CCCTC-binding factor; CBS, CTCF binding site; ChIP, chromatin immunoprecipitation; BM-DC, bone marrow-derived dendritic cell; TNP-KLH, 2,4,6-trinitrophenyl-keyhole limpet hemocyanin; DN, double negative; DP, double positive; ISP, immature single positive; SP, single positive; WT, wild type.

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cytokine expression defect, despite normal induction of the tran-

The 11-zinc finger protein CCCTC-binding factor (CTCF) is a ubiquitously expressed and highly conserved transcriptional regulator implicated in many key processes within the nucleus, including promoter activation and repression, hormone-responsive gene silencing, and genomic imprinting (for review, see Ref. 22). CTCF often binds in the vicinity of insulators, elements that affect gene expression by preventing the spread of heterochromatin (acting as “barrier”) and inhibiting inappropriate interactions between regulatory elements on adjacent chromatin domains (acting as “enhancer blocker”) (23). It has been shown that CTCF is required for the enhancer-blocking activity of insulators (24). Consistent with a role for CTCF as an insulator protein, we have shown that in the mouse β-globin locus, CTCF mediates long-range chromatin looping and regulates local histone modifications (25). However, CTCF binding is not always required for chromatin insula
tion (26, 27). Combined, these data establish CTCF as an important protein involved in long-range DNA interactions and the regulation of active and repressive chromatin marks.

Genome-wide mapping of CTCF binding sites (CBS) in the human genome identified ~14,000 sites, whose distribution correlated with genes but not with transcriptional start sites (28, 29). Domains with few or no CTCF sites tend to include clusters of transcriptionally coregulated genes, whereby these regions are often flanked by CTCF binding sites (28, 30). The genome-wide analyses also revealed CTCF binding sites near genes displaying extensive alternative promoter usage, including protocadherin γ, the Ig λ L chain, and the TCR α/δ- and β-chain loci. In mice, CTCF binding was observed downstream of the TCR α/δ and the Ig H chain loci (31, 32). Very recently, CTCF was found to control MHC class II gene expression and long-range chromatin interactions between MHC class II promoter regions (33). These data imply an important role for CTCF in lymphocytes, in particular in the regulation of gene transcription in complex loci. We have recently found that conditional inactivation of Ctcf early in thymocyte development resulted in a severe arrest of early T cell development (34). Our findings indicated that CTCF regulates cell cycle progression of αβ T cells in the thymus (34).

In this report, we investigated whether CTCF is important for Th2 cytokine expression. Chromatin immunoprecipitation (ChIP) assays revealed the presence of multiple CTCF binding sites in the Th2 cytokine locus. We show that conditional deletion of the Ctcf gene in the thymus, using CD4-Cre mice, allowed the generation of peripheral T cells, albeit with reduced numbers. In vitro polarization cultures of CTCF-deficient CD4+ T cells revealed a Th2 cytokine expression defect, despite normal induction of the transcription factors GATA3 and SATB1.

Materials and Methods

Mice

T cell-specific deletion of Ctcf was achieved by breeding Ctecflox mice (34), which were crossed on the C57BL/6 background for >10 generations to CD4-Cre mice (35), which were provided by Dr. C. Wilson (University of Washington, Seattle, WA). OT-II transgenic mice have been described previously (36). Mice were genotyped by Southern blotting or by PCR using Cre-specific primers. Mice were bred and maintained in the Erasmus MC animal care facility under specific pathogen-free conditions and analyzed at 6–10 wk of age. Experimental procedures were reviewed and approved by the Erasmus University committee of animal experiments.

RNA and protein analyses

Total RNA was extracted using the GeneElute mammalian total RNA miniprep system (Sigma-Aldrich). Primers spanning at least one intron-exon junction were designed either manually or using the ProbeFinder software (Roche Applied Science). Probes were chosen from the universal probe library (Roche Applied Science) or designed manually (GATA3, Gapdh) and purchased from Eurogentec. Quantitative real-time PCR was performed using an Applied Biosystems Prism 7700 sequence detection system. To confirm the specificity of the amplified products, samples were separated by standard agarose gel electrophoresis. Threshold levels were set and further analysis was performed using the SDS version 1.9 software (Applied Biosystems). Obtained cycle threshold values were normalized to cycle threshold values of Gapdh or β-actin. Each PCR was performed at least in triplicate. Primer sequences and PCR conditions used are available on request.

Nuclear extracts were prepared and analyzed by Western blot (37). Abs specific for CTCF (N3) and fibrillarin have been previously described (34). Alternatively, anti-CTCF antisera was purchased from Millipore. Anti-DNMT1 was from Abcam and anti-Ub, anti-SATB1, and anti-fibrillarin were from Santa Cruz Biotechnology. Primary Ab incubation was done overnight at 4°C in TBS containing 5% (w/v) BSA and 0.15% (v/v) Nonidet P-40. Blots were incubated with secondary goat anti-rabbit or mouse Abs coupled to HRP (GE Healthcare). Signal detection was performed using ECL (Amersham Biosciences). Western blots were scanned and quantified using the gel macro function in ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

ChIP assay

ChIP followed by ultrahigh-throughput DNA sequencing on H11 erythroid cells was performed using a Solexa 1G Genome Analyser (38). ChIP analyses of the Th2 cytokine locus were performed as described in the ChIP Assay Kit protocol (Upstate Biotechnology) using the anti-CTCF Abs listed above or as IgG as control. Quantitative real-time PCR (Bio-Rad iQ5) on immunoprecipitated DNA was performed using SYBR Green (Sigma-Aldrich) and Platinum Taq DNA Polymerase (Invitrogen). Enrichment was calculated relative to Necdin and values were normalized to input measurements. The sequences of the primers used were as follows: CBS-1F, 5′-GGCTTACAGCCGTTCCAAA-3′; CBS-1R, 5′-CGTTGGG TAAAGCAAGAGC-3′; CBS-2F, 5′-CAGTCTGAGCCTGAGCCTTC-3′; CBS-2R, 5′-CCTGGCGTAAATGATCAGT-3′; CBS-3F, 5′-AGGCC ACACTGTGAAGATGT-3′; CBS-3R, 5′-GTCCTCTTCTGTCCAGTCATT-3′; CBS-4F, 5′-GGCCATCTTGAAGCCTCTA-3′; CBS-4R, 5′-CCCCCTG ACCAACATCTCCCAA-3′; CBS-5F, 5′-ATTGGAAGGCTGAGCAAG-3′; CBS-5R, 5′-GGTGGGATCCCAATGAAGT-3′; CBS-6F, 5′-5′-CCAATCGGAGCTCCTATCCTTACAGC-3′; CBS-7F, 5′-CAGGGTGTTATCATAACCAC-3′; and CBS-8R, 5′-TTGCAGGAGCACGACT-3′.

Flow cytometric analyses

Preparation of single-cell suspensions and mAb incubations for four-color cytometry has been previously described (39). All mAbs were purchased from BD Biosciences except for PE-conjugated anti-granzyme B (GB12; Caltag Laboratories), anti-GATA3 (Hg-3;31; Santa Cruz Biotechnology), allophycocyanin-conjugated anti-Foxp3 (FJK-16s; eBioscience), biotinylated anti-IL-13 (R&D Systems), and allophycocyanin-conjugated IL-10 (JES5-16E3; eBioscience).

For intracellular detection of cytokines, cells were restimulated with plate-bound anti-CD3 (10 μg/ml in PBS; 145-2C11) or PMA (50 ng/ml; Sigma-Aldrich) plus ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of GolgiStop (BD Pharamingen) for 4 h. Cells were harvested and stained intracellularly, followed by standard intracellular staining using parafformaldehyde and saponin. Foxp3 expression was evaluated by intracellular staining using a Foxp3 buffer set (eBioscience).

Cell cycle status of T cell cultures was determined after fixing in ice-cold ethanol and subsequent staining in PBS containing 0.02 mg/ml propidium iodide, 0.1% v/v Triton X-100, and 0.2 mg/ml RNase. Doublet cells were excluded by measuring peak area and width. CFSE labeling of cells was performed as described elsewhere (40). Samples were acquired on a FACS-Calibur or FACS LSRII flow cytometer and analyzed using CellQuest (BD Biosciences) or FlowJo (Tree Star) research software.
In vitro T cell cultures

For in vitro T cell stimulations and Th1/Th2 polarization cultures, naïve CD62L<sup>−</sup>CD4<sup>+</sup> or CD8<sup>+</sup> T cells were purified by cell sorting using a FACSVantage VE equipped with Diva Option and BD FACSDiva software (BD Biosciences). CD4<sup>+</sup> T cells from C57BL/6 mice were obtained through incubation with biotinylated mAbs (BD Phamrmingen) specific for CD4<sup>+</sup> (RM4-5), CD8α<sup>+</sup> (2.43), and CD122<sup>+</sup> (YTS191.1). Stimulation was performed with plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) mAbs coated at 10 ng/ml; IL-2 (5 ng/ml); IFN-γ (10 ng/ml); IL-4 (10 ng/ml). Treg- and Th17-polarizing conditions in vivo consisted of immature and mature BM-DC were harvested. For in vitro T cell stimulations and Th1/Th2 polarization cultures, naïve OT-II-transgenic CD4<sup>+</sup> T cells were cocultured with 0.2×10<sup>6</sup> BM-DC previously pulsed with OVA peptide 323–339 (50 μg/ml) in complete IMDM. At day 4, cells were restimulated with PMA (50 ng/ml) plus ionomycin at day 3, supplemented with TGF-β (3 ng/ml), anti-IFN-γ, and anti-IL-4. Th17-polarizing conditions included IL-6 (20 ng/ml). For Th0 conditions, no cytokines or mAbs were added. For differentiation of CD8<sup>+</sup> effector T cell cultures, only rIL-2 (5 ng/ml) was added to the medium. For Th0, Th1, and Th2 cultures, cells were supplemented with IL-2 (5 ng/ml) on day 3 after activation and expanded up to day 7 under the same cytokine conditions as the primary cultures. In Th17 cultures, cells were restimulated with PMA plus ionomycin at day 3, supplemented with TGF-β and IL-6 and expanded up to day 5. All cytokines were from R&D Systems.

Stimulation of OT-II-transgenic CD4<sup>+</sup> T cells was conducted in the presence of bone marrow-derived dendritic cells (BM-DC). Briefly, BM single-cell suspensions were prepared from C57BL/6 femurs and seeded at 2×10<sup>5</sup> per petri dish in complete IMDM and 200 ng/ml murine GM-CSF (BioSource International). On days 3 and 6, 200 ng/ml murine GM-CSF was added in 10 ml of fresh IMDM. On day 8, the nonadherent cells consisting of immature and mature BM-DC were harvested. For in vitro T cell proliferation studies, 0.2×10<sup>5</sup> CFSE-labeled OT-II-transgenic naive CD4<sup>+</sup> T cells were cocultured with 0.2×10<sup>5</sup> BM-DC previously pulsed with OVA peptide 233–339 (50 μg/ml) in complete IMDM. At day 4, cultured T cells were harvested for proliferation analysis.

ELISA

In vitro-polarized Th2 cells were harvested after 7 days in culture and washed twice with culture medium. Cells were resuspended (1.5×10<sup>6</sup> cells/ml) in fresh culture medium containing PMA (50 ng/ml) plus ionomycin (300 ng/ml). Three days later, supernatants were harvested and analyzed for the presence of cytokines using eBioscience (IL-4 and IL-5) and R&D (IL-13) ELISA systems.

Total serum Ig levels were determined by subclass-specific sandwich ELISA, as described; IgE was induced by i.p. injection of 10 μg of 2,4,6-trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) precipitated on alum (41).

Statistical evaluations

All statistical evaluations were done with Student’s t test.

Results

CTCF binding sites in the Th2 cytokine locus

CTCF binding sites in the human Th2 locus have recently been identified in CD4<sup>+</sup> T cells (30). Taking into account that CTCF binding sites are largely invariant between cell types (28), we first used ChIP coupled to ultrahigh-throughput DNA sequencing data obtained in mouse I11 erythroid cells (S. Krpic and F. Grosveld, manuscript in preparation) to gain insight into CTCF binding in the murine Th2 cytokine locus. We identified four CTCF binding sites (CBS-1, CBS-3, CBS-6, and CBS-7) encompassing the 200-kb region containing the Il15, Rad50, Il113, Il4, and Kit3a genes (Fig. 1A). These sites, as well as three other CTCF binding sites (CBS-2, CBS-4, and CBS-5), have been reported to be occupied in mouse embryonic stem cells (42). We subsequently analyzed CTCF-binding to CBS-1 to CBS-7 in cultured Th1 and Th2 cells by ChIP. For these experiments, MACS-purified CD4<sup>+</sup> T cells from spleen and lymph nodes were stimulated with anti-CD3/CD28 under Th1-polarizing conditions with (IL-12 and anti-IL-4 Abs) or Th2-polarizing conditions with IL-4, anti-IL-12, and anti-IFN-γ Abs) for 7 days. Quantitative real-time PCR analyses showed that CTCF binding to CBS-1, CBS-3, CBS-6, and CBS-7 was significantly increased when compared with IgG control, both in Th1- and Th2-polarized cells (Fig. 1B). A similar enrichment at these sites was also found in PMA/ionomycin-activated Th1- and Th2-polarized cells (data not shown). Thus, the Il-4, Il-5, and Il-13 genes in the Th2 locus are flanked by CBS-6 and CBS-7 upstream of the Il-5 gene and CBS-1 downstream of the Il-4 gene, within the Kit3a gene. Interestingly, CBS-3 is located in the intergenic region between the Il-13 and Il-4 genes, close to a conserved noncoding sequence, designated CNS-1, which has been shown to be critical...
for GATA3 binding and Th2 cytokine expression (43, 44). Our data indicate that the CTCF protein binds to CBS-1, CBS-3, CBS-6, and CBS-7, irrespectively, of Th1 or Th2 polarization.

Conditional deletion of the Ctcf gene in T lymphocytes

To study CTCF function in vivo, we generated a conditional Ctcf allele (Ctcff) by inserting a loxP site upstream of exon 3 and a loxP site along with a lacZ reporter downstream of exon 12 (Fig. 2A) (34). We bred Ctcff mice to mice carrying a Cre-encoding transgene under the control of the CD4 promoter (35). Southern blotting showed almost complete deletion of the Ctcf gene in the thymus of CD4-Cre Ctcff mice, whereas in the spleen Ctcf deletion was only partial, reflecting the presence of many non-T lineage cells (Fig. 2B).

**FIGURE 2.** Conditional targeting of the mouse Ctcf gene. A, Simplified map of the WT murine Ctcf locus and modified alleles. Top line, WT Ctcf allele, with exons shown as solid boxes, and position of HindIII sites (H) and the Southern blot probe (arrow) indicated. Middle line, Floxed Ctcf allele (Ctcff). Two targeting cassettes were inserted into the Ctcf gene via consecutive rounds of homologous recombination. The first cassette contained LoxP sites (▲) flanking a PMCl-driven neomycin resistance gene (neo'), the second cassette contained LoxP sites flanking a PGK-driven puromycin resistance gene (puro'), followed by a splice acceptor-lacZ cassette (SA-LacZ). Bottom line, deleted Ctcf gene (Ctcff) generated after complete Cre-mediated recombination at the outermost loxP sites. For details on constructs and targeting, see Heath et al. (34). The small horizontal lines underneath each allele represent HindIII fragments recognized by the probe in the Southern blot analysis of B below. B, Southern blot analysis of CD4-Cre recombinase activity. HindIII-digested genomic DNA from thymus and spleen of mice of the indicated genotypes was analyzed by hybridization with a CTCF locus-specific probe (see A). The positions of the WT, Ctcf<sup>+/+</sup> (flox), and Ctcf<sup>-/-</sup> (del) alleles are indicated (asterisk indicates a polymorphic WT allele from the FVB background). C, Western blot analysis of sorted naive CD62L<sup>-</sup>CD4<sup>+</sup>CD8<sup>-</sup> and CD8<sup>+</sup> T cell fractions from nontransgenic (-) or CD4-Cre-transgenic (+) Ctcf<sup>+/+</sup> mice were analyzed for CTCF. DNMT1 and UBF were used as loading controls. D, Flow cytometric analysis of lacZ expression in CTCF conditionally deleted mice. LacZ expression was analyzed in conjunction with cell surface markers. The indicated cell populations were gated and lacZ expression data are displayed as histogram overlays of CD4-Cre Ctcf<sup>+/+</sup> mice (green) on top of the background signals in WT mice (black). ISP, Immature SP.
in conjunction with cell surface markers. Consistent with the reported CD4-Cre activity at the double-negative (DN) stage (45), Ctcf deletion was initiated in DN cells (DN2 to DN4) and completed from the immature single-positive (ISP) cell stage onward (Fig. 2D). Despite efficient deletion of the Ctcf gene in the thymus, residual CTCF protein was still detectable in purified fractions of peripheral naive CD62L<sup>−</sup>CD4<sup>+</sup> or CD8<sup>+</sup> T cells (~25% of control; Fig. 2C), indicating that CTCF is a remarkably stable protein in resting naive T cells.

To examine the effects of Ctcf deletion on T cell development, thymocyte subpopulations from 6- to 8-wk-old CD4-Cre <em>Ctcf</em><sup>fl/fl</sup> mice and wild-type (WT) littermates were analyzed by flow cytometry. CD4-Cre <em>Ctcf</em><sup>fl/fl</sup> mice displayed low thymic cellularity, with reduced numbers of double-positive (DP), CD4<sup>+</sup> and CD8<sup>+</sup> single-positive (SP) cells, when compared with WT controls (Fig. 3, A and B). The CD<sup>+</sup>CD8<sup>+</sup> thymocyte fraction had a relative increase of CD3<sup>lo</sup>CD69<sup>lo</sup> ISP cells and a decrease of CD3<sup>hi</sup>CD8<sup>+</sup> SP cells. Consistent with impaired thymic SP cell production, the numbers of mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells in spleen and lymph nodes of CD4-Cre <em>Ctcf</em><sup>fl/fl</sup> mice were significantly reduced (Fig. 3, A and C, and data not shown). Furthermore, the numbers of γδ T cells in the spleens of CD4-Cre <em>Ctcf</em><sup>fl/fl</sup> mice were increased (Fig. 3C).

In summary, in CD4-Cre <em>Ctcf</em><sup>fl/fl</sup> mice, αβ T cell development is partially arrested at the DP stage, resulting in a significant reduction of the numbers of peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

**Defective TCR/CD3-mediated proliferation of CTCF-deficient CD4<sup>+</sup> and CD8<sup>+</sup> T cells**

To investigate cellular activation of CTCF-deficient T cells, we performed in vitro stimulation experiments with highly purified naive CD62L<sup>−</sup>CD4<sup>+</sup> and CD8<sup>+</sup> peripheral T cells. We evaluated their ability to go through sequential cell divisions by CFSE labeling and observed severely reduced proliferation of anti-CD3/CD28-activated CTCF-deficient CD4<sup>+</sup> T cells at day 3 (Fig. 4A). Proliferation was not only defective when standard conditions of 10 μg/ml anti-CD3/anti-CD28 were used, but also when we increased either of the two (or both) Ab concentrations to 50 μg/ml (data not shown). Next, the capacity of CTCF-deficient CD4<sup>+</sup> T cells to proliferate upon a more physiological, Ag-specific stimulation was investigated by crossing CD4-Cre-<em>Ctcf</em><sup>fl/fl</sup> mice with OT-II-transgenic mice, which harbor a TCR specific for OVA peptide. Upon activation by OVA peptide<sub>323–339</sub>-pulsed APC, survival of nondividing (WT and CTCF-deficient OT-II-transgenic CD4<sup>+</sup>) T cells was similar, but we noticed a severe proliferation defect in the absence of CTCF (Fig. 4A). Although anti-CD3/CD28-activated

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**FIGURE 3.** Defective TCRαβ lineage development in CTCF-deficient mice. A, Flow cytometric analyses of the indicated cell populations in thymus or spleen from WT and CD4-Cre <em>Ctcf</em><sup>fl/fl</sup> mice. Expression profiles of surface markers are shown as dot plots and the percentages of cells within the quadrants or gates are given. B and C, Absolute numbers of the indicated thymic and splenic T cell subpopulations. Each symbol represents one individual animal and lines indicate average values. Values of <em>p</em> of significant differences between WT and CD4-Cre <em>Ctcf</em><sup>fl/fl</sup> mice are indicated.
CTCF-deficient CD8\(^+\) T cells were able to undergo cell division, they lagged behind WT cells by approximately one cell cycle, and cell recovery was reduced when compared with WT CD8\(^+\) T cells (Fig. 4A).

PMA bypasses proximal TCR signaling events and directly activates protein kinase C signaling (46). Under conditions where PMA was added as a costimulatory signal with anti-CD3, CTCF-deficient cells showed defective proliferation. However, when T cells were stimulated by PMA and the calcium ionophore ionomycin (which raises the intracellular level of Ca\(^{2+}\)), we observed significant proliferation of CTCF-deficient CD4\(^+\) and CD8\(^+\) T cells (Fig. 4A).

Consistent with limited cell division observed in the CFSE experiments, anti-CD3/CD28-stimulated CTCF-deficient CD4\(^+\) T cell cultures showed diminished cell recovery and an almost complete lack of cells in the S-G2-M phase of the cell cycle (Fig. 4B). Although CTCF-deficient PMA/ionomycin-stimulated CD4\(^+\) or CD8\(^+\) T cell cultures exhibited lower expansion rates, their cell cycle profiles at day 3 were similar to those from WT cells (Fig. 4B). In vitro proliferation of CD4-Cre Ctcff\(^{fl}\) T cells did not reflect a specific expansion of rare cells that have escaped CTCF deletion, as CTCF protein levels were severely reduced both in CD8\(^+\) (Fig. 4C) and in CD4\(^+\) T cell cultures (see Fig. 9C).

Taken together, these findings demonstrate that conditional deletion of the Ctcf gene in DP thymocytes allows development of peripheral T cells, but their activation and proliferation upon anti-CD3/anti-CD28 stimulation in vitro is severely impaired. Nevertheless, when TCR signaling is circumvented with phorbol ester and ionomycin, CTCF-deficient T cells have the capacity to proliferate, indicating that in this context loss of CTCF can be compensated for by signaling molecules or nuclear factors induced by PMA/ionomycin. These results therefore indicate that CTCF is not absolutely required for cell proliferation.

Defective TCR/CD3-mediated activation of CTCF-deficient CD4\(^+\) and CD8\(^+\) T cells

Next, we investigated whether defective proliferation of CTCF-deficient T cells was caused by impaired cellular activation. Binding of IL-2 to its receptor is a critical event in the initiation of T cell proliferation, since it regulates transition of the cell cycle from G\(_1\) into S phase (47). Therefore, we investigated...
whether CTCF-deficient T cells had an IL-2 production defect or impaired IL-2R induction. We found that upon stimulation with anti-CD3/CD28 or PMA/ionomycin, the absence of CTCF did not affect IL-2 production in CD4<sup>+</sup> or CD8<sup>+</sup> T cells, as analyzed by intracellular cytokine staining at day 3 (Fig. 5A). By contrast, induction of the IL-2 receptor CD25 on CTCF-deficient CD4<sup>+</sup> and CD8<sup>+</sup> T cells was severely impaired, when activated by plate-bound anti-CD3/CD28 (Fig. 5B). When activated by PMA/ionomycin, CTCF-deficient CD4<sup>+</sup> T cells displayed a partial defect in CD25 up-regulation, while in CD8<sup>+</sup> T cells CD25 induction was normal.

Expression of CD69, an ~30-kDa glycoprotein induced in activated T cells, was hampered in CTCF-deficient CD4<sup>+</sup> T cells upon TCR stimulation, but was normal in CD8<sup>+</sup> T cells or in PMA/ionomycin-stimulated CTCF-deficient T cells. Finally, analyses of forward scatter values of stimulated cells showed that cell size increases in CTCF-deficient cells were limited, particularly upon anti-CD3/CD28 stimulation, whereby CD4<sup>+</sup> cells were more affected than CD8<sup>+</sup> cells (Fig. 5B).

From these findings, we conclude that anti-CD3/CD28 treatment does not elicit proper activation of CTCF-deficient peripheral T cells, in terms of induction of CD25, CD69, and cell size increase, whereby CD4<sup>+</sup> T cells are somewhat more affected than CD8<sup>+</sup> T cells. However, when T cells are stimulated by PMA/ionomycin, expression levels of CD25 and CD69 are largely in the normal ranges.

CD4-Cre Ctcff<sup>−/−</sup> mice have severely reduced serum levels of IgG1 and IgE

Concentrations of Ig subclasses in the serum of CD4-Cre Ctcff<sup>−/−</sup> mice were reduced, except for IgM and IgG3, which are T cell

FIGURE 6. Serum Ig analysis in CTCF-deficient mice. A. Serum concentrations of Ig isotypes displayed as average values plus SD. Mice were 2 mo of age. B. Total IgE serum concentrations in nonimmunized mice (left) and in immunized mice 7 days after i.p. injection with 10 μg TNP-KLH (right).
independent and IgA which requires TGF-β expression (Fig. 6A). This does not necessarily mean that CTCF-deficient CD4+ T cells produce normal levels of TGF-β, since this cytokine is secreted by many cell types.

Interestingly, serum levels of the IL-4-dependent isotype IgG1 were more affected than those of the IFN-γ-dependent isotype IgG2c (~10 and ~60% of WT, respectively). Serum concentrations of the IL-4-dependent isotype IgE were also lower in the absence of CTCF (Fig. 6B). Moreover, when Th2-mediated responses were tested in vivo by injection of a low dose of TNP-KLH in alum (10 μg), we observed an increase in the concentration of total IgE in WT mice at day 10, but not in CD4-Cre Ctcff/f animals (Fig. 6B). Thus, the absence of CTCF resulted in a severe deficiency for the Th2-dependent subclasses IgG1 and IgE in the serum, whereas the Th1-dependent subclass IgG2c was only moderately affected. CD4-Cre Ctcff/f mice also had very low levels of IgG2b in the serum. Because class switch recombination to IgG2b is thought to be regulated by various cytokines including IFN-γ and TGF-β, but not by Th2 cytokines, the IgG2b deficiency cannot be explained by a selective Th2 defect.

**FIGURE 7.** CTCF regulates Th2 cytokine expression. A, Expansion of Th0, Th1, and Th2 cultures 7 days after stimulation with anti-CD3/CD28 or PMA/ionomycin. Symbols represent the expansion values of cultures from individual mice, whereby cell numbers at the start of the culture were set to 1. Lines indicate average values. Values of p of significant differences between WT and CD4-Cre Ctcff/f mice are indicated. B, Flow cytometric analysis for intracellular expression of IFN-γ and IL-4 in the indicated T cell cultures after stimulation with PMA/ionomycin. CD4+ T cells were gated and expression profiles are displayed as dot plots. The percentages of cells within the quadrants are given. C, Flow cytometric analysis for intracellular expression of the indicated cytokines after stimulation with PMA/ionomycin. Total cells and CD4+ T cells were gated and expression profiles are displayed as CD4/IL-5 and IL-10/IL-13 dot plots, respectively. D, Cytokine levels of WT (□) and CD4-Cre Ctcff/f (■) Th2 cultures supernatant after stimulation with PMA/ionomycin. Mean values and SD are given for two seven mice analyzed per group; *, p < 0.05 and **, p < 0.01. E, Quantitative RT-PCR analysis of expression of the indicated cytokines in different T cell cultures (WT, □; CD4-Cre Ctcff/f, ■). Expression levels were normalized for GAPDH and are expressed as arbitrary units, whereby the values in WT Th2 cells were set to 1. Mean values and SD are given for four mice analyzed per group; *, p < 0.05 and **, p < 0.01.

**Th2 cytokine defect in CD4-Cre Ctcff/f mice**

Next, we performed in vitro polarization cultures to investigate whether CTCF is specifically required for differentiation of Th2 effector cells. Sorted naive CD62L+CD4+ T cells from spleen and lymph nodes were stimulated with anti-CD3/CD28 or PMA/ionomycin under Th0 conditions (without additional cytokines or Abs), Th1-polarizing conditions (with IL-12 and anti-IL-4), or Th2-polarizing conditions (with IL-4, anti-IL-12, and anti-IFN-γ) for 7 days. Under these conditions, anti-CD3/CD28-stimulated WT CD4+ T cell fractions manifested a ~10- to 100-fold expansion (Fig. 7A). Consistent with the observed severely defective proliferation of anti-CD3/CD28-stimulated CTCF-deficient CD4+ T cells, we found that the expansion of these cells at day 7 was negligible. Importantly, replacing anti-CD3/CD28 by PMA/ionomycin stimulation resulted in significant expansion of CTCF-deficient T cell cultures (~2–10 times at day 7; Fig. 7A), enabling the analysis of Th1 and Th2 development in vitro. Under these conditions, WT Th cultures showed an ~10- to 30-fold expansion (Fig. 7A).
In Th1-polarized cultures, CTCF-deficient T cells produced moderately reduced levels of IFN-γ when compared with WT T cells, as determined by intracellular flow cytometry (Fig. 7B). The proportions of IFN-γ+ cells were 55 ± 15% (n = 7) and 23 ± 6% (n = 4; p < 0.005) in WT and CTCF-deficient Th1 cultures, respectively. Remarkably, CTCF-deficient T cells showed a more severe IL-4 production defect in Th2-polarized cultures. Both the frequency of IL-4+ T cells (51 ± 11% (n = 7) in WT and 9 ± 4% (n = 4; p < 0.00005) in CTCF-deficient cultures) and intracellular IL-4 signals per cell were significantly reduced in the absence of CTCF (Fig. 7B). Additional intracellular flow cytometry analyses showed that in CTCF-deficient Th2 cultures IL-5 production was not detectable and IL-10 and IL-13 were severely reduced (Fig. 7C). Consistent with these findings, in the supernatants of Th2-polarized cultures, production of IL-4, IL-5, and IL-13 was significantly reduced in the absence of CTCF, as determined by ELISA (Fig. 7D). Finally, quantitative RT-PCR analysis of day 7 cultures showed that in CTCF-deficient Th2 cultures transcription of the IL-4, IL-5, and IL-13 cytokines was strongly reduced (Fig. 7E). We also analyzed transcription of the IL-10 gene, which is not located within the Th2 locus, and found that in the absence of CTCF IL-10 expression was very low, both in Th1 and Th2 cultures.

Thus, in CTCF-deficient Th2 polarization cultures in vitro, transcription of IL-4, IL-5, and IL-13 was strongly reduced. By contrast, CTCF deficiency had a moderate effect on IFN-γ production in Th1 cultures.

**Th17 and Treg differentiation in CD4-Cre Ctcff^f/f mice**

The observed Th2 cytokine defect in CTCF-deficient Th2 cells prompted us to investigate whether differentiation toward alternative CD4 T cell fates, including Th17 and Treg cells, was possible in the absence of CTCF. We activated sorted naive CD62L^-CD4^+ T cells with PMA/ionomycin and cultured them under Th17 conditions (in the presence of TGF-β, IL-6, anti-IFN-γ, and anti-IL-4) or Treg conditions (with TGF-β, anti-IFN-γ, and anti-IL-4). As examined in CSFE experiments, we observed significant proliferation of CTCF-deficient CD4^+ T cells under Th17 and Treg conditions at day 3 (Fig. 8A). When Th17 cultures were analyzed for intracellular cytokines at day 5, we found that the expression of IL-17 in CTCF-deficient T cells reached values that were similar to those found in WT T cells (~8%, n = 6; Fig. 8B). In addition, we noticed a small but consistent population of IFN-γ+ cells in CTCF-deficient Th17 cultures, which was not detected in WT Th17 cultures.

When naive WT CD62L^-CD4^+ T cells were cultured under Treg conditions for 3 days, a large majority of cells (85 ± 2%,...
the proportions of naturally occurring CD25+ H11001/H11006 proportions were slightly lower (62% present in Treg cultures of CTCF-deficient T cells, albeit that the GATA3 and SATB1 expression in CD4-Cre Ctcff/f mice does not result in a global defect in effector T cell and CD8+ cells and lack of CTCF has limited effects on differentiation of Treg-deficient T cells into IL-17-producing Th17 cells is apparently not shown).

FIGURE 9. GATA3 and SATB1 are induced in CTCF-deficient Th2 cultures. A, Flow cytometric analysis for intracellular GATA3 protein expression in PMA/ionomycin-stimulated T cell cultures. CD4+ T cells were gated and for the indicated mice expression profiles are displayed as histograms overlays of Th1 (gray histograms) and Th2 cultures (bold lines). B, Quantitative RT-PCR analysis of GATA3, T-bet, and STAT4 expression in different T cell cultures from WT (□) or CD4-Cre Ctcff/f mice (■). Expression levels were normalized for GAPDH and are expressed as arbitrary units, whereby expression in WT Th1 cells (T-bet, STAT4) or Th2 cells (GATA3) was set to 1. Mean values and SD are given for four mice analyzed per group. C, Western blotting analysis of SATB1 and CTCF protein levels in PMA/ionomycin-stimulated T effector cell cultures at day 7. Fibrillarin was used as a loading control.

Discussion

The differentiation process of naive CD4+ T cells to Th1 or Th2 cells is critically dependent on coordinated transcriptional regulation of cytokine gene loci (18). To investigate whether CTCF regulates transcription of cytokine-encoding genes, we studied mice in which CTCF was conditionally deleted in the T cell lineage. Our data indicate that CTCF deficiency affects differentiation of Th2 effector cells by impairing T cell activation as well as Th2 cytokine production. Activation and proliferation of CTCF-deficient CD4+ and CD8+ cells upon anti-CD3/anti-CD28 stimulation in vitro was severely hampered. However, when TCR signaling was circumvented with phorbol ester and ionomycin, we observed proliferation of CTCF-deficient T cells, enabling the analysis of Th cell differentiation. We found that in CTCF-deficient Th2 polarization cultures, transcription of IL-4, IL-5, and IL-13 was strongly reduced. By contrast, CTCF deficiency had only a modest effect on IFN-γ production in Th1 cultures and did not appear to affect Th17 differentiation and IL-17 production. In CTCF-deficient Th2 cultures, cells were polarized toward the Th2 lineage, as the key transcriptional regulators GATA3 and SATB1 were induced and T-bet and STAT4 were down-regulated. Nevertheless, expression of IL-4, IL-5, and IL-13 remained strongly inhibited in these otherwise Th2-polarized cells. We therefore propose that CTCF plays a major role in the GATA3- and SATB1-dependent regulation of the expression of genes within the Th2 cytokine locus.

We found that CTCF-deficient CD4+ and CD8+ T cells have severely defective activation and proliferation in response to anti-CD3/CD28 stimulation. Nevertheless, one of the most important downstream events, production of IL-2, was not noticeably affected, ruling out global defects in TCR clustering, actin polymerization, or activation of NFAT, NF-κB, and JNK cascades (50, 51).

The finding that PMA/ionomycin stimulation bypassed the CD3/CD28-signaling defect suggests that the presence of CTCF is important for signaling events downstream of the TCR which are necessary for full T cell activation. In this context, we found that PMA/ionomycin partially (in CD4+ T cells) or completely (in CD8+ T cells) rescued the defective induction of IL-2R expression in CTCF-deficient T cells observed upon anti-CD3/CD28 stimulation (Fig. 5B). Additional experiments are required to clarify whether CTCF controls gene expression of specific proteins involved in proximal signaling events (induced upon TCR stimulation) that regulate IL-2R induction.
The in vitro Th1 polarization cultures showed that CTCF-deficient Th1 effector cells produced significant amounts of IFN-γ, but expression was reduced when compared with WT. Also, in anti-CD3/CD28-stimulated CTCF-deficient CD8⁺ T cells, we found that the proportion of IFN-γ⁺ cells was ~60% of that in WT cells (C. R. de Almeida, unpublished data). In contrast, we observed a small cloud of IFN-γ⁺ cells in Th17 cultures from CTCF-deficient T cells, which was not detectable in WT Th17 cultures, indicating that IFN-γ expression is not reduced under all culture conditions. Therefore, we conclude that the observed reduction of IFN-γ/H9253 site HSS-3, which has been shown to be present both in naive region. that CTCF binding to CBS-3 influences Th2 cytokine expression, distance between CNS-1 and CBS-3 is modification. CBS-3 is located near the conserved noncoding sequence CNS-1, which is critical for Th2 cytokine expression and binds the C-terminal zinc finger of GATA3 (43, 44). However, because the distance between CNS-1 and CBS-3 is ~1 kb, it is not very likely that CTCF binding to CBS-3 influences Th2 cytokine expression by direct interaction with nuclear proteins recruited to the CNS-1 region.

Interestingly, CBS-3 is located at the constitutive hypersensitive site HSS-3, which has been shown to be present both in naive CD4⁺ cells and Th1 and Th2 cells (43). Although nine SATB1 sites were identified in the mouse Th2 locus (21), none of these is located near the CNS-1 region. Thus, SATB1 and CTCF binding sites in the Th2 locus are interspersed. One of the possible explanations for defective Th2 cytokine expression in the absence of CTCF would be that CTCF is involved in chromatin organization of the Th2 locus. In such a model, loss of CTCF would affect SATB1-mediated looping of the Th2 locus and, consequently, Th2 cytokine expression. Indeed, studies in the chicken β-globin locus have led to a model of CTCF-dependent enhancer-blocking function based on the interaction between CTCF and the nucleolar protein nucleophosmin, whereby tethering of the insulator to a nuclear structure prevents enhancer-promoter communication (52). Moreover, SATB1 was originally identified as a matrix attachment region DNA-binding protein (53), which possibly contributes to chromatin loop organization (54). It would be interesting to investigate whether CTCF binding sites at the Th2 cytokine locus are involved in the tethering of this 200-kb DNA region to subnuclear sites, thereby allowing coordinated expression of Th2 cytokine genes from a SATB1-dependent, transcriptionally active chromatin structure.

However, mechanisms other than formation of chromatin loops can also account for CTCF function and explain the defective Th2 cytokine expression observed in the absence of CTCF. Similar to its role in the mouse β-globin locus (25), CTCF could function to direct local histone modifications at the Th2 cytokine locus. Indeed, high-resolution profiling of histone methylation in the human genome showed that CTCF marks boundaries of histone methylation domains (30) and CTCF interaction with histones or histone-modifying proteins have been reported (30, 52, 55, 56). Additionally, CTCF has also been shown to interact with the large subunit of RNA polymerase II (57), shown to be recruited to the Th2 cytokine locus upon Th2 cell activation (21). Finally, it remains possible that the effect of CTCF on Th2 cytokine expression is indirect. For example, CTCF could be an essential regulator of the expression 1) of nuclear regulators other than GATA3 or SATB1 that are required for Th2 cytokine transcription or 2) of enzymes involved in posttranslational modifications of Th2-specific transcription factors.

The role for CTCF in the GATA3/SATB1-mediated regulation of the Th2 cytokine locus may well parallel its recently described role in the control of MHC class II gene expression and the formation of long-distance chromatin interactions involving the CIITA (33). In contrast, we reported that deletion of one CTCF binding site in the mouse β-globin locus did not affect expression of the β-globin genes (25). Thus, it is clear that CTCF has cell type-specific functions. It was proposed that CTCF remains bound to its ~14,000 cognate binding sites irrespective of cell type (28). It will be interesting to determine how CTCF performs cell type-specific roles while remaining bound to its cognate sites. Equally interesting are the questions how and which chromosomal interactions, both in cis-and in trans, persist in the absence of CTCF. Importantly, our experiments in mature CTCF-negative T cells show they can proliferate and differentiate under appropriate conditions and it is therefore feasible to address these issues using CTCF knockdown or conditional targeting approaches.

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Disclosures
The authors have no financial conflict of interest.

References
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