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RESEARCH ARTICLE

The DNA-binding factor Ctcf critically controls gene expression in macrophages

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Macrophages play an important role in immunity and homeostasis. Upon pathogen recognition *via* specific receptors, they rapidly induce inflammatory responses. This process is tightly controlled at the transcriptional level. The DNA binding zinc-finger protein CCCTC-binding factor (Ctcf) is a crucial regulator of long-range chromatin interactions and coordinates specific communication between transcription factors and gene expression processes. In this study, the *Ctcf* gene was specifically deleted in myeloid cells by making use of the transgenic Cre-LoxP system. Conditional deletion of the *Ctcf* gene in myeloid cells induced a mild phenotype *in vivo*. *Ctcf*-deficient mice exhibited significantly reduced expression of major histocompatibility complex (MHC) class II in the liver. *Ctcf*-deficient macrophages demonstrated a normal surface phenotype and phagocytosis capacity. Upon Toll-like receptor (TLR) stimulation, they produced normal levels of the pro-inflammatory cytokines IL-12 and IL-6, but manifested a strongly impaired capacity to produce tumor-necrosis factor (TNF) and IL-10, as well as to express the IL-10 family members IL-19, IL-20 and IL-24. Taken together, our data demonstrate a role of Ctcf that involves fine-tuning of macrophage function.
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INTRODUCTION

Cells belonging to the myeloid lineage take a central role in homeostasis and immunity, and are involved in the initiation, maintenance and resolution of immune responses. Myeloid cells include granulocytes, monocytes, dendritic cells and macrophages.[1] These cells recognize bacteria, viruses or apoptotic cells *via* a broad array of pattern recognition receptors to trigger their effector functions leading to elimination of bacteria and viruses or removal of apoptotic cells.[1] Recognition *via* specific receptors, such as Toll-like receptors (TLRs), and phagocytic uptake of pathogens by macrophages or dendritic cells (DCs) generally induces the production and secretion of pro-inflammatory cytokines, such as tumor-necrosis factor (TNF), IL-6 or IL-12, which initiate and promote host defense. Cytokine expression is generally inducible and can be cell type-specific.[2] Inflammatory responses required for pathogen elimination are extremely complex and tightly controlled at the level of gene transcription. Transcriptional programs need to initiate an efficient effector response that controls the harmful challenge to the host.

The DNA-binding CCCTC binding factor (Ctcf) plays an important role in the regulation of expression of numerous genes, and approximately 14 000–40 000 binding sites have been identified genome-wide.[3,4] Ctcf is a highly conserved 11-zinc finger protein involved in the regulation of gene expression in a cell type-specific manner at complex gene clusters, such as the β-globin, major histocompatibility complex (MHC) class and the Ig gene loci.[3,4] Moreover, Ctcf is important in a variety of regulatory functions, including genomic imprinting, X-chromosome inactivation and long-range

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chromatin interactions, hormone-responsive gene silencing, enhancer blocking and/or barrier gene insulation and transcriptional activation or repression.[3,4] The distinct functions of Ctcf are exerted by combinatorial use of 11-zinc fingers allowing it to bind highly divergent sequences.[3] Ctcf regulates chromatin architecture together with cohesion proteins, which are enriched at Ctcf-binding sites.[5–7] In addition, multiple functions of Ctcf are enabled by interaction with different binding partners like transcription factors (Yy1, Kaiso and YB-1),[8–10] chromatin modifying proteins (Sin3a, CHD8, Suz12),[11–13] RNA polymerase II[14] and poly(ADP-ribose) polymerase-1.[15]

Recently, Ctcf was found to control MHC class II gene expression,[16] and it was reported that enforced overexpression of Ctcf in DC caused increased apoptosis, reduced proliferation and impaired differentiation.[17] Conditional targeting experiments in mice showed that Ctcf controls both T-cell development[18] and differentiation into effector subsets, particularly into type T helper (Th)-2 cells.[19] We previously reported that mice with a Ctcf defect in $CD4^+$ T cells exhibit reduced Th2 development and production of the Th2 cytokines IL-4, IL-5 and IL-13, despite the expression of normal levels of the key Th2 transcription factors Gata3 and Satb1.[19] Interestingly, it was demonstrated that cooperation between T-bet and Ctcf is required for Th1 cell-specific expression of IFN-γ.[20] In addition, Ctcf also plays a role in the regulation of V(D)J recombination events and V gene usage at the Ig H and L chain loci in B cells.[21,22] The role of Ctcf in development and function of myeloid cells *in vivo* has not been investigated. Here, we used the transgenic Cre-loxP system to generate conditional myeloid-specific *Ctcf*-knockout mice, allowing analysis of the role of Ctcf in the development and function of macrophages *in vivo* and *in vitro*. We demonstrate that deletion of the *Ctcf* gene in cells with active Cre expression driven by the M lysozyme (LysM) promoter affected the numbers of macrophages generated from monocytes. Interestingly, we observed that Ctcf-deficient macrophages from *LysM-Cre Ctcf$^{f/f}$* mice retained the capacity to produce IL-6 and IL-12 upon TLR ligation, but manifested impaired expression or production of the regulatory cytokines of the IL-10 family as well as TNF.

MATERIALS AND METHODS

To obtain mice with conditional knockout of Ctcf in macrophages, mice bearing the Ctcf allele flanked with loxP sites (Ctcf^f mice)[18] were crossed with mice expressing Cre recombinase under the LysM promoter (LysM-Cre mice).[23] To investigate the efficiency of Cre-mediated deletion in the various myeloid cell lineages, we used a Cre-reporter strain harboring a targeted insertion of enhanced yellow fluoresencent protein (EYFP) into the ROSA26 locus.[24] Genotyping of mice for the presence of transgenic constructs was performed as previously described.[18,23,24] Crosses of LysM-Cre transgenic and Ctcf$^{f/f}$ mice yielded mice with myeloid-specific deletion of the *Ctcf* gene (LysM-Cre Ctcf$^{f/f}$) mice, as well as littermates that did not have the LysM-Cre transgene or a floxed *Ctcf* gene, both of which were referred to as 'wild-type' mice. Mice were bred and maintained at the Erasmus MC animal facility under specific pathogen-free conditions, and used for experiments at 6–12 weeks of age. Experimental procedures were approved by the Erasmus University committee of animal experiments.

Preparation of single cell suspensions

Single-cell suspensions were prepared using standard methods and filtered through a 100-μm cell strainer. Livers were removed without perfusion; small pieces were incubated for 30 min in RPMI 1640 containing 30-μg/ml Liberase TM (Roche, Woerden, Netherlands) and 20 μg/ml DNAse type I (Sigma, St. Louis, USA), and passed through a 100-μm cell strainer. Cells were resuspended in phosphate-buffered saline (PBS) containing 1% serum and 2.5 mM EDTA. Parenchymal cells were removed by low-speed centrifugation at 300 r.p.m. for 3 min, and erythrocytes were lysed with 0.8% NH_4Cl. Remaining liver cells were resuspended in culture medium.

Phenotypic analysis by flow cytometry

Aliquots of 2×10^6 cells were incubated with a cocktail of monoclonal antibodies. Each incubation step was performed at 4 °C for 30 min and cells were subsequently washed two times in FACS buffer: PBS supplemented 1% fetal calf serum, 2.5 mM EDTA and 0.1% sodium azide. Prior to acquisition, labeled cells were incubated for 1 min with propidium-iodide (Sigma), 7-AAD (Invitrogen, Bleiswijk, Netherlands) or DAPI (Molecular Probes, Bleiswijk, Netherlands) at the final concentration of 1 μg/ml and washed with FACS buffer. Ly6G-PE, Ly6C-FITC/biotin antibodies were purchased from BD Pharmingen, Breda, Netherlands. Antibodies against CD4-FITC, CD86-FITC, CD8α-PE, CD40-PE, CD31-PECy7, CD11b-PeCy5/-PerCPCy5.5/-PECy7, CD45R(B220)-FITC/-PE/-PECy7, CD11c-PETxRed/-APC/-APCCy7, MHC class II-PE, F4/80-FITC/-APCCy7/-APC, CD16/32-AF700, CD45-PacificBlue and CD206-APC were purchased from eBioscience Hatfield, UK. Polyclonal anti-Ctcf-biotin antibody (http://antibodies-online.com) was used for intracellular detection of Ctcf protein. Biotinylated antibodies were detected by streptavidin-PacificBlue (eBioscience, Hatfield, UK).

In freshly isolated spleen, peritoneal wash or liver cells, leukocytes were defined as follows: lymphocytes ($CD11b^-$ $CD11c^-$ and $B220^+$, $CD4^+$ or $CD8^+$), neutrophils (SSC^{lo} $CD11b^{hi}Ly6G^+$), monocytes ($SSC^{lo}CD11b^{hi}Ly6G^-Ly\text{-}6C^+$), myeloid DC ($B220^-CD11c^{hi}$) and macrophages ($CD11c^{low}$ $CD11b^{hi}F4/80^+$). Detection of the LacZ reporter was performed using fluorescein-di-β-D-galactopyranoside substrate (Invitrogen) as previously described.[25] Samples were acquired on LSR-II or Calibur (BD Bioscience, Breda, Netherlands) and analyzed using FlowJo software (TreeStar, Olten, Switzerland).

Cell cultures

In vitro differentiation of bone marrow (BM) cells into macrophages was performed using 10% L929 cell culture medium (conditioned medium), as previously described.[26] Briefly, BM cells were isolated and seeded in a petridish (Sarstedt, Etten-Leur, Netherlands) at 0.5×10^6 cells/ml, in a volume of 8 ml. At

day 4, 10 ml conditioned medium was added. On day 7, adherent cells were harvested. Purity of the F4/80$^+$CD11b$^+$ cells was always >85%. Next, 0.5×10^6/ml BM derived macrophages were stimulated with lipopolysaccharide (LPS) (100 ng/ml, from *S. Minnesota* (Invivogen) or *E. coli* 026:B6 (Sigma)), R848 (1 μg/ml; Alexis, Antwerp, Belgium) or CpG-1668 (5 μg/ml; Invitrogen). Following overnight incubation, supernatants were harvested and measured by ELISA for IL-10, IL-6, TNF and IL-12p40 (eBioscience) according to manufacturers' protocol.

Multilamellar liposomes labeled with DiI in the aqueous phase were prepared as described previously.[27,28] Liposomes consisted of phosphatidyl choline and cholesterol in a 6 : 1 molar ratio. After washing, the liposomes were resuspended in PBS. For the *in vitro* phagocytosis test, DiI-liposomes (1%) were added to the macrophage cultures, and labeled cells were detected using FACSCalibur.

Protein analysis by western blotting

For western blotting, macrophages were lysed with 2× Laemmli buffer (whole-cell extracts), and nuclear extracts of cultured cells were isolated as described before.[29] Polyclonal anti-Ctcf antibody (Bioke, Leiden, Netherlands) and anti-RCC1 (Santa Cruz, Heidelberg, Germany) was incubated overnight at 4 °C in Tris-buffered saline containing 5% bovine serum albumin and 0.15% (v/v) NP-40. Blots were incubated with secondary goat anti-rabbit antibody coupled to horseradish peroxidase (1 : 50 000; GE Healthcare UK Ltd, Buckinghamshire, UK). Signal detection was performed using ECL (Amersham, Buckinghamshire, UK).

Immunohistochemistry

Liver was fixed in 4% formaldehyde or snap-frozen after removal. Tissue was embedded in paraffin or TissueTek and fixed with cold acetone for 2 min. For paraffin embedded tissue, F4/80 and Ctcf antigens were retrieved by proteinase K and TE buffer, respectively. Endogenous peroxidase activity was removed by 20 min incubation with 0.3% H_2O_2. Tissue sections were further blocked with 10% rabbit serum and 5% bovine serum albumin in PBS, 0.1% avidin and 0.01% biotin (DAKO, Heverlee, Belgium) consecutively for 15 min for each blocking step. Next, tissue sections were incubated with the primary antibody (F4/80, Ctcf or MHC II), with or without biotin conjugated-rabbit-anti-rat Ig (DAKO) and streptavidin HRP (DAKO) or goat-anti-rabbit HRP for 1 h with proper washing after each step. The staining was visualized using diaminobenzidine (Invitrogen), and counterstained with hematoxylin (Sigma). Digital images of four randomly selected high-power fields (×20 magnification) were captured using NIS-Elements D 3.0 software (Nikon Digital Sight DS-U1). The average of the number of MHC II and F4/80-positive cells from four high-power field was determined and expression of MHC II was graded as 1 (<20 positive cells) until 4 (>120 positive cells).

Isolation of RNA, generation of cDNA, quantitative PCR and gene expression analysis

RNA was isolated using the Total RNA purification kit (Ambion; Life Technologies, Bleiswijk, Netherlands) or NucleoSpin RNAII kit (Bioke) as described in the manufacturer's protocol. The quantity and quality of RNA were determined using a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, USA). Total RNA (0.5–1.0 μg) was used as a template for cDNA synthesis by iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Veenendaal, Netherlands) or Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. Quantitative real-time PCR was performed using the Bio-Rad optical 96-well plates with a MyIQ5 detection system (Bio-Rad Laboratories) or ABI Prism 7700 sequence detection system (Applied Biosystems). The probe in the master mix (TaqMan Gene Expression Master Mix) was an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye. Primers for housekeeping gene 18S (Hs99999901_s1), TLR4 (Mm00445274_m1), TLR8 (Mm01157262_m1) and TLR9 (Mm00446193_m1) were purchased from Applied Biosystems. The nucleotide sequences of other primers used are listed in Supplementary Table 1. The expression of genes was normalized to 18S or GAPDH.

For microarray gene expression analysis, labeling and hybridization with GeneChip MouseGene 1.0 ST arrays was performed according to the manufacturer's protocol and scanned with Affymetrix GeneChip Command Console software. In total, eight arrays were analyzed (five Ctcf-knockout and three wild-type samples from three independent experiments). Data were filtered using a multistep filtration method, which involves the application of receiver operating characteristic analysis for the estimation of cutoff signal intensity values. Only probe set identifiers having gene assignments (annotation date: 21 July 2008; Affymetrix, Santa Clara, USA) were used for analysis. A relative gene expression value was calculated by normalization to the median expression value for the gene across samples. Efron-Tibshirani's test uses 'maxmean' statistics to identify gene sets differentially expressed. The threshold of determining significant gene sets was set to 0.005.

Data analysis and statistics

For all experiments, the difference between groups was calculated using the Mann–Whitney *U* test or Wilcoxon *t*-test for unpaired data (GraphPad Prism version 4.0; GraphPad Software, La Jolla, USA). Differences were considered significant when $P<0.05$. Results are presented as the mean±s.e.m., unless otherwise indicated.

RESULTS

Deletion of *Ctcf* gene in the macrophage subpopulations

To examine the role of Ctcf in the myeloid cell lineage *in vivo*, we crossed mice carrying a *Ctcf*-floxed allele[18,23] with *LysM-Cre* transgenic mice, which express the *Cre* recombinase under the control of the LysM promoter,[18,23] thereby confining *Ctcf* gene deletion to myeloid cells.

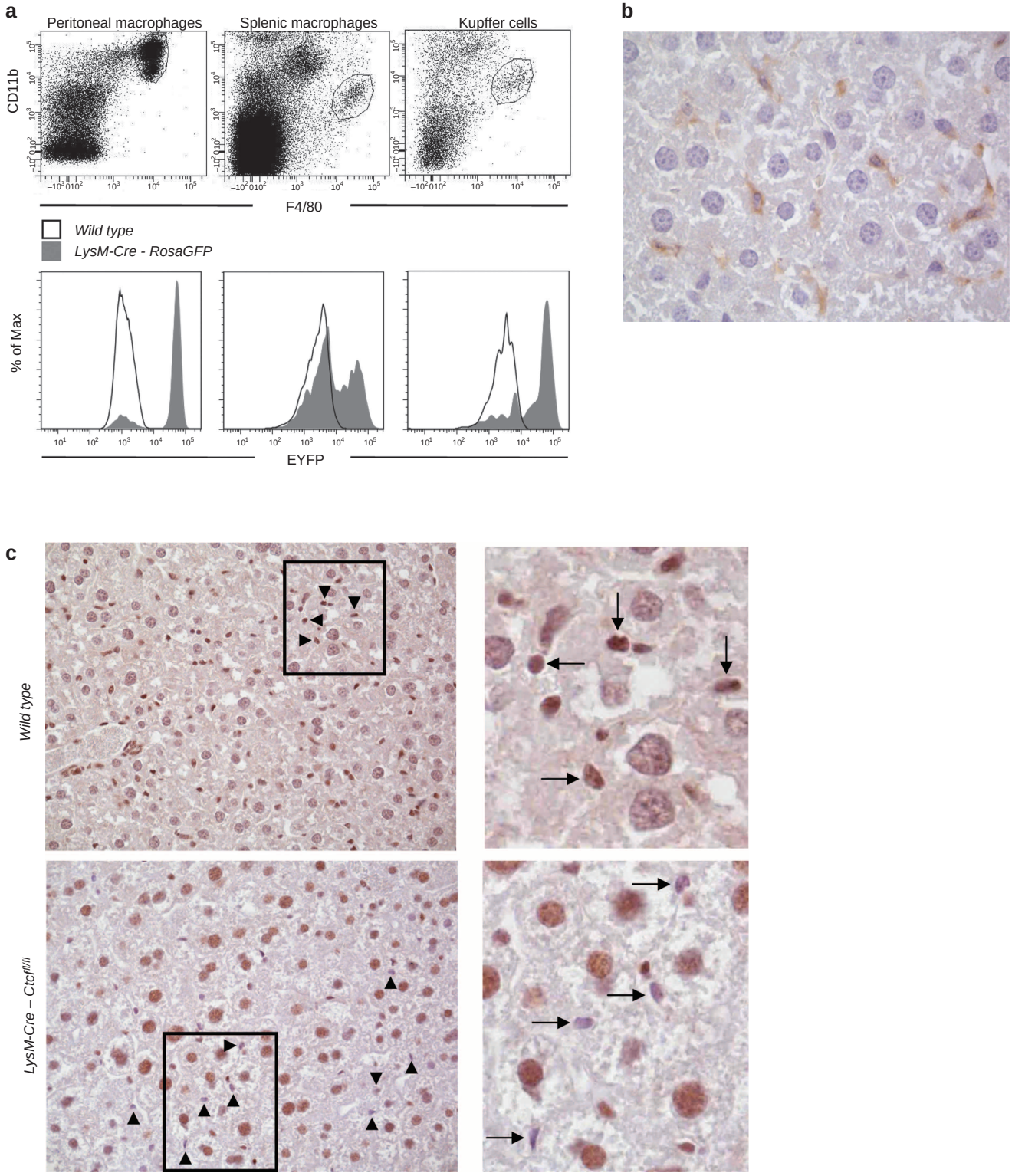

Figure 1 LysM promoter is active in macrophages and drives *Ctcf* deletion in macrophages in *LysM-Cre Ctcf* $^{f/f}$ mice. (**a**) Representative flow cytometric plot and histogram to visualize the activity of LysM promoter in LysM-Cre Rosa-EYFP mice. Peritoneal and splenic macrophages, and Kupffer cells were identified as $CD11c^{low}CD11b^{+}F4/80^{+}$ cells. EYFP expression results from LysM-driven Cre-recombinase deletion of 'floxed-stop' fragment upstream EYFP. Flow cytometric analysis shows that ~80%, ~35% and ~70% of peritoneal and splenic macrophages, and Kupffer cells, respectively, are $EYFP^{+}$, indicative for LysM activity in these macrophage populations. (**b**) Representative F4/80 and hematoxylin stainings of the liver of wild-type animals. The nuclei of hepatocytes are characterized by their large size and round shape. Additionally, small and elongated nuclei, of which ~70% are associated with F4/80 expression, are observed. (**c**) Representative nuclear Ctcf and hematoxylin stainings of the livers of wild-type and *LysM-Cre Ctcf* $^{f/f}$ animals. Nuclear expression of Ctcf, observed as brown staining, is weaker in the non-hepatocyte cells of *LysM-Cre Ctcf* $^{f/f}$ compared to the wild-type animals. Ctcf, CCCTC-binding factor; EYFP, enhanced yellow fluoresencent protein; LysM, lysozyme M.

First, we confirmed that the *LysM-Cre* transgene is functionally expressed in various macrophage populations, using a mouse Cre-reporter strain harboring a targeted insertion of EYFP into the ROSA26 locus.[24] We found substantial EYFP expression in $CD11b^+F4/80^{high}$ peritoneal and splenic macrophages, as well as in $CD11b^+F4/80^{high}$ Kupffer cells in the liver, although in all of these compartments EYFP-negative cells were also present (Figure 1a).

Homozygous *LysM-Cre Ctcf$^{f/f}$* mice appeared normal and were fertile and born at the expected frequencies on the basis of Mendelian inheritance. Deletion of the *Ctcf* gene was monitored by the expression of the bacterial β-galactosidase (*lacZ*) reporter present in the floxed *Ctcf* allele.[18] As expected,[23] we found *lacZ* expression, detected by fluorescein-di-β-D-galactopyranoside in conjunction with cell-specific surface markers, in substantial fractions of myeloid cell populations, including granulocytes, monocytes and macrophages of *LysM-Cre Ctcf$^{f/f}$* mice (not shown). To assess whether deletion of the *Ctcf* allele resulted in the lack of Ctcf protein expression, we performed immunohistochemical analyses in the liver. Kupffer cells can be identified by expression of the F4/80 markers and differ from hepatocytes present in the liver by their smaller and more elongated cell nucleus (Figure 1b). When we analyzed expression of Ctcf, we noticed that Kupffer cells manifested a dense nuclear staining, whereas hepatocytes show a less intense nuclear staining (Figure 1c). Immunohistochemical analysis of liver specimens from *LysM-Cre Ctcf$^{f/f}$* mice demonstrated that Kuffper cells were still present in apparently normal frequencies. In a large fraction of Kupffer cells, the expression of Ctcf was lost, although also Ctcf-expressing Kupffer cells were detected (Figure 1c).

To assess whether deletion of *Ctcf* influenced the size of the macrophage compartment, we used flow cytometry to compare the proportions of individual myeloid subpopulations in peritoneum and spleen. In the peritoneal cavity of *LysM-Cre Ctcf$^{f/f}$* mice the proportions of macrophages were moderately reduced and the proportions of lymphocytes and myeloid DC were increased, compared with wild-type controls (Supplementary Figure 1a). In the spleen of *LysM-Cre Ctcf$^{f/f}$* mice, we observed a reduced frequency of monocytes, but there were no significant differences in frequencies of macrophages, when compared with wild-type controls (Supplementary Figure 1b).

Taken together, although *LysM*-promoter mediated *Cre* expression resulted in deletion of the *Ctcf* gene in a substantial proportion of macrophages, Ctcf deficiency had only moderate effects on the frequencies of these cell populations in peritoneum, spleen and liver of *LysM-Cre Ctcf$^{f/f}$* mice.

Reduced MHC class II expression in the liver of *LysM-Cre Ctcf$^{f/f}$* mice

Previous studies indicated that Ctcf plays an important role in regulation of MHC class II expression in human B cells.[30] Despite significant deletion of Ctcf (Figure 1), flow-cytometric analyses of peritoneal or splenic macrophages did not show evidence for reduced surface MHC class II expression in *LysM-Cre Ctcf$^{f/f}$* mice, compared with wild-type littermates (Figure 2a). In contrast, flow cytometric and histological analysis of the liver of *LysM-Cre Ctcf$^{f/f}$* mice demonstrated substantial reduction of MHC class II expression (Figure 2a and b). Quantification of MHC class II expression in histological samples of the liver showed a highly significant reduction in gene-targeted mice, compared with the control mice (Figure 2c, $P<0.0001$). Since the majority of MHC class II-expressing cells in the liver are $F4/80^+$ Kupffer cells, we assessed their numbers and observed that the proportion of F4/80-expressing cells was not affected (Figure 2b and c), demonstrating that deletion of Ctcf leads to lower levels of expression of MHC class II and not to deletion of MHC class II-expressing Kupffer cells.

These findings indicate that Ctcf plays an important role in the regulation of MHC class II expression in Kupffer cells, but not in the other macrophage populations analyzed.

Reduced *in vitro* macrophage differentiation from *LysM-Cre Ctcf$^{f/f}$* BM

To study the effects of Ctcf on macrophage activation and function, we generated macrophages by *in vitro* differentiation from BM precursors using L-929 conditioned medium (Figure 3a). The yield of $CD11b^+F4/80^+$ macrophages from total BM of *LysM-Cre Ctcf$^{f/f}$* mice generated after 7 days of culture was significantly reduced compared with wild-type littermates mice ($P<0.05$; Figure 3bB). Analysis of *Ctcf* mRNA expression by quantitative RT-PCR showed a reduction to ~33% of wild-type levels ($n=6$), which was also reflected by substantial reduction of Ctcf protein as analyzed in western blotting experiments (Supplementary Figure 2). Since we observed efficient Ctcf deletion by *lacZ* expression in mature macrophage populations *in vivo* (not shown), these findings point to a long half-life of Ctcf protein or a specific survival of Ctcf-expressing cells.

LysM-Cre Ctcf$^{f/f}$ and wild-type macrophages did not differ in surface expression of lineage-associated markers F4/80 and CD11b (Figure 3a) or the activation markers CD86 and MHC class II (Figure 3c and d). We observed lower CD206 and CD16/32 expression on *LysM-Cre Ctcf$^{f/f}$* than on wild-type macrophages, but these differences were not statistically significant. However, surface CD40 expression was significantly reduced on *LysM-Cre Ctcf$^{f/f}$* macrophages, when compared with wild-type macrophages, both unstimulated and upon LPS stimulation (Figure 3c and d).

We found that BM-derived macrophages from *LysM-Cre Ctcf$^{f/f}$* mice and wild-type mice had a similar capacity to phagocytose DiI-labeled liposomes (Figure 3e). Finally, the viability of *LysM-Cre Ctcf$^{f/f}$* and wild-type macrophages was similar, both with and without LPS stimulation (not shown).

Therefore, the absence of Ctcf was associated with reduced *in vitro* differentiation of BM-derived macrophages, both in cell numbers and in terms of surface CD40 expression.

LysM-Cre Ctcf$^{f/f}$ macrophages exhibit reduced TLR-induced IL-10 and TNF production

Next, we analyzed the ability of *LysM-Cre Ctcf$^{f/f}$* macrophages to produce cytokines upon induction by different TLR

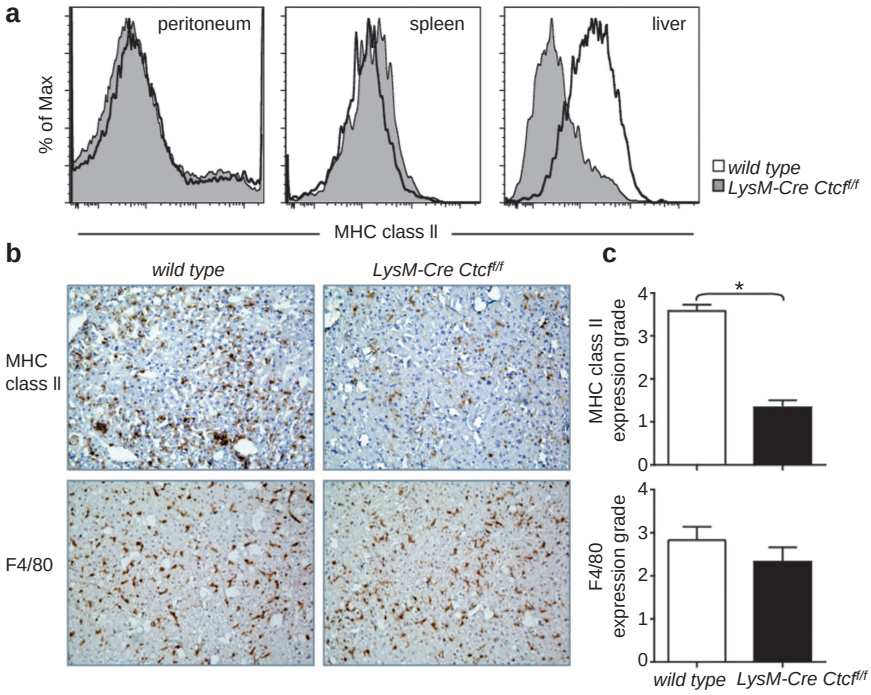

Figure 2 *Ctcf* deletion results in lower MHC class II expression on Kupffer cells in the liver. (**a**) Representative histograms showing MHC class II expression by macrophages in peripheral organs. Gray histograms represent *LysM-Cre Ctcf* $^{f/f}$ and black-line histograms represent wild-type macrophages. Total non-parenchymal cells from the liver were isolated as described in the section on 'Materials and methods'. Macrophages in the liver were identified as $CD45^{+}CD11c^{low}CD11b^{+}F4/80^{high}$. The staining was performed on at least five wild-type and five *LysM-Cre Ctcf* $^{f/f}$ mice with similar results. (**b**) Representative immunohistochemical staining for MHC class II and F4/80 on liver tissue sections from wild-type and *LysM-Cre Ctcf* $^{f/f}$ mice. MHC class II expression (upper panel) of *LysM-Cre Ctcf* $^{f/f}$ mice was lower than in wild-type mice, while the expression of $F4/80^{+}$ cells was similar (lower panel). The staining is representative of 9 wild=type and 9 *LysM-Cre Ctcf* $^{f/f}$ mice. (**c**) Quantitative analysis of MHC class II and F4/80 expression. Liver tissue sections from nine *LysM-Cre Ctcf* $^{f/f}$ and nine wild-type mice were scored (1–4) for the degree of MHC class II and F4/80-positive staining. The grading for MHC class II positivity, but not for F4/80, in the livers of *LysM-Cre Ctcf* $^{f/f}$ mice was significantly lower than in wild-type livers ($P<0.0001$). Ctcf, CCCTC-binding factor; MHC, major histocompatibility complex.

agonists. Upon stimulation with LPS and R848, *LysM-Cre Ctcf*$^{f/f}$ macrophages produce lower levels of IL-10 than did wild-type macrophages ($P<0.05$), whereas upon CpG stimulation differences in IL-10 production were not significant (Figure 4). Stimulation with all three distinct TLR agonists showed a significant reduction of TNF production by macrophages from *LysM-Cre Ctcf*$^{f/f}$ mice, when compared with controls ($P<0.05$). In contrast, the production of IL-12p40 or IL-6 by *LysM-Cre Ctcf*$^{f/f}$ or wild-type macrophages was similar upon TLR ligation (Figure 4).

The IL-10 family of cytokines includes IL-19, IL-20 and IL-24. The genes encoding these cytokines are all located in a cluster together with the IL-10 gene on chromosome 1q31-32 [31,32] (Figure 5a). In parallel to the Th2 cytokine locus,[19] we hypothesized that also the IL-10 locus may contain several Ctcf sites, which would enable long-range chromatin interactions between regulatory elements and promoter regions for the individual cytokine genes. Since Ctcf-binding sites are generally common to different cell types,[33,34] we made use of our reported dataset of Ctcf-binding sites identified in cultured primary pre-B cells by chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-Seq).[22] We found that the IL-10 locus contains several Ctcf binding sites, which are not located in the promoter regions of individual cytokine genes, but rather between the cytokine genes (Figure 5a). This would be consistent with a role for Ctcf in looping and enabling long-range DNA interactions that are essential for coordinated expression of the individual genes in the IL-10 locus.

We therefore set out to determine whether besides IL-10 protein production, also transcription levels of the *Il10* gene and the closely linked *Il19*, *Il20* and *Il24* genes were reduced in *LysM-Cre Ctcf*$^{f/f}$ macrophages upon TLR ligation. In line with the observation at the protein level, IL-10 mRNA expression was reduced in *LysM-Cre Ctcf*$^{f/f}$ macrophages, particularly upon R848 stimulation ($P<0.05$; Figure 5b). Interestingly, *LysM-Cre Ctcf*$^{f/f}$ macrophages exhibit lower expression of the other three IL-10 locus cytokines as well, whereby significance was reached for IL-19 upon R848 stimulation ($P<0.05$) and for IL-20 upon LPS or CpG stimulation ($P<0.05$; Figure 5b). The observed reduced cytokine production in Ctcf-deficient macrophages cannot easily be explained by an effect of Ctcf on TLR expression, as *LysM-Cre Ctcf*$^{f/f}$ BM-derived macrophages did not manifest reduced levels of Tlr4, Tlr8 or Tlr9 mRNA (Supplementary Figure 3).

Taken together, these data show that the absence of Ctcf in macrophages does not appear to affect their capacity to

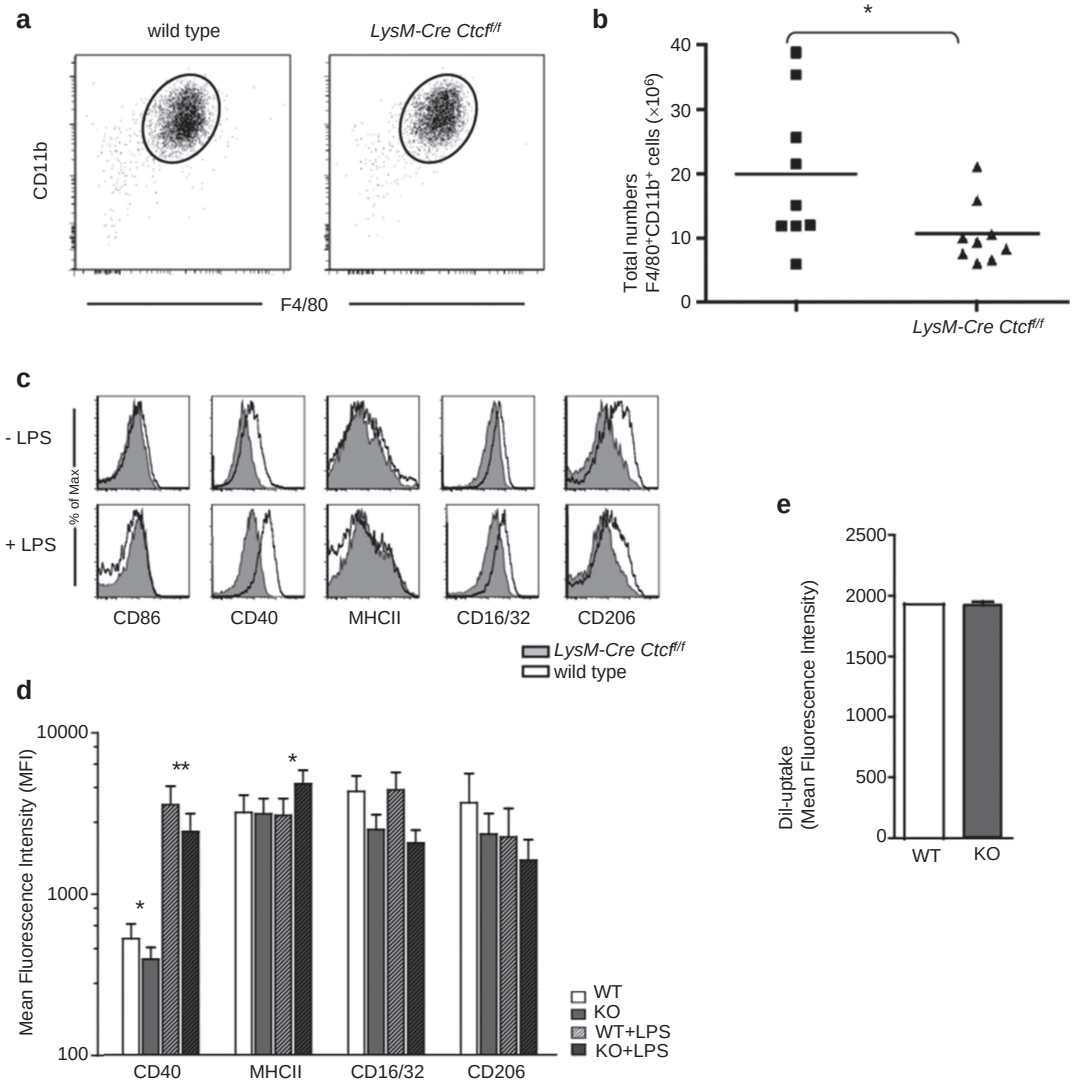

Figure 3 *Ctcf* deletion impairs *in vitro* macrophage differentiation from bone marrow cells. (**a**) FACS plots that define bone marrow derived macrophages as $CD11b^+F4/80^+$ cells. Cultured macrophages from *LysM-Cre Ctcf* $^{f/f}$ mice express similar levels of CD11b and F4/80 as wild-type macrophages. The assay was performed with cells from nine wild-type and nine *LysM-Cre Ctcf* $^{f/f}$ mice with similar results. (**b**) Macrophages were generated *in vitro* as described in Materials and Methods and the cell yields were determined at the end of the culture (day 7). The number of macrophages derived from *LysM-Cre Ctcf* $^{f/f}$ mice was significantly lower than from wild-type mice (P=0.0235). (**c**) Representative histograms of CD86, CD40, MHC class II, CD206 and CD16/32 expression by cultured macrophages from wild-type (black line histogram) and *LysM-Cre Ctcf* $^{f/f}$ mice (gray histogram) unstimulated (upper row) or after LPS stimulation (lower row). (**d**) Average MFI expression of analyzed molecules in wild-type and *LysM-Cre Ctcf* $^{f/f}$ (KO) macrophages before or after stimulation with LPS. Data are presented as average MFI±s.e.m. from seven wild-type and 10 *LysM-Cre Ctcf* $^{f/f}$ mice. *P<0.05, **P<0.01. (**e**) Phagocytosis capacity of cultured macrophages was measured by incubating them overnight DiI-liposomes. Uptake of liposomes was quantified by flow cytometry. Data represent MFI±s.d. from two independent cultures from each genotype. Ctcf, CCCTC-binding factor; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MHC, major histocompatibility complex.

produce IL-6 or IL-12, but is associated with reduced expression of TNF-α and IL-10 locus cytokines upon stimulation with various TLR ligands.

Gene expression profiling in BM-derived *LysM-Cre Ctcf* $^{f/f}$ macrophages upon LPS stimulation

Finally, we aimed to investigate the effect of *Ctcf* deletion in macrophages in a genome-wide fashion. To this end, we performed gene expression profiling of *LysM-Cre Ctcf* $^{f/f}$ and wild-type macrophages upon overnight LPS stimulation. Of 23 500 detected genes, 617 genes (212 up and 405 down) were differentially expressed between *LysM-Cre Ctcf* $^{f/f}$ and wild-type macrophages (threshold of twofold). The 100 most downregulated and 100 most upregulated genes are shown in Table 1. As expected, *Ctcf* was among these most downregulated genes. We analyzed the expression of genes that were downregulated in Ctcf-deficient macrophages as compared to wild-type macrophages. These downregulated genes included CCL8 and CCL12 (also known as monocyte chemotactic protein-2 and monocyte chemotactic protein-5 and Cxcl10 (also known as IP-10),

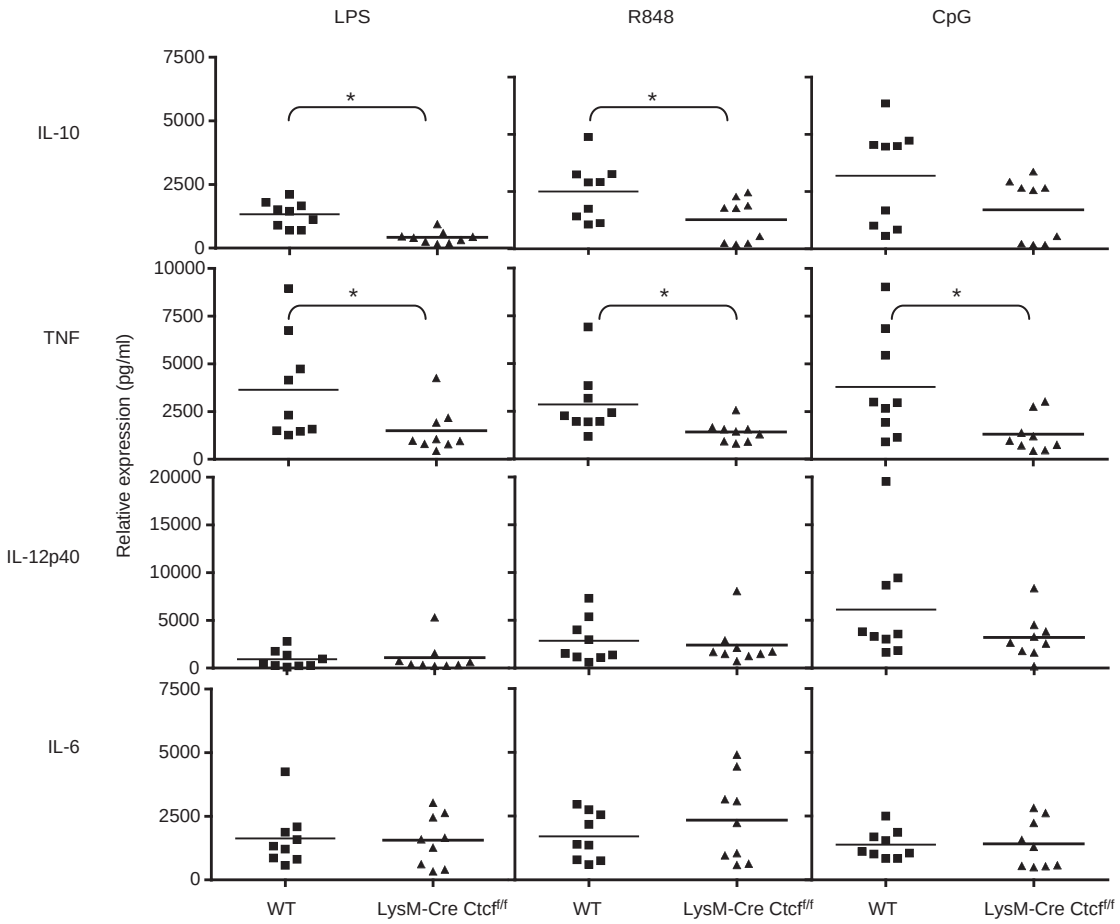

Figure 4 Impaired cytokine induction by bone marrow-derived macrophages from *LysM-Cre Ctcf* $^{f/f}$ mice upon TLR4, TLR7/8 and TLR9 ligation. 1×10^5 bone marrow-derived macrophages from wild-type and *LysM-Cre Ctcf* $^{f/f}$ mice were stimulated with LPS, R848 and CpG for 24 h. The levels of IL-10, TNF, IL-12p40 and IL-6 in supernatant were measured by ELISA. Data show concentrations of produced cytokines (pg/ml). The experiments were performed using independent cultures from nine wild-type and nine *LysM-Cre Ctcf* $^{f/f}$ mice. Ctcf, CCCTC-binding factor; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; TLR, Toll-like receptor; TNF: tumor-necrosis factor.

which are chemotactic for and activate numerous immune cells, such as monocytes, T cells and natural killer cells. In addition, genes involved in antibacterial (Nos2) or antiviral responses (Mx1, Mx2, ISG20 and Rsad2) were strongly downregulated, suggesting weaker responses to eliminate pathogens. Furthermore, we observed reduced expression of molecules known to negatively regulate immunity, including IL1rn, cd274 (PD-1 ligand) and fgl-2 (fibrinogen-like protein 2), which may have the same functional consequence as the lower levels of the immunosuppressive cytokine IL-10 as we observed following stimulation with LPS or R848. We also found reduced expression of the nitric oxide synthetase Nos2, which catalyzes the production of nitric oxide and contributes to the anti-microbial or anti-tumor function of macrophages as part of the oxidative burst.

Many of the genes that were significant upregulated in Ctcf-deficient macrophages, including meiotic nuclear divisions 1 homolog (Mnd1, ~14×), the CD69 antigen (~13×) and integral membrane protein 2a (Itm2a, ~9×), were previously found to be controlled by Ctcf in precursor B cells (Mnd1, CD69, Itm2a)[22] or during mammalian limb development (Mnd1, Itm2a)[35] and may reflect direct cell-lineage independent targets of Ctcf. Other genes are expected to be more indirectly regulated by Ctcf, e.g., the sugar transport facilitator Slc2a3 (~11.5×), perhaps through crosstalk between the Ctcf-regulated imprinted growth demand gene *Igf2*.[36] Among genes upregulated in Ctcf-deficient macrophages, were also genes with a more macrophage-specific function, including the 5-lipoxygenase (Alox5, ~7×) enzyme that is involved in the generation of leukotriens that enhance phagocytosis and NADPH oxidase Nox1 (~5×), an important source of reactive oxygen species in macrophages. In addition, Ctcf-deficient macrophages expressed, e.g., higher levels of the chemokine receptor CX3CR1 (~3×), which is known to regulate intestinal macrophage homeostasis[37] and phosphodiesterase Pde2a (~3×), which is induced during macrophage colony-stimulating factor differentiation of macrophages.[38]

DISCUSSION

Ctcf has been identified as an important regulator of long-range chromatin interactions in lymphocytes,[4] but the role of Ctcf in macrophages cells has not been investigated. Macrophages are crucial cells in immune responses to bacteria

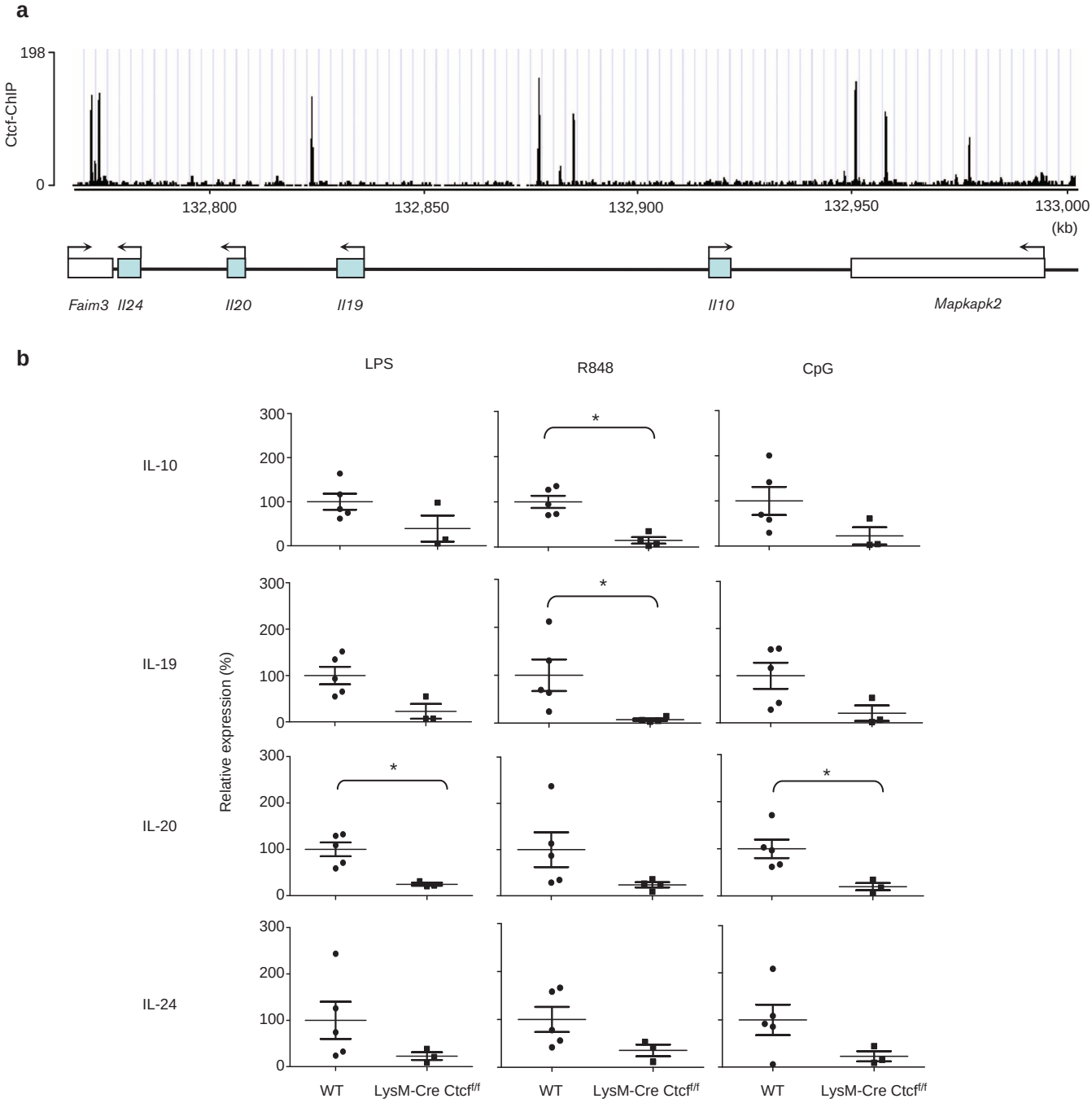

Figure 5 Impaired TLR-induced mRNA expression of IL-10 family members in bone marrow derived macrophages from *LysM-Cre Ctcf* $^{f/f}$ mice. (**a**) Mouse genomic region containing the IL-10 gene family locus. Ctcf ChIP-Seq data are depicted above the localization of the IL-10 family (IL-10, IL-19, IL-20 and IL-24) and flanking genes. ChIP-Seq data were obtained from cultured pre-B cells.[21,22] kb=kilobases. (**b**) Bone marrow derived macrophages from wild-type and *LysM-Cre Ctcf* $^{f/f}$ mice were stimulated for 5 h with LPS, R848 or CpG. IL-10, IL-19, IL-20 and IL-24 mRNA expression in macrophages were quantified by real time PCR, and expressed relative to GAPDH. The values depicted show of 3–5 mice per experimental group. Statistical analysis: unpaired *t*-test, $*P<0.05$. ChIP-Seq, chromatin immuno-precipitation coupled to high-throughput sequencing; Ctcf, CCCTC-binding factor; IL, interleukin; LPS, lipopolysaccharide; TLR, Toll-like receptor.

and viruses, and for removing apoptotic cells or cellular debris. Triggering of macrophages *via* a broad array of pattern recognition receptors is the initiating step, and this quickly leads to uptake of the pathogen and debris, and the release of effector molecules, such as cytokines. This study describes for the first time the effect of specific Ctcf deletion in macrophages in mice. *In vivo*, deletion of Ctcf resulted in a mild phenotype. Furthermore, there was a strongly reduced expression of MHC class II in the liver of *LysM-Cre Ctcf*$^{f/f}$ mice, and to a lesser extent in the spleen. Finally, macrophages generated *in*

Table 1 Genes modulated in Ctcf-deficient macrophages

Genes downregulated in Ctcf-deficient macrophages				*Genes upregulated in Ctcf-deficient macrophages*			
Gene	*Ratio*	*Gene*	*Ratio*	*Gene*	*Ratio*	*Gene*	*Ratio*
Lipg	0.072	Cmpk2	0.269	Uchl1	16.58	Cldn12	3.41
Ccl8*	**0.073**	**Cxcl11**	**0.270**	**Mnd1**	**13.71**	Clec4a2	3.40
Htr2b	0.130	Phf11	0.271	**Cd69**	**12.77**	Ptpla	3.39
Nos2	0.136	Serpinb1c	0.277	Slc2a3	11.50	Ephx1	3.37
Iqgap2	0.156	Timp1	0.280	**Itm2a**	**9.22**	Klhl6	3.36
C1rb	0.161	Ctsk	0.287	Dner	8.96	Fbxl21	3.34
Gbp5	0.167	Fabp5	0.292	Fam171b	7.50	Dusp6	3.34
Dhfr	0.168	Rhoc	0.294	**Alox5**	**7.28**	Fh1	3.32
Ctcf	**0.178**	Ifi205	0.297	Sqle	7.21	Ccr1	3.32
Ednrb	0.179	Ppap2b	0.299	Clec4b1	6.80	Pgm2l1	3.32
Thbs1	0.183	Treml2	0.299	Opn3	6.52	Apol7c	3.31
Cd300lf	0.184	**Cd274**	**0.300**	Ptrf	6.11	H2-M2	3.29
Slfn1	0.189	Csprs	0.302	Prps2	5.75	Tfrc	3.27
Mertk	0.195	Plk2	0.308	Asrgl1	5.53	Clec7a	3.26
Fgl2	**0.201**	Ccdc99	0.311	Emb	5.07	Ppap2a	3.22
Mmp27	0.205	Nupr1	0.312	**Nox1**	**5.05**	Zswim7	3.21
Dcn	0.206	Tnfrsf26	0.313	Slc25a4	4.96	Padi2	3.21
Slc40a1	0.213	Nr1d2	0.313	Slc35e3	4.95	Ahi1	3.20
Lrrc14b	0.214	Csf3r	0.316	Glt25d2	4.94	Ccl22	3.20
Trib3	0.215	E430029J22Rik	0.320	Ppbp	4.81	Gmds	3.20
Ch25h	0.223	Gbp6	0.320	C1s	4.77	Fscn1	3.20
Kcnab1	0.226	Sectm1a	0.320	Anp32e	4.71	Gprc5c	3.19
Slc28a2	0.229	Tgtp	0.320	Clec4n	4.62	Rgs18	3.18
Ccrl2	0.229	Mxd1	0.321	Vcan	4.58	Zdhhc2	3.16
Vegfa	0.229	Stoml1	0.322	Htra1	4.39	Prune2	3.16
Tspan13	0.231	Il1rn	0.322	L1cam	4.25	Prkar2b	3.14
Soat2	0.239	Gbp1	0.323	Gpr183	4.23	Nme1	3.14
Slco3a1	0.242	Slamf7	0.324	Clec2i	4.22	Ccna2	3.12
Gprc5b	0.243	Igsf9	0.326	Gbgt1	4.11	Gria3	3.10
Ifitm6	0.244	**Ccl12**	**0.326**	Fads3	4.07	Cep78	3.10
Ddit3	0.248	Nt5c3	0.331	Uck2	4.03	Adrb2	3.07
Gbp2	0.249	Mthfd2	0.331	Zcwpw1	3.86	Mthfd1	3.06
Tmem140	0.249	EG634650	0.334	Nme4	3.73	Acot7	3.06
Tmod1	0.253	Mpa2l	0.335	Gcsh	3.72	Tomm20	3.06
Gadd45b	0.253	**Cxcl10**	**0.337**	Rasgrp3	3.69	Igf2bp3	3.05
Cd5l	0.254	Olfr1444	0.339	**Myc**	**3.68**	Rhof	3.01
Hal	0.256	**Isg20**	**0.342**	Lifr	3.67	Dock1	3.00
Klra3	0.257	Clec2d	0.342	Nudt15	3.67	Pik3cg	3.00
Rhov	0.260	Iigp1	0.343	Fads2	3.63	Paf1	2.99
Gtpbp2	0.261	Plac8	0.343	Scd1	3.62	Lpl	2.98
Hyal1	0.262	Tsc22d3	0.344	Emr4	3.58	Mettl1	2.98
Gadd45a	0.264	Tmem26	0.346	Vcl	3.51	Fchsd2	2.97
Sp140	0.264	Carhsp1	0.347	Ebpl	3.51	Gnai1	2.97
Mx1	**0.265**	S100a1	0.349	Grk4	3.51	Pde2a	2.97
Tlr6	0.266	Gbp3	0.353	Cd109	3.49	**Cx3cr1**	**2.95**
Mx2	**0.266**	Sell	0.354	Pf4	3.49	Plaur	2.93

Abbreviation: Ctcf, CCCTC-binding factor.
* Bold genes are discussed in the text.

vitro from BM of *LysM-Cre Ctcf$^{f/f}$* mice showed a normal phenotype, but a significantly reduced capacity to induce the expression of IL-10 family genes, and the production of IL-10 and TNF upon stimulation with different TLR ligands, while IL-12p40 and IL-6 levels were not affected.

The importance of Ctcf in controlling MHC class II expression has been reported before.[30,39] In line with this, we found specific reduction of MHC class II expression on Kupffer cells in the liver in *LysM-Cre Ctcf$^{f/f}$* mice. Since the number of Kupffer cells in the liver was not affected, our findings corroborate the

previously established participation of Ctcf in the expression of genes within the MHC locus. However, we did not observe a change of the MHC class II expression in splenic, peritoneal or in cultured macrophages. Interestingly, three genes located within the MHC locus (*Cfb*, *Daxx* and *Tap2*) were downregulated in LPS stimulated *LysM-Cre Ctcf$^{f/f}$ versus* wild-type macrophages (ratio: 0.49, 0.43 and 0.49, respectively), suggesting that the absence of modulation of MHC class II expression could reflect defective long-range interactions in the MHC locus in the absence of Ctcf in Kuppfer cells in mice. In addition, differential expression levels of LysM in distinct tissues may also cause the differences in the observed phenotype. Reduced numbers of peritoneal and cultured macrophages as we observed in *LysM-Cre Ctcf$^{f/f}$* mice may be the result of upregulation of the proto-oncogene c-Myc compared to controls, as we observed in our microarray analysis (~3.6×; Supplementary Table 1), which is also in line with previously reported findings.[40] Overexpression of c-Myc may result in enhanced proliferation but may also induce apoptosis. Next to c-Myc, other differentially expressed genes in *LysM-Cre Ctcf$^{f/f}$* macrophages were significantly represented in cell cycle, cell death and proliferation networks, and may consequently modulate macrophage numbers. Myeloid cells downregulate Ctcf expression during maturation[41] and overexpression of Ctcf in myeloid progenitors resulted in strongly impaired development and survival of DC,[17] suggesting that a fine regulation of Ctcf expression may be important for competent myeloid cell development.

A set of genes downregulated during macrophage differentiation but induced upon TLR stimulation are the Schlafen (Slfn) genes, especially *Slfn4*.[42] In our data set, the expression levels of *Slfn1*, *Slfn4* and *Slfn9* genes were lower after LPS stimulation in *LysM-Cre Ctcf$^{f/f}$* than in control macrophages (ratio: 0.21, 0.38 and 0.38, respectively). Since we showed that the deletion of Ctcf does not significantly affect the expression levels of TLR4, TLR8 and TLR9 mRNA, and since these genes are not direct targets of Ctcf, the observed effect may have been indirectly caused through type I IFN.[42] Regulation of TNF expression by Ctcf is in agreement with the literature.[43] Additionally, we show diminished IL-10 production by macrophages as a result of Ctcf depletion, similarly to low IL-10 production upon Ctcf deletion in Th2 cells as we previously observed.[19] In macrophages, TLR4 stimulation induces IL-10 production through TRAF3 and NF-κB[26,44] and—indeed—we found downregulation of genes downstream of the NF-κB complex in *LysM-Cre Ctcf$^{f/f}$* macrophages. We found that the IL-10 gene harbors Ctcf-binding sites on both the 5′ and 3′ end in pre-B cells. These data demonstrate that Ctcf binding sites are present in the complex IL-10 cytokine locus, although it is unclear whether Ctcf binds to this locus in macrophage. Nevertheless, since Ctcf sites are relatively invariant across diverse cell types and since Ctcf has an essential role in chromatin architecture, one may appreciate the possible importance of Ctcf in the reorganization of the IL-10 locus that occurs upon TLR stimulation.[45] In line with this, our data demonstrate that reduced Ctcf expression in macrophages modulated the expression of the IL-10 homologs IL-19, IL-20 and IL-24, which are expressed within a highly conserved cytokine gene cluster. Our findings identify Ctcf as an important regulator of the IL-10 family gene locus.

In this study, we show that deletion of Ctcf in cells with active LysM in mice resulted in a mild decline in numbers of neutrophils, monocytes and macrophages in some peripheral tissues. Our observation that gene expression differences were more pronounced in macrophage cultures with lower Ctcf mRNA levels support the notion that a mild phenotype observed *in vivo* may be due to residual Ctcf protein. It is unclear what may cause possible retention of the Ctcf protein in myeloid cells. Expression of lacZ reporter demonstrated specific deletion of Ctcf in myeloid cells, but this does not exclude the possibility that in some cells incomplete deletions of *Ctcf* on both alleles may have occurred. The literature showing complete deletion of floxed transgenes using the LysM-Cre system[23,46] together with our data showing reduction of Ctcf expression down to 25% of the wild-type levels do not support this notion. We rather envisage that due to the fast turnover of myeloid cells in mice leading to a short time between the deletion event and analysis combined with a long half-life of the Ctcf protein in cells could underlie the observed differences in residual Ctcf protein levels.

Macrophages of *LysM-Cre Ctcf$^{f/f}$* mice demonstrated normal phagocytosis capacity and production of inflammatory cytokines IL-12 and IL-6, but decreased production of the cytokines TNF and IL-10, pointing to a confined change in functionality as a result of deletion of Ctcf. Our Ctcf ChIP-seq analysis provided evidence for strong Ctcf binding in the ~10 kb region encompassing *Tnf*, *Lta* (lymphotoxin-α) and *Ltb* genes, as well as in the loci encoding IL-6 and IL-12p40 (RWH, unpublished). Further experiments are required to investigate if Ctcf acts as direct regulator of the *Tnf-Lta-Ltb* locus or why Ctcf does not appear to regulate expression of the *Il6* or *Il12b* genes. Implications of these findings may be important in pathological conditions to influence production of cytokines without affecting other macrophage functions. Regulation of IL-10 or TNF produced by macrophages could improve immunotherapy of tumors by reducing unwanted IL-10 production and induction of immunosuppressive macrophages.[47,48] Likewise, controlled induction of IL-10 or TNF in diabetes could improve wound healing or the regulation of the autoimmune response.[49,50]

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Supplementary Information accompanies the paper on *Cellular & Molecular Immunology* website.

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