CTCF: A Crucial Regulator of Gene Expression in Lymphocytes

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CTCF: een cruciale regulator van genexpressie in lymfocyten

Thesis

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Table of contents

LIST OF ABBREVIATIONS

3C-Seq	Chromosome Conformation Capture coupled to high-throughput Sequencing
7-AAD	7-Amino-actinomycin D
BCR	B-cell receptor
BM	Bone marrow
Bp	base pairs
BrdU	Bromodeoxyuridine
CFSE	Carboxy Fluorescein Succinimidyl Ester
ChIP-Seq	Chromatin Immuno Precipitation coupled to high-throughput Sequencing
CTCF	CCCTC-binding factor
DC	Dendritic cell
DN	Double Negative
DP	Doule Positive
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescent Activated Cell Sorting
FDG	Fluorescein-di-β-D-galactopyranoside
FISH	Fluorescent *in situ* hybridization
GFP	Green Fluorescent Protein
HS	Hypersensitive Site
ICR	Imprinting Control Region
IFN-γ	Interferon-γ
IgH/Igμ	Immunoglobulin heavy-chain
IgL	Immunoglobulin light-chain
Igκ	Immunoglobulin kappa light-chain
Igλ	Immunoglobulin lambda light-chain
IL	Interleukin
ISP	Immature Single Positive
(c)KO	(conditional) knock-out
LacZ	Bacterial β-galactosidase
LCR	Locus Control Region
LN	Lymph Nodes
MACS	Magnetic Activated Cell Sorting
MAR	Matrix Attachment/Associated Region
MHCI/II	Major Histocompatibility Complex class I/class II
PC	Peritoneal Cavity
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PMA	Phorbol Myrisate Acetate
Pre-BCR	precursor-B cell receptor
RAG	Recombination-activating gene
RHS	*Rad50* Hypersensitive Site
RT-PCR	Reverse transcriptase-Polymerase Chain Reaction
Sp	Spleen
SP	Single Positive
TCR	T-cell receptor
Tg	Trangene
Th	T helper
V(D)J	Variable(Diversity)Joining
WT	Wild-type

1

General Introduction

In vertebrates, the immune system is responsible for the protection against pathogens such as viruses, bacteria, fungi or parasites. This remarkably effective defense system depends upon the white blood cells or leukocytes, which mediate both innate and adaptive immune responses.

Innate immunity provides an immediate but non-specific front line of host defense against many pathogens and involves granulocytes (neutrophils, eosinophils and basophils), mast cells, macrophages and natural killer (NK) cells. Germ line-encoded surface receptors to common pathogens constituents on innate effector cells trigger the elimination of pathogens by phagocytosis and the release of inflammatory mediators, such as cytokines and chemokines. These receptors are referred to as pattern recognition receptors (PRRs), which recognize a limited set of molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs).

Acquired or adaptive immunity is characterized by gradual, though highly specific and effective immune responses against pathogens. Three major cell types are involved in adaptive immunity: B-lymphocytes, T-lymphocytes and antigen presenting cells (APCs), the most potent of which are dendritic cells (DCs). DCs act as messengers between the innate and adaptive immune system. Their main function is to take up, process and present pathogen constituents (also called antigens) with major histocompatibility complex (MHC) proteins to T lymphocytes. B- and T-lymphocytes are considered to be the central players of the adaptive immune system. Their unique and virtually limitless capacity to specifically recognize antigens relies on the generation of a wide repertoire of antigen receptors – B-cell receptor (BCR) in B-lymphocytes and T-cell receptor (TCR) in T-lymphocytes – during lymphocyte development. B-lymphocytes respond to pathogens by producing large quantities of antibodies (secreted form of BCR) with pathogen neutralizing capacities, when terminally differentiated into plasma cells. In response to antigens, T-lymphocytes produce cytokines that direct the immune response (T-helper cells) or toxic granules that induce the death of pathogen infected cells (cytotoxic T-cells). Following pathogen elimination, lymphocytes leave a lasting legacy of the antigens they have encountered in the form of memory cells, which are able to mount faster and stronger immune responses in subsequent challenges with the same antigen – a process know as immunological memory.

This thesis focuses on the role of specific transcription factors during lymphocyte development and differentiation into functional effector subsets.

PART I – LYMPHOCYTE DEVELOPMENT

Models for early hematopoiesis

All blood cells, including lymphocytes, originate from a small number of self-renewing and pluripotent hematopoietic stem cells (HSCs) in the mouse bone marrow (BM) by successive expansions in cell number in concert with progressive restrictions in lineage potential[1-4] (**Fig. 1**). A primary event in HSC differentiation is loss of the potential to self-renew, while retaining the capacity for multilineage differentiation, thereby generating multipotent progenitors (MPPs). The initial view was that the first and decisive lineage commitment step of MPPs resulted in a strict separation into two major lineage-restricted progenitors: common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs)[5, 6]. Progeny derived from CLPs would segregate into the B-lymphocyte, T-lymphocyte, NK cell or dendritic cell lineage and CMPs would give

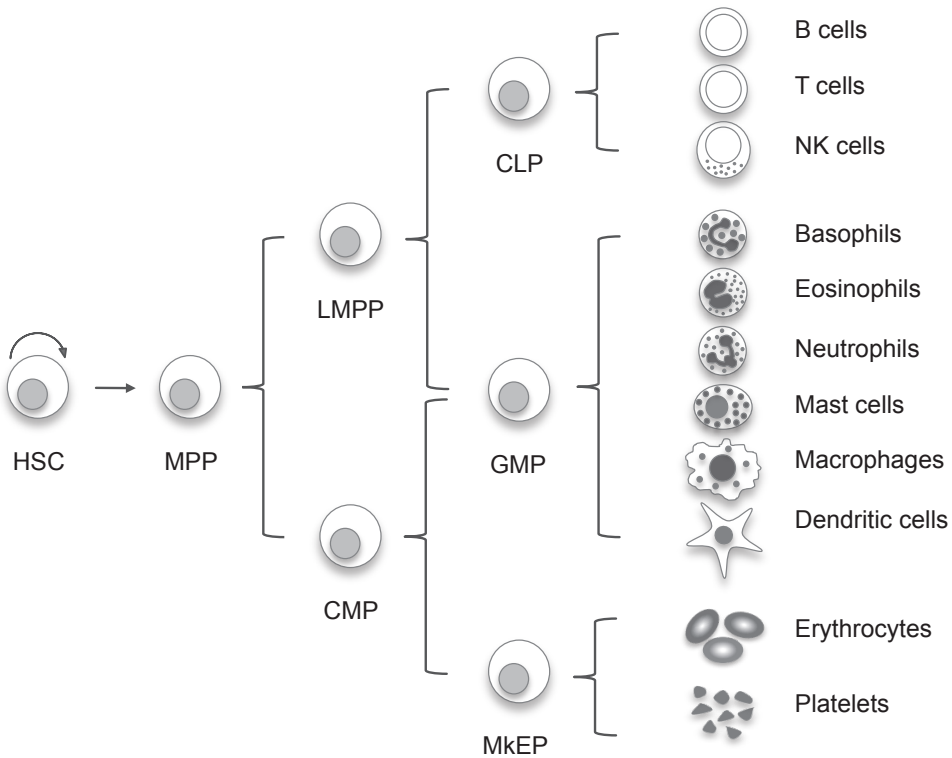

Figure 1. Schematic overview of hematopoiesis. Hematopoietic stem cells (HSCs) can give rise to all blood cells while retaining the capacity for self-renewal. The main lineage restriction steps during HSCs differentiation are indicated. MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-macrophage progenitor; MkEP, megakaryocyte-erythroid progenitor. Model is according to Adolfsson *et al*, 2005[7].

rise to either granulocyte-macrophage progenitors (GMPs) or megakaryocyte-erythroid progenitors (MkEPs). However, the identification of lympho-myeloid restricted MPPs (LMPPs, initially identified as lymphoid-primed multipotent progenitors or common myelolymphoid progenitor), which lack megakaryocyte-erythroid potential, questioned CMPs as obligate intermediates in HSC lineage commitment[7, 8]. Genome-wide gene expression profile analyses have been used to identify lineage-affiliated transcriptional programs in HSC and early lineage-restricted progenitors[9, 10]. These studies have shown that multilineage transcriptional priming present at the MPPs stage is quickly resolved upon erythroid lineage restriction, with rapid extinction of both lymphoid and myeloid transcriptional programs. In contrast, early lymphoid and myeloid transcriptional programs remain associated past lymphoid and myeloid lineage restrictions, revealing a common transcriptional backbone utilized during the development of both the adaptive and innate immune systems. Therefore, both differentiation and transcriptional studies support a revised road map for early hematopoiesis whereby MPPs lose their megakaryocyte-erythroid potential (MkEPs) early in development, giving rise to LMPPs, which retain both granulocyte-macrophage (GMPs) and lymphoid (CLPs) potential.

B cell development in the bone marrow

In the BM, early B-cell progenitors arise from CLPs[5]. Recent work led to the identification of the transmembrane protein Ly6D as a marker for the earliest stage of B-cell specification (BLPs, B-cell biased lymphoid progenitors) within the CLP compartment[11]. The processes of B-cell lineage specification (induction of a lineage-specific gene expression program) and commitment (repression of alternative lineages gene-expression programs) are dependent on the coordinate actions of signalling cascades and transcription factors networks[12-14]. Both the interleukin-7 receptor (IL-7R) and Fms-like tyrosine kinase 3 receptor (Flt3) are critical for B-cell development[15-19]. Likewise, developmental control of early B lymphopoiesis is exerted by a regulatory network of key transcription factors that include PU.1, Ikaros, Bcl11a, Foxo1, basic helix-loop-helix (bHLH) E-protein E2A, early B cell factor-1 (Ebf1) and Pax5.

PU.1 and Ikaros are required for further specification of LMPPs into the lymphoid pathway probably because both are needed for proper expression of Flt3 and IL-7R[20-25]. Similarly, Bcl11a and E2A are necessary for lymphoid-lineage priming in early hematopoietic progenitors[26, 27]. Foxo1 has been linked to the expression of the IL-7R and lymphoid lineage-specific recombination-activating gene (RAG) proteins (RAG1 and RAG2) on early B-cells[28, 29]. Specification of the B-cell developmental program (pre-pro-B cells, **Fig. 2**) is initiated by the induction of Ebf1, downstream of E2A and IL-7R signalling[18, 19, 30, 31]. Ebf1 and E2A cooperatively regulate most B cell-specific genes

including those encoding B lineage commitment factor Pax5 and the precursor-B cell receptor (pre-BCR) components λ5, VpreB, Igα (mb-1, CD79a) and Igβ (B29, CD79b)[32-34]. Commitment to the B-lymphoid lineage (pro-B cells) depends on continuous expression of the transcription factor Pax5, which represses B lineage-inappropriate genes and activates B lineage-specific genes[35-38]. Striking examples of Pax5-mediated repression includes genes encoding essential receptors for the differentiation of progenitors into myeloid, DC or T cell lineages, such as MCSF-R (macrophage colony stimulating factor receptor), Flt3 or Notch1, respectively[35, 37, 39, 40]. Notably, Ebf1 can also to some extent restrict alternative lineage options and promote B cell fate commitment in the absence of Pax5[41]. Some of the Pax5-activated genes code for many components of the pre-BCR and associated molecules, such as CD19, Slp-65, Igα and λ5, and transcription factors important for B-cell differentiation, including SpiB, Aiolos, Id3, Lef1, Irf4 and Irf8[38]. In a feedback loop mechanism, Pax5 also induces the expression of Ebf1[31] thereby amplifying B cell-specific gene expression while reinforcing B-cell commitment and subsequent B cell differentiation. Although it is clear that Pax5 acts as a determinant of B cell lineage commitment, recent studies have suggested that collaborative DNA-binding of PU.1, Foxo1, E2A, Ebf1 and Pax5 on a genome-wide scale is on the basis of a global network of transcription factors that orchestrates B cell fate[42-44].

During the initial stages of B lineage commitment, ongoing differentiation is additionally dependent on successful assembly of a BCR. The BCR is composed of identical pairs of Ig heavy (H) and light (L, κ or λ subtype) chains encoded by the *Igh*, *Igκ* and *Igλ* loci. A wide range in BCR antigen specificities is mostly achieved by a mechanism of gene rearrangement at the *Ig* loci initiated by RAG1 and RAG2, a process also known as V(D)J recombination (see below). During B cell development, sequential rearrangement of *Igh* and *Igκ* or *Igλ* loci is essential for the production of a functional BCR (**Fig. 2**). Rearrangement of the *Igh* locus occurs at the pro-B cell stage. Upon successful rearrangement of the *Igh* locus, Igμ H chain protein is deposited on the surface of pre-B cells together with other components of the pre-BCR complex, the invariant surrogate light chain (SLC) constituents λ5 and VpreB, and the transmembrane signal transduction proteins Igα and Igβ[45]. The pre-BCR acts as a key checkpoint in B cell development to monitor the expression of a functional IgH chain[46, 47]. Signalling through the pre-BCR and the IL-7R drives clonal expansion and survival of $Ig\mu^+$ pre-B cells (large pre-B cells). In large pre-B cells, the expression of *Rag1* and *Rag2* is transiently downregulated to terminate further *Igh* rearrangements[48], thus ensuring that only one functional Igμ is synthesized, a phenomenon referred to as allelic exclusion. In mice with a targeted disruption of the Igμ membrane exon, the pre-B cells cannot deposit the Igμ chain on cell surface and therefore proliferation and allelic exclusion are lost[49].

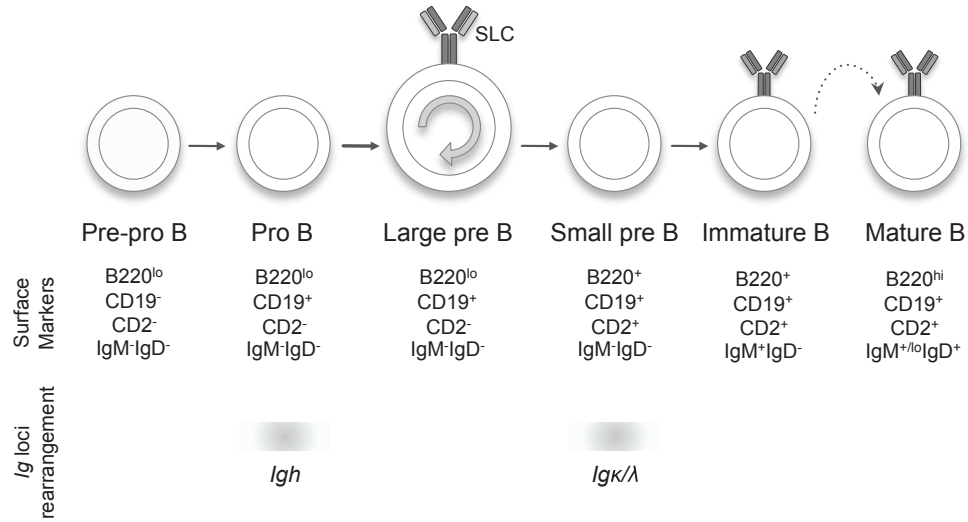

Figure 2. B-cell developmental stages. B-cells develop in the BM through five distinct stages identified on the basis of cell surface markers expression and the rearrangement status of *Igh* and *Igκ/λ* loci. Large pre B-cells are distinguished from pro B-cells by intracellular expression of Igμ heavy chain protein. Immature B-cells with a functional BCR migrate into the periphery where they further mature into naïve mature B-cells that recirculate throughout the body, including the BM. SLC, surrogate light chain.

Pre-BCR signals are also required for developmental progression into small pre-B cells, which occurs concomitantly with cessation of proliferation (**Fig. 2**). Although pre-B cell signalling has been shown to downregulate the transcription of the genes encoding λ5, Vpreb and IL-7R[50-54], silencing of SLC is not critical for limitation of pre-B cell expansion[55]. Small pre-B cells are characterized by the reactivation of the recombination machinery for the initiation of *Igκ* or *Igλ* loci rearrangement and by significant changes in cell-surface-marker profiles: c-Kit, CD43, SLC and IL-7R expression is terminated, and CD2, CD25, and MHC class II expression is induced[46, 47]. The observation that Igκ$^+$ B cells mostly retain their *Igλ* locus in germline configuration whereas Igλ$^+$ B cells mostly carry non-productive *Igκ* locus rearrangements on both alleles, indicates that the Igκ locus is rearranged earlier or faster than the Igλ locus[56-59]. In fact, the *Igκ* locus is five times more frequently rearranged than the *Igλ* locus in mice and, due to selection, Igκ$^+$ B cells are 15-20 times more frequent in the periphery than those expressing Igλ. Nevertheless, successful rearrangement of the *Igκ* or *Igλ* locus results in the assembly of the BCR on the membrane of immature B cells, provided that the IgL chain is able to pair with the IgH chain. The BCR acts as a second key checkpoint in B cell development not only to monitor for expression of a functional IgL chain but also to prevent the formation of autoreactive B cells[60]. It is estimated that more than half of all newly generated BCRs

are capable of binding autoantigen[61]. Therefore, mechanisms to enforce self-tolerance have to be in place in order to prevent autoimmune disease. These include secondary IgL chain loci rearrangements (other alleles of the *Igκ* or *Igλ* loci) to change the specificity of a previously autoreactive BCR (receptor editing) or clonal deletion by apoptosis[62]. As a result of negative selection mechanisms, only 10-20% of the newly formed immature B cells leave the bone marrow via the bloodstream to enter the spleen[63], where they further mature into naïve mature B cells. Despite these mechanisms of central tolerance in the bone marrow many self-reactive B cells escape to the spleen, where they are silenced by an induced state of antigen unresponsiveness known as anergy.

T cell development in the thymus

T cells are unique among blood cells, as they require a dedicated organ for their development, the thymus. Hematopoietic progenitors must therefore leave the BM, traffic through the circulation and settle the thymus before developing into the T cell lineage. Early T cell precursors in the thymus (lacking the cell surface markers CD4 and CD8 and therefore termed double-negative (DN) cells) are classically subdivided into four distinct developmental stages (DN1–DN4) by differential expression of the cell surface markers CD25 and CD44[64] (**Fig. 3**). Further examination of the DN1 population revealed that efficient T lineage progenitor activity resides in a c-Kit^{+}CCR9^{+} subset termed early thymic progenitors (ETPs)[65], which have very limited B cell potential and still retains the capacity to diverge into NK, DC and myeloid lineages. Although the precise origin of ETPs remains controversial, it is likely that ETPs arise from the LMPP compartment in the BM[66].

Similarly to early B-cell pathway, the processes of T cell lineage specification and commitment are dependent on the coordinate actions of signalling cascades and transcription factors networks. However, the establishment of a T cell lineage program is remarkable for its dependence on a balance among multiple factors, none of which appears to act as a T lineage "master regulator"[67, 68]. Development through the "pro-T cell" stages – that is from the ETP to DN3 stage – is characterized by extensive proliferation, initiation of the T cell developmental program and restriction of alternative cell lineages. Coordinated migrations of pro-T cells through distinct thymic microenvironments[69], provides changes in the combinations of receptor ligands (for example, Delta-like and Jagged family of Notch ligands and the c-Kit ligand stem cell factors (SCF)) and growth factors (such as IL-7) expressed by the thymic epithelium, which drive pro-T cell proliferation, survival and differentiation. Both c-Kit and IL-7R sustain most pro-T cell proliferation and survival[15, 16, 70, 71]. Notch signalling is essential for several distinct aspects of early T cell development[72], including the onset of the T cell lineage program[73,

[74], suppression of B, NK, DC and myeloid lineages potentials[74-78], survival, proliferation and/or differentiation of DN3 cells[78].

Although Notch signals are critical to promote T cell fate, they most likely act in concert with a core group of transcription factors in the T cell specification and commitment process including: GATA-binding protein 3 (Gata3); avian myeloblastosis virus oncogen homolog (Myb); runt-related transcription factor 1-core binding factor β (Runx1-CBFβ) complexes; bHLH E-proteins E2A and HeLa E-box binding factor (HEB); T-cell factor 1 (Tcf1); growth factor independent 1 (Gfi1) and Ikaros[67, 68]. Other transcription factors which expression is more dynamically regulated during T cell developmental stages collaborate with the core group of transcription factors to induce stage-specific gene expression programs. These include: 1) pre-thymically inherited non-T cell transcription factors (PU.1, stem cell leukemia (SCL)/Tal1, Gata2 and CCAAT-enhancer binding protein α (C/EBPα))[79] that most likely sustain the lineage plasticity in early stages of T cell development and which expression is downregulated before the DN3 stage, when T cell precursors are committed to the T cell lineage and can no longer develop into alternative cell types; 2) transcription factors expressed at the ETP to DN2 transition (Bcl11b, HEBalt (an alternative promoter isoform of HEB) and glioma-associated oncogene 2 (Gli2))[79, 80], when most T cell identity genes (encoding for the precursor-TCR (pre-TCR) assembly complex - CD3ε, CD3γ and pre-TCRα chain (pTα), signalling components - kinases, phosphatases and adaptor proteins such as LCK, ζ-chain associated protein kinase of 70 kDa (Zap70) and linker of activated T cells (Lat), RAG proteins and IL-7Rα) begin to be upregulated[79-81]; 3) and transcription factors expressed at the DN2 to DN3 transition (Ets1, Ets2, lymphoid-enhancer-binding factor 1 (Lef1), SpiB and Hes1)[79-82], when most T cell identity genes are fully activated and commitment to the T cell lineage is completely and stably established.

Further differentiation of T cells depends on successful assembly of the TCR. Each receptor consists of a TCRα and a TCRβ chain, encoded by *Tcr*α and *Tcr*β loci, respectively (**Fig. 3**). Most mature T cells express a TCRαβ on their cell surface (αβ T-cells) and these are the T cells involved in the classic adaptive immune response. However, a distinct and minor subset of T cells (γδ T-cells) with largely elusive functional role in the immune system bears an alternative TCR made up of TCRγ and TCRδ chains, encoded by the *Tcr*γ and *Tcr*δ loci, respectively. Similar to the BCR, a large variety of TCR antigen specificities is achieved by gene rearrangement of the *Tcr* loci (see below). During T cell development, sequential rearrangement of *Tcrδ*, *Tcrγ* or *Tcrβ* and *Tcrα* loci is essential for the production of a functional TCR. During the DN2 to DN3 transition, rearrangements at the *Tcrδ, Tcrγ* and *Tcrβ* loci are initiated[83]. Cells that productively rearrange the *Tcrδ* and *Tcrγ* loci may express a functional TCRγδ on the membrane,

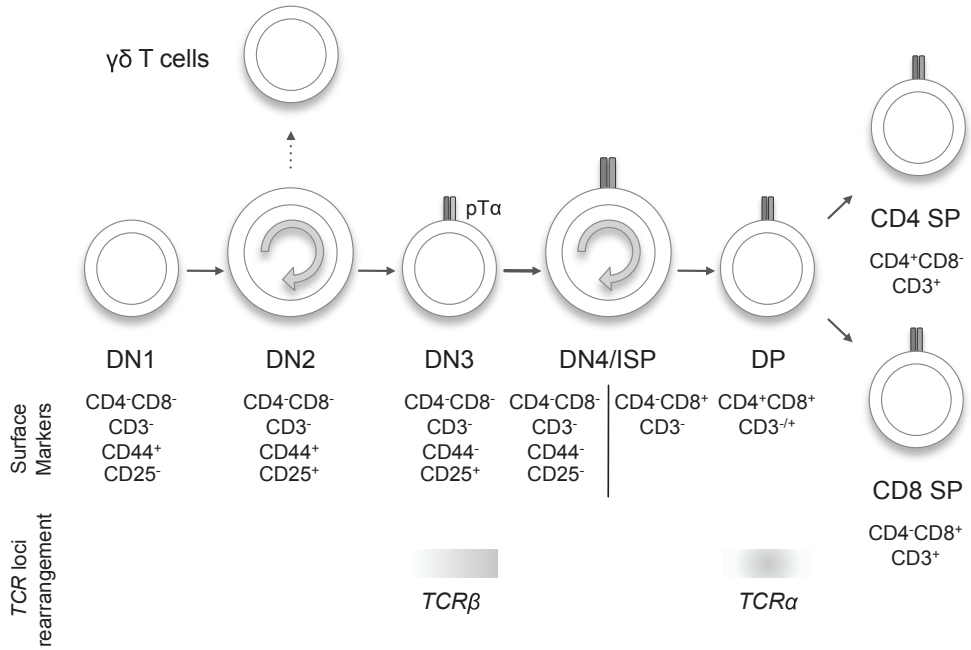

Figure 3. T-cell developmental stages. T-cells develop in the thymus towards αβ or γδ T-cell lineages. Eight distinct αβ thymocyte developmental stages are identified on the basis of cell surface markers expression and the rearrangement status of *TCRβ* and *TCRα* loci. DN, double-negative; ISP, immature single positive; DP, double-positive; SP, single positive; pTα, pre-TCRα chain.

undergo a small proliferative burst and develop into γδ T-cells. Upon productive rearrangement of the *Tcrβ* locus, the TCRβ chain associates with the invariant pTα chain and CD3 molecules on the cell surface and forms the pre-TCR complex, which qualifies DN3 cells to enter the αβ T cell lineage. Recent studies suggest that stronger signals delivered by the TCRγδ and relatively weaker pre-TCR signals determine the αβ/γδ-lineage choice[84-86].

Pre-TCR signalling tests for the functionality of the TCRβ chain, a process called β-selection[87]. Cells that have passed this checkpoint are termed β-selected cells (or late DN3 cells, to distinguish them from early DN3 cells that are not yet selected)[88]. The pre-TCR complex signals for *Tcrβ* locus allelic exclusion and DN3 cells survival, proliferation and differentiation. In particular, pre-TCR signals result in the downregulation of CD25 expression and progression of DN3 cells into the DN4 stage[83] (**Fig. 3**). Cells subsequently acquire both CD4 and CD8 coreceptors to become double-positive (DP) cells, with CD8 usually being expressed first in most mouse strains generating immature SP (ISP) cells. Therefore, the late DN3, DN4 and ISP stages consist of large cycling “pre-T cells”. At the DP cell stage, proliferation is ceased before rearrangement at the *Tcrα* locus is

initiated, which leads to deletion of the *Tcrδ* locus located in between V_α and J_α gene segments. If *Tcrα* gene recombination is productive, TCRαβ is expressed on the cell surface of DP cells. TCRαβ-bearing DP cells are tested for self-MHC recognition during the process of positive selection[89]. TCRαβ receptors recognizing MHC class I or class II molecules will develop into CD8 single positive (SP) or CD4 SP T cells, respectively[90]. Two models have been elaborated to explain CD4/CD8 lineage commitment. In the stochastic/selective model, lineage commitment is determined randomly, followed by a selection step that eliminates thymocytes that express the inappropriate thymocytes. The instructive/kinetic model proposes that qualitatively distinct signals initiated upon TCR engagement by MHC class I or class II ligands and duration of TCR signalling, instruct CD8 or CD4 lineage choice, respectively. DP thymocytes that fail to recognize MHC molecules die "by neglect", whereas potential self-reactive T lymphocytes are eliminated by apoptosis, a process called negative selection. When combined, these selection processes result in the generation of CD4 and CD8 SP thymocytes with TCRαβ receptors that can recognize non-self-antigens presented by MHC class II and I proteins, respectively. Mature SP cells exit the thymus and migrate via the bloodstream to peripheral lymphoid organs – such as the spleen, lymph nodes and mucosa associated lymphoid tissue (MALT; for example, Peyer's Patches) - as naïve recirculating $CD4^+$ T helper and $CD8^+$ cytotoxic T cells.

Gata3 and thymocyte development

Gata3 is a transcription factor which expression is confined to T- and NK-cell lineages among all hematopoietic cell types[91-96]. Initial experiments using antisense oligonucleotides strongly suggested that Gata3 plays an essential role in the initiation of the earliest steps of T cell development in the fetal thymus together with Tcf1[97]. Analyses in *Gata3-LacZ* knock-in mice show that Gata3 expression is already detectable at the earliest stages of T cell development in the adult thymus[91] and in the absence of Gata3 development of T cell precursors is arrested, even before the DN1 stage[91, 98]. More recently it has been shown that Gata3 is required for ETP generation[99]. Importantly, impairment of T-lymphopoiesis in Gata3 deficient hematopoietic progenitor cells can only be rescued when both Notch signals and Gata3 are present but not either alone[100]. This shows that Notch signalling and Gata3 act in concert at the earliest stages of T cell development to promote T cell fate specification.

Gata3 expression in thymocytes peaks during proliferation at β-selection checkpoint and again later in cells undergoing positive selection to the CD4 SP T cell fate[91, 101, 102]. Therefore, low Gata3 expression during the two waves of *Tcr* gene recombination are separated by a stage of high Gata3 expression (β-selection), which

suggests that Gata3 may function as a regulator of proliferation events associated with the essential coupling of *Tcr* loci rearrangement to the cell cycle. In agreement with this, enforced expression of Gata3 during T cell development induces thymic lymphoma in transgenic mice[103]. Conditional deletion of *Gata3* at the DN stage shows that Gata3 is needed for normal β-selection and proliferation in the DN3 to DP transition[102]. Gata3 deficient DN3 cells can rearrange the *Tcrβ* locus and express the pre-TCR but fail to increase their cell size and do not downregulate CD25, although there is no evidence for a survival defect. These findings indicate that Gata3 functions as a key mediator for β-selection. Conditional deletion of *Gata3* after β-selection, demonstrates that at the DP stage Gata3 has a prominent role during positive selection, promoting the development of CD4 SP cells and blocking the development of CD8 SP cells[101, 102, 104]. The finding that enforced Gata3 expression results in increased cell size and TCR expression levels in DP cells also suggests that Gata3 may influence the kinetics of positive selection[105]. However, whether Gata3 is important for commitment to the CD4 lineage or for survival or development of CD4 lineage committed cells still remains elusive[101, 102]. In this context, TCR signal strength and duration at the DP stage has been shown to affect CD4 versus CD8 lineage decision, with strong and lasting TCR signals directing commitment towards the CD4 lineage[89]. Conversely, in DP cells Gata3 enhances the expression of TCR and reduces the expression of CD5, a negative regulator of TCR signalling[105]. Because Gata3 is induced by TCR signalling[101], these results implicate Gata3 in a key regulatory positive feedback loop that increases TCR expression and signal strength/duration at the DP cell stage. Two recent studies have further elucidated the role of Gata3 in directing the differentiation of DP cells towards the CD4 lineage. *Gata3* disruption blocks the differentiation into CD4 T cells before commitment to the CD4 lineage but acts as a critical regulator of ThPOK expression at this stage[106]. ThPOK is a transcription factor that promotes differentiation into the CD4 lineage in a Gata3-dependent fashion, while inhibits Runx3-dependent differentiation towards the CD8 lineage independently of Gata3[106, 107]. Based on these finding it was proposed that Gata3 works as a specification factor for the CD4 lineage 'upstream' of the ThPOK-controlled CD4 commitment checkpoint.

Interestingly, the timing and dosage of Gata3 expression is essential in T cell development. Forced expression of Gata3 in nonthymic hematopoietic precursors or early T lineage progenitors (DN1 and DN2 stage) does not enhance or accelerate T cell specification. Instead it completely aborts T cell specification, resulting in non-lymphoid hematopoietic cell fates[108-110]. In developing thymocytes, overexpression of Gata3 clearly has adverse effects and is associated with reduced cell survival[104, 105, 109].

Taking together, these findings indicate that Gata3 function is associated with

T cell lineage fate specification, β-selection and positive selection towards the CD4 lineage.

V(D)J recombination

One of the hallmark characteristics of the adaptive immune system is its practically endless capacity to recognize a great variety of different antigens. This relies on the generation of a repertoire of antigen receptors with a wide range of antigen-binding specificities during B and T lymphocyte development. The Ig and TCR chains - which constitute the BCR and TCR, respectively - are different in nature but similar in structure, having both variable (V) and constant (C) regions[111]. The C region is mostly involved in membrane expression and signalling functions of the antigen receptors. In B cells, the

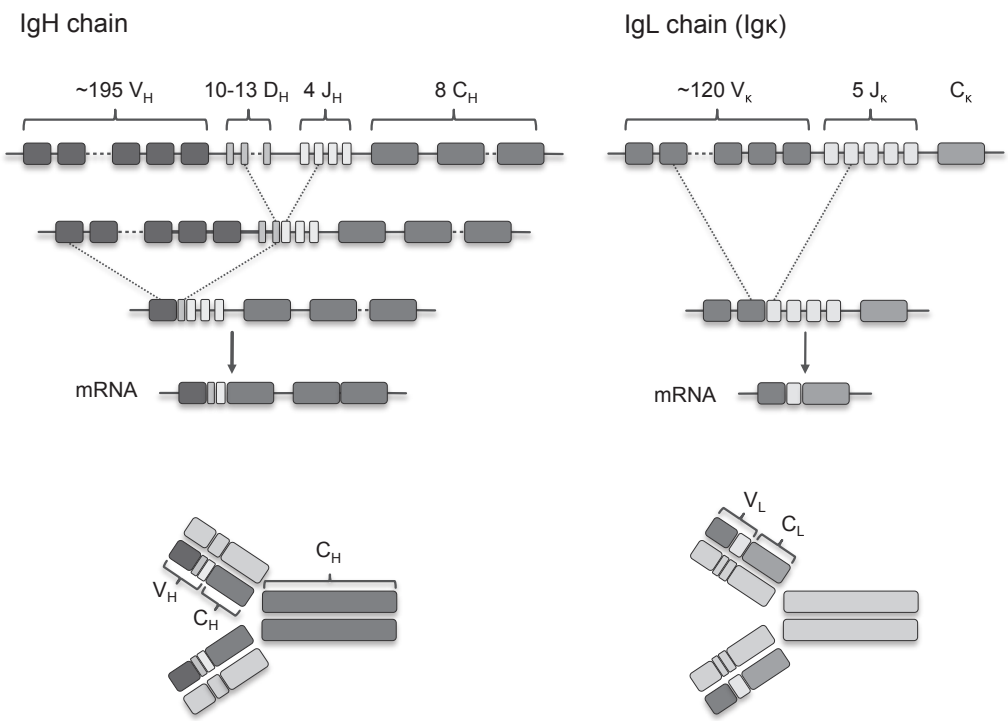

Figure 4. Schematic representation of V(D)J recombination at *Ig* loci. The BCR is composed of identical pairs of Ig heavy (H) and light (L) chains, both with a constant (C) and a variable (V) region. The IgH V region (V_H) is encoded in multiple variable (V), diversity (D) and joining (J) gene segments. First a D_H gene segment is joined to a J_H gene segment and, subsequently, a V_H gene segment is coupled to the DJ_H joint. The VDJ exon is transcribed and spliced to the C_H exons. The IgL V region (V_L) is encoded in multiple V and J gene segments (Igκ type). Only one recombination event is required to join a $V_κ$ gene segment to a $J_κ$ gene segment. The $V_κJ_κ$ exon is transcribed and spliced to the $C_κ$ exon.

C region of IgH chains is additionally involved in determining the effector function of antibodies (secreted Ig). The V region is the part of the antigen receptor that specifically recognizes the antigen and consists of three hypervariable complementary determining regions (CDR1-3). A wide range in CDR specificities is achieved by the many possible different combinations of Ig or TCR chains V regions, which in turn are encoded in multiple V (variable), D (diversity, only for *Igh*, *Tcrδ* and *Tcrβ* loci) and J (joining) gene segments. These are assembled through a somatic DNA recombination mechanism to form a complete V-region exon, a process also known as gene rearrangement or V(D)J recombination[112-114] (**Fig. 4**). Selection of a V, (D) and J gene segment during gene rearrangement occurs at random and the large number of possible different combinations accounts for most of the diversity of the antigen receptor repertoire. The C region is in most antigen receptor loci encoded in a separate single gene segment that is joined to the V region exon by RNA splicing. In the *Igh* locus the C region is encoded by several gene segments, each of them corresponding to the different classes or isotypes of the IgH chain involved in distinct antibody effector functions. In naïve B cells only the first two of these C region gene segments, Cμ and Cδ, are expressed in conjunction with an assembled V region exon by alternative RNA splicing, and thus naïve B cells have both IgM and IgD on their surface. Further somatic DNA recombination events known as isotype switching or class switch recombination (CSR) occur in B cells that have become activated by antigen, thereby allowing a switch to downstream C region gene segments (see below).

V(D)J recombination mechanism

DNA rearrangements are targeted to the *Ig* and *Tcr* loci by short, conserved, non-coding sequences at the flanks of coding V, D and J gene segments, which are termed recombination signal sequences (RSS) and serve as recognition sites for the recombination machinery[111] (**Fig. 5**). RSS consist of a relatively conserved heptamer (always contiguous with the coding sequence) and nonamer separated by a non-conserved spacer of either 12 or 23 base pairs (bp). Gene segments of a certain type in a particular locus are all flanked by RSS with the same spacer length, sometimes on both sides (D gene segments). Efficient recombination is only permitted between gene segments flanked by a 12 bp- and a 23 bp-spacer RSS. This restriction, referred to as "12/23 rule" ensues that the appropriate gene segments are joined within a given locus. For example in the *Igh* locus, V and J gene segments are flanked by 23 bp RSS and D segments are flanked on both sides by 12 bp RSS. The "12/23 rule" therefore dictates that rearrangements involving V gene segments will contain a D gene segment placed between a V and a J.

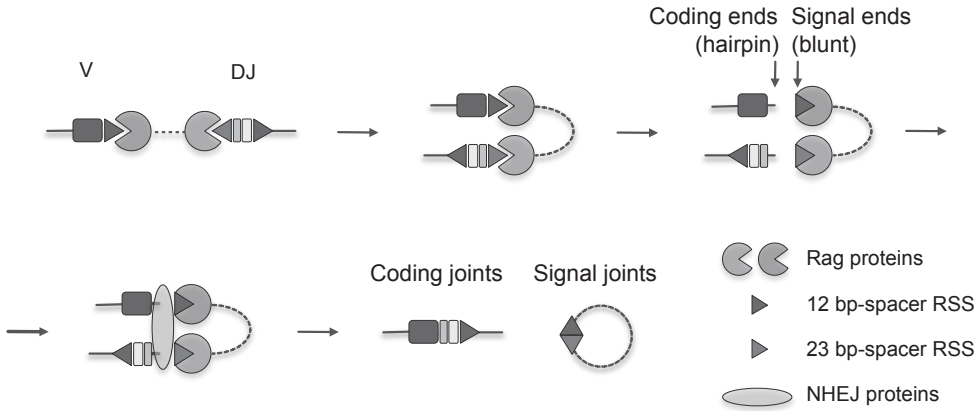

Figure 5. Schematic representation of V(D)J recombination mechanism. Rag proteins bind to 12 bp- and 23 bp-spacer recombination signal sequences (RSS) and bring together the segments to be joined. The DNA is cleaved to create coding (hairpin) and signal (blunt) ends, where non-homologous end-joining (NHEJ) proteins bind. Coding ends hairpins are cleaved at random and sequences can additionally be modified by insertion or deletion of nucleotides. NHEJ proteins are responsible for joining coding and signal ends, thereby forming coding and signal joints.

Recombination is initiated by the lymphoid-specific RAG1 and RAG2 proteins, which cooperate to induce double-strand breaks (DSB) precisely at the 5' border of the heptamer sequence in a 12 bp- and a 23 bp-spacer RSS[111]. This DSB generates coding ends that are covalently closed DNA hairpins (in V, D and J coding sequences) and signal ends that are blunt and 5' phosphorylated (in the intervening DNA between two gene segments). The DSB generated by the RAG endonucleases are joined by a ubiquitously expressed set of non-homologous end-joining (NHEJ) proteins: Ku70, Ku80, DNA-dependent protein kinase (DNA-PK), X-ray repair cross-complementing factor 4 (Xrcc4), Cernunnos (also called XLF or Xrcc4-like factor), Artemis and DNA ligase IV[111, 115]. The DNA binding proteins Ku70 and Ku80 bind as a complex to DSB and recruit DNA-PK, which in turn recruits Artemis, Xrcc4, Cernunnos and DNA ligase IV. Activated DNA-PK activates the nuclease activity of Artemis leading to imprecise cleavage of coding ends hairpins thus generating palindromic sequences when filled in the complementary strand by DNA polymerases λ and μ (P-nucleotides addition). Opened hairpins can be additionally modified by insertion of non-template nucleotides (N-nucleotides addition) by terminal deoxynucleotidyl transferase (TdT) or deletion of nucleotides at coding ends. Finally, Xrcc4 and Cernunnos increase the activity of DNA ligase IV, responsible for sealing signal and coding joints.

Of note, the DJ or VJ junctional sequences encode the CDR3 region of Ig and TCR chains[111] and therefore, imprecisely joined borders between recombined gene segments contributes considerably to the diversity of BCR and TCR antigen-binding

specificities. However, removal and/or addition of nucleotides often disrupt the reading frame of the coding sequence beyond the joint. Such frameshifts might prevent protein expression or expression of a non-functional protein and therefore these rearrangements are termed out-of-frame or non-productive. Deficiency in any of the proteins involved in V(D)J recombination leads to incompletion of the process and results in severe combined immune deficiency (SCID), characterized by complete arrest of B and T cell development[116].

V(D)J recombination regulation

Recombination at *Ig* and *Tcr* loci is regulated at three different levels: lineage-specificity, temporal order within a lineage and allelic exclusion. Allelic exclusion refers to a process by which each individual cell only expresses one allele of each antigen receptor loci, thus assuring the expression of a unique antigen receptor[117]. Several studies have suggested that regulation relies on developmental-stage specific changes in the availability of the recombination machinery and accessibility of appropriate antigen receptor loci for recombination[118-120].

Transcriptional regulation limits expression of RAG proteins to B- and T-lineage cells within the hematopoietic system[121-127], which accounts for lymphoid lineage specificity of recombination *per se*. During developmental progression, RAG expression is restricted to nonproliferating cells undergoing active rearrangement of antigen receptor loci. Particularly, initial productive *Igh* or *Tcrβ* rearrangement results in the cell-cycle dependent downregulation of RAG expression during the clonal expansion stage that follows[128-132]. RAG expression is upregulated again once cells arrest proliferation for rearrangement of *Igκ*, *Igλ* or *Tcrα* loci. In the thymus, positive selection also results in the loss of RAG expression in CD4 SP or CD8 SP thymocytes[133]. In immature B cells, it is likely that BCR basal signalling inhibits RAG expression[134]. Nevertheless, in the presence of self antigens surface BCR expression is downregulated, which results in loss of basal signal that probably allows re-induction of RAG expression and edition of IgL chain loci[135]. Cell-cycle dependent regulation of RAG expression during lymphocyte development might be crucial for genomic stability and allelic exclusion at productively rearranged loci.

Fully rearranged *Ig* and *Tcr* loci are only generated in developing B- and T-lymphocytes, respectively[112-114]. Moreover, rearrangement is ordered in a given lineage and, sometimes, within the locus – in the *Igh* or *Tcrβ* locus, D-J rearrangements precedes V-DJ rearrangement, followed by rearrangement of *Igκ*/*Igλ* or *Tcrα* locus. Finally, each individual B- or T-cell only generates one functional allele at each locus. Given that all rearranging gene segments are flanked by conserved RSS and use a

common RAG recombinase, regulation of RAG expression by itself cannot entirely account for the complete regulation of the V(D)J recombination process. Based on the observation that rearranging gene segments are concomitantly undergoing germline transcription[136], it was proposed that changes in locus accessibility are on the basis of lineage-, developmental-stage- and allelic-specific regulation of V(D)J recombination. Later experiments confirmed this hypothesis by showing that isolated nuclei from cells at different developmental stages show appropriate locus sensitivity to *in vitro* cleavage by recombinant RAG proteins[137]. More recently, developmentally regulated antisense germline transcription in the *Igh* locus was implicated in the regulation of chromatin accessibility over the V_H region, further supporting the accessibility hypothesis[138]. Whether germline transcription itself can direct V(D)J recombination targeting by increasing locus accessibility was elegantly demonstrated for the *Tcrα* locus[139, 140]. These studies have demonstrated that germline transcription can instruct positive and negative alterations in J_α chromatin structure and target RAG proteins to the J_α region. In combination, these effects target initial V_α-J_α recombination to proximal J_α gene segments, thereby promoting the ordered usage of the J_α array and the selection of a diverse TCRα repertoire.

The molecular basis of locus accessibility likely involves subnuclear relocation, histone acetylation and/or methylation, DNA demethylation and locus contraction[118-120]. Accessibility is controlled through interactions between local *cis*-regulatory elements, such as promoters and enhancers, and developmental stage-specific transcription factors. Target deletion of these *cis*-regulatory elements *in vivo* have shown their important role in *Ig* and *Tcr* loci rearrangement maybe by initiating locus specific histone modifications and DNA demethylation[141-145]. Cytokine signalling can also result in the activation of locus accessibility. For example, IL-7R signalling has previously been implicated in histone acetylation at the *Tcrδ*[146, 147], *Tcrβ*[148] and *Igh* loci[149, 150], although the role of IL-7R signalling in the control of chromatin accessibility at the *Igh* locus has recently been questioned[151].

To become accessible for rearrangement, the antigen receptor loci must undergo a series of chromatin changes that is best exemplified by the behaviour of *Igh* and *Igκ* loci in developing B cells. In early pro-B cells, the two alleles of *Igh* and *Igκ* loci are recruited from the periphery to the centre of the nucleus[152]. At the *Igh* locus, stepwise activation of the independently regulated D_H-J_H and V_H domains likely accounts for ordered rearrangement. In pro-B cells poised to undergo D_H-J_H rearrangement, the D_H and J_H regions exhibit active histone acetylation and methylation marks[153-155]. At the late pro-B cell stage when V_H-DJ_H joining occurs, accessibility over the V_H region is achieved by histone acetylation[149, 150, 153] and removal of inactive histone methylation marks[156]. In pre-B cells, one *Igκ* allele becomes preferentially packaged into an active chromatin structure

characterized by histone acetylation and methylation, while the other allele is recruited to repressive pericentromeric heterochromatin[157-159]. These events may actually underlie the process of *Igκ* allelic exclusion by rendering only a single allele available to DNA demethylation and, consequently, susceptible to rearrangement[157-159]. It was proposed that allelic differences in the timing of DNA replication randomly established at the early embryo direct the initial choice of the *Igκ* allele for rearrangement, as rearrangement takes place preferentially on the early-replicating allele in B cells[160]. Asynchronous, early replication of one *Igh* allele is also closely correlated with rearrangement of that

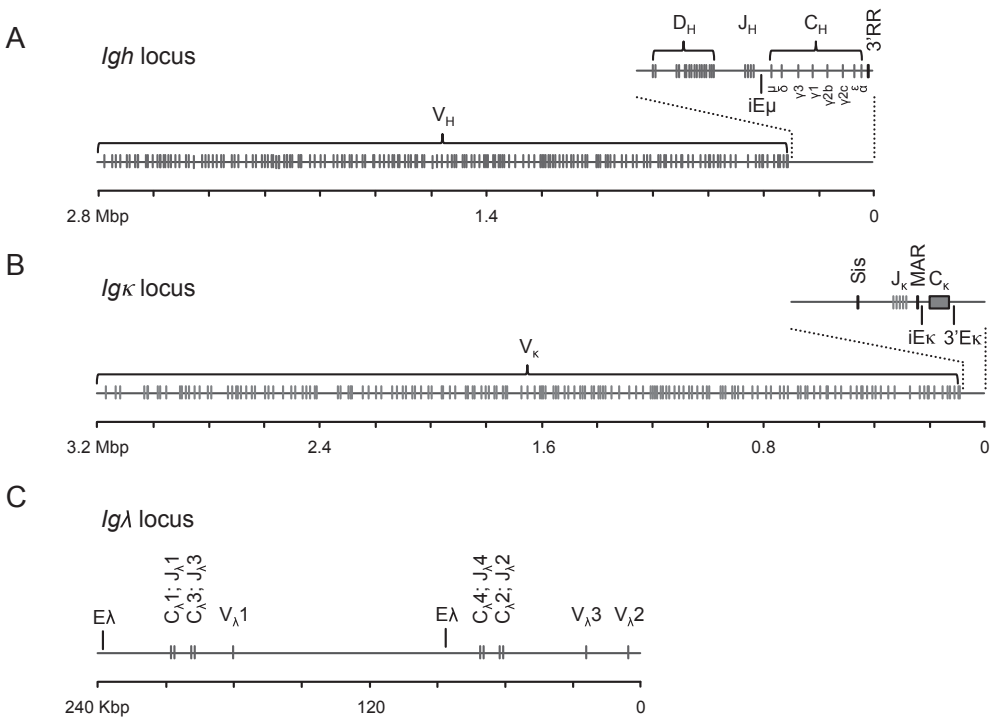

Figure 6. Schematic representation of the mouse *Igh*, *Igκ* and *Igλ* loci. (**A**) and (**B**) The *Igh* and *Igκ* loci are composed of > 100 variable (V) gene segments that span a 2.5 and 3.1 Mbp region, respectively. In contrast, diversity (D), joining (J) and constant (C) gene segments only comprise a relatively small region, 0.2 Mbp in the *Igh* locus and 0.1 Mbp in *Igκ* locus. In the *Igh* locus, the C region is encoded by eight different gene segments, each of them corresponding to the different classes or isotypes of the Ig heavy chain involved in different antibody effector functions. iEμ, intronic μ enhancer; iEκ, intronic κ enhancer; 3'Eκ, 3' κ enhancer; MAR, matrix attachment region; Sis, silencer in the intervening sequence. (**C**) The organization of the *Igλ* locus is quite distinct from that of the *Igh* and *Igκ* loci. Each of the four C_λ gene segments contains their own unique J_λ gene segment and only two V_λ region gene segments, $V_\lambda1$ and $V_\lambda2$, are frequently utilized. $V_\lambda2$ is located approximately 60 kbp from $J_\lambda2$, and it will generally not recombine with other J_λ gene segments, while $V_\lambda1$, located 22 kbp from the $J_\lambda1$, will form joints with either $J_\lambda1$ or $J_\lambda3$.

allele, although the mechanistic link between the two processes remains unclear[160]. In contrast, recent studies propose that monoallelic V_β-to-$D_\beta J_\beta$ recombination events at the *Tcrβ* locus occur due to frequent stochastic, rather than directed, interactions of *Tcrβ* alleles with repressive nuclear compartments[161].

Following central relocation in the nucleus, the *Igh* and *Igκ* loci undergo large-scale contraction by looping of individual chromatin domains in rearranging pro-B and small pre-B cells, respectively[152, 162-164]. In the *Igh* locus, approximately 195 V_H gene segments span a 2.5 Mbp region and D_H, J_H and C_H clusters located downstream only comprise 0.2 Mbp (**Fig. 6**). Likewise, the *Igκ* locus (3.2 Mbp) is composed of approximately 120 V_κ gene segments that cover 3.1 Mbp, a J_κ cluster and a single constant region. Therefore, locus contraction may function to ensure that the multiple V gene segments scattered over a vast genomic region have equal opportunities to recombine with the DJ_H or J_κ region[165]. In support of this hypothesis, the transcription factors Pax5, Yy1 and Ikaros have been shown to play a role in *Igh* locus contraction and in their absence, only the D_H proximal V_H genes recombine[166-168]. Successful rearrangement of a *Igh* allele ultimately leads to pre-BCR signalling which engages allelic exclusion mechanisms via downregulation of IL-7R signalling, such as locus decontraction[162], rapid repositioning of the non-productive *Igh* allele to repressive pericentromeric heterochromatin[162] and histone deacetylation in the V_H region[149, 150]. At this stage, also one *Igκ* allele is recruited to the same repressive subnuclear compartment, even before the onset of recombination at the euchromatic *Igκ* allele. Recently it was shown that interchromossomal association between the *Igκ* and *Igh* loci at the pre-B cell stage is required to direct pericentromeric recruitment and locus decontraction of the non-productive *Igh* allele[169]. At later stages of B cell development productively rearranged *Igh* and *Igκ* alleles remain spatially discrete from heterochromatin, whereas non-productively or unrearranged alleles are positioned in pericentromeric heterochromatin thus ensuring monoallelic expression of *Ig* loci and single BCR specificity[170]. Reversible long-range interactions by chromatin looping in *Tcrβ* and *Tcrα/δ* loci and pericentromeric heterochromatin positioning of the non-productive *Tcrβ* allele has also been observed in thymocytes[171]. Therefore, locus contractions and subnuclear repositioning constitute general mechanisms of accessibility regulation at antigen receptor loci.

PART II – LYMPHOCYTE FUNCTION

B cell functional subsets

Naïve B cells are generally divided into three functionally distinct mature B cell subsets: marginal zone (MZ) B cells, follicular (FO) B cells and B-1 B cells[172, 173]. Different subsets vary in terms of their origins and development, their location, their ability to migrate, and in the likelihood that they will be activated in a T cell-dependent or T cell-independent fashion.

Marginal zone and Follicular B cells

Immature B cells migrating from the BM can further mature in the spleen, through transient transitional – T1 and T2 – B cell stages, into either MZ or FO B cells. Because MZ and FO B cells have a common pathway of development they are also referred to as conventional or B-2 B cells.

MZ B cells are sessile and reside in the vicinity of the marginal sinus at the interface between the non-lymphoid red pulp and the lymphoid white-pulp of the spleen, a region known as marginal zone. These cells are especially well positioned to rapidly respond to blood-born pathogens by forming short-lived IgM-secreting plasma cells in response to T cell-independent antigens such as polysaccharides[174, 175]. MZ B cells have the ability to self-renew and therefore have an unlimited lifespan.

FO B cells have a defined lifespan that is measured in weeks, comprise most mature B cells and are recirculating cells that home mainly to B cell follicles in secondary lymphoid organs – spleen, lymph nodes and Peyer's Patches. Follicles are always adjacent to T cell zones and therefore follicular B cells are particularly well suited to participate in T cell-dependent immune responses to protein antigens[176, 177]. Upon antigen exposure and help from cognate $CD4^+$ T cells, FO B cells have the ability to differentiate into long-lived plasma cells that secrete high-affinity and class-switched (this is, IgG, IgE or IgA) antibodies. This occurs in discrete structures within the B cell follicles known as germinal centres, where B cells undergo extensive antigen-dependent proliferation, class switch recombination (CSR) and somatic hypermutation (SHM). CSR refers to the somatic DNA recombination events between switch regions located upstream of each C_H gene segment in the *Igh* locus that allow B cells to switch from expressing IgM to expressing a different class or isotype of IgH chain[178]. Cytokines secreted by T helper (Th) cells direct CSR to certain switch regions in the *Igh* locus, thereby influencing the antibody isotypes produced by B cells during a germinal centre reaction. In the mouse, IL-4 preferentially induces switching to Cγ1 and Cε (IgG1 and IgE), whereas transforming growth factor (TGF)-β induces switching to Cγ2b (IgG2b) and Cα (IgA).

Th2 cells make both of these cytokines and also IL-5, which promotes IgA secretion from cells that have already undergone switching. Th1 cells are relatively poor initiators of antibody responses and IFN-γ preferentially induces switching to Cγ2c/a (IgG2c/a) and Cγ3 (IgG3). Because different IgH chain isotypes confer different antibody effector functions, the process of CSR is important to generate functional diversity among antigen-specific antibodies in the course of an immune response. Additional diversity within the expanding clone of B cells responding to antigen is generated by SHM, a unique mutation mechanism that is targeted to the V regions of rearranged Ig genes[179]. Combined with the selection for B cells that produce antibodies with highest affinity for antigen, SHM leads to affinity maturation of B cells in germinal centres.

B-1 B cells

B-1 B cells likely arise from precursors in the fetal liver and neonatal bone marrow and can be found predominantly in peritoneal and pleural cavities, were they are maintained by self-renewal. B-1 B cells are typically subdivided in B-1a and B-1b B cells. B-1a B cells spontaneously secrete the so called “natural antibodies” that constitute most of the serum IgM present in non-immunized mice. These “natural antibodies” display extensive polyreactivity, with low affinity to both self-antigens and common bacterial polysaccharides. Previously it was shown, using a mouse model of naturally generated autoreactive B cells, that the presence of self-antigen promotes B1a B cell accumulation in the peritoneal cavity and serum autoantibody secretion[180]. Importantly, this study provides evidence for positive seletion of B1a B cells on the basis of their autoreactivity. Studies on the relative contributions of B1a and B2 B cells during influenza virus infection highlight the importance of naturally occuring IgM antibodies as crucial and nonredudant components of the antiviral response[181-183]. In contrast to B1a B cells, B1b cells confer long-lasting and highly protective antibody responses by specifically recognizing T cell-independent antigens present in many pathogenic bacteria[184, 185].

Differentiation of effector T cell subsets

Mature $CD4^+$ or $CD8^+$ T cells that have not yet encountered their specific antigen recirculate between blood and peripheral lymphoid organs as naïve T cells. At sites of infection DCs take up antigen and are activated as part of the innate immune response, through receptors that signal the presence of pathogen constituents or by cytokines produced during the inflammatory response[186]. This induces their migration to local lymphoid tissue and their maturation into cells that are highly effective at presenting antigen to naïve T cells in the form of an antigen:MHC complex. Peptide antigens from intracellular pathogens that multiply in the cytoplasm are carried to the cell surface of

DCs by MHC class I (MHCI) molecules and presented to $CD8^+$ T cells. Peptide antigens from pathogens multiplying in intracellular vesicules and those derived from extracellular pathogens, are carried to the cell surface by MHC class II (MHCII) molecules and presented to $CD4^+$ T cells. Mature DCs are distinguished by the expression of high levels of surface co-stimulatory molecules, such as CD80 and CD86 that bind to the CD28 receptor on T cells. Simultaneous delivery of two independent signals by the same APC - an antigen-specific TCR signal and a co-stimulatory signal – is absolutely required for the activation of naïve T cells. This means that only activated APCs can initiate T cell responses, which is important because not all potentially self-reactive T cells are deleted in the thymus. Naïve T cells recognizing self-antigens in the absence of co-stimulation are not activated and instead are induced to enter a state of non-responsiveness or anergy.

T cell activation is characterized by clonal expansion of antigen-specific T cells and differentiation of $CD4^+$ or $CD8^+$ T cells into functionally distinct T helper or cytotoxic effector subsets, respectively. It is crucially dependent on the synthesis of IL-2 along with the α chain of the IL-2 receptor (also known as CD25) by activated T cells. Association of the IL-2 receptor α chain to the constitutively expressed β and γ chains in resting T cells confers high affinity to IL-2 and allows activated cells to respond to very low concentrations of IL-2. IL-2 signals through activation of Stat5 (signal transducer and activator of transcription 5) proteins to promote T cell proliferation and survival[187]. $CD4^+$ T cell differentiation into different T helper lineages is determined by the cytokine milieu present during the initial proliferative phase of T cell activation[188], whereas $CD8^+$ T cells are already predestined to become cytotoxic T cells[189, 190].

T helper cell differentiation into subsets with unique cytokine expression profiles

T helper cell differentiation is classically regarded as a dichotomy between two main cell types, termed Th1 and Th2[188, 191] **Fig. 7**). Th1 cells produce IFN-γ as their signature cytokine and are predominantly involved in cell-mediated immunity against intracellular pathogens, through activation of macrophages and directing the production of opsonising antibodies by B cells (i.e. antibodies that trigger the elimination of pathogens by phagocytosis). In contrast, Th2 cells fail to produce IFN-γ and their signature cytokines are IL-4, IL-5 and IL-13 encoded by genes clustered in a Th2 cytokine locus, also containing the constitutively expressed *Rad50* gene (**Fig. 8**). Th2 cells are the most effective activators of B cell proliferation and antibody production, thereby mediating humoral immunity essential for the eradication of extracellular pathogens.

The differentiation process of naïve $CD4^+$ T cells into distinct T helper effector

subsets involves both TCR- and cytokine-mediated signalling[188, 191], which are critical for early cytokine production and induction of master transcription factor expression. Cytokine-mediated signalling results in the activation of Stat proteins, which collaborates with master transcription factors in the regulation of distinct patterns of cytokine gene expression, selection of differentiated lineage cells by positive feedback loops and active repression of alternative lineage fates. Two major signalling pathways facilitate Th1 development, one involving IL-12/Stat4 and the other involving IFN-γ/Stat1/T-bet. Activated Stat4 can directly induce IFN-γ production and expression of IL-12Rβ2 and the T-box transcription factor T-bet during Th1 differentiation[192, 193]. Activation of Stat1 by IFN-γ is also important for the induction of T-bet during Th1 differentiation[194, 195]. T-bet is a major transcription factor for inducing IFN-γ production and T-bet-deficient CD4$^+$ T cell have severe defects in Th1 cell differentiation[196, 197]. T-bet induces IFN-γ and IL12Rβ2 expression, thus promoting selective Th1 cell expansion in response to IL-12[198, 199]. Th2

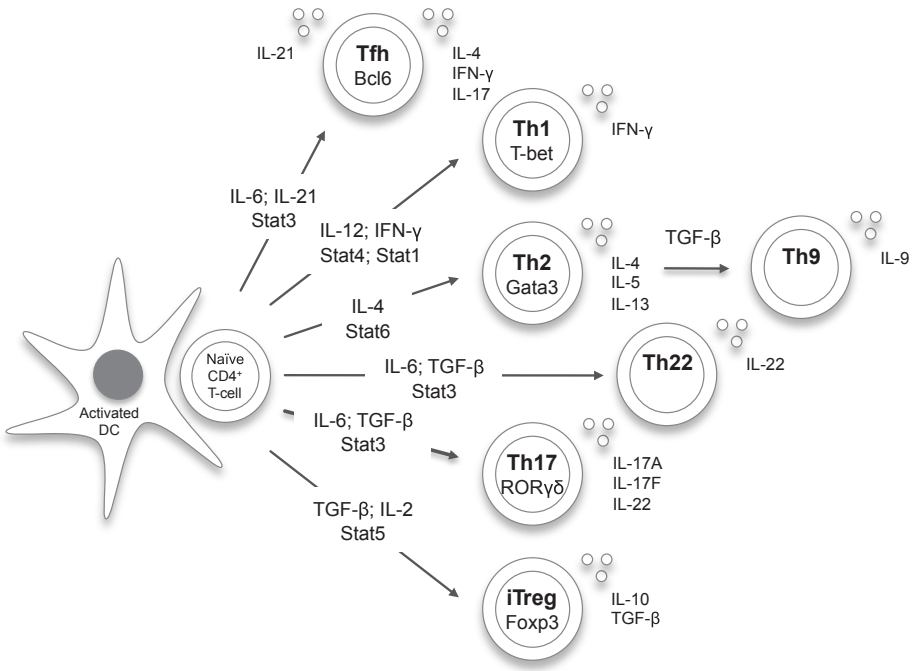

Figure 7. Differentiation of effector CD4$^+$ T-cells subsets in the mouse. During activation in a particular cytokine milieu, naïve CD4$^+$ T-cells may differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17, and inducible regulatory T-cells (iTregs). Different differentiation programs triggered by TCR and cytokine signalling are mainly based on the activation of Stat proteins and induction of master transcription factors that collaborate in the regulation of unique patterns of cytokine production. However, cytokine production by Th cells might be more flexible than previously though and recently T follicular helper (Tfh), Th9 and Th22 cells have been described. Whether these subsets represent distinct lineages remains to be elucidated. **See Appendix for full-color figure.**

cytokine production is dependent on the transcription factor Gata3[200, 201], which is rapidly induced by IL-4 through Stat6[202-206]. During the induction phase of Th2 polarization, early TCR-dependent and IL-4/Stat6-independent IL-4 transcription requires induction of Gata3 expression by TCR-signalling and activated Stat5 in response to IL-2[207]. Gata3 expression is further enhanced by IL-4/Stat6 signalling and an auto-regulatory positive feedback loop[208]. In the absence of Gata3 Th2 differentiation is totally abolished[209-211]. In contrast, deletion of *Gata3* from fully differentiated Th2 cells has only a modest effect on IL-4 production but completely blocks the production of IL-5 and IL-13[210]. Importantly, master regulators and Stat proteins collaborate with other transcription factors (for example, Runx1, Runx3, IFN regulatory factor (Irf) 4, Irf1, Gfi-1, Ikaros and c-Maf) and signalling pathways (Ca^{2+}/Nfat, NF-κB and Notch signalling) in the fine-tuning of T helper cell differentiation and function[188]. Of interest, different Notch ligands on APCs have been proposed to instruct distinct T helper cell fates[212]. Particularly, Notch appears to direct Th2 differentiation by directly regulating the expression of *Gata3* and *Il4*[212-214].

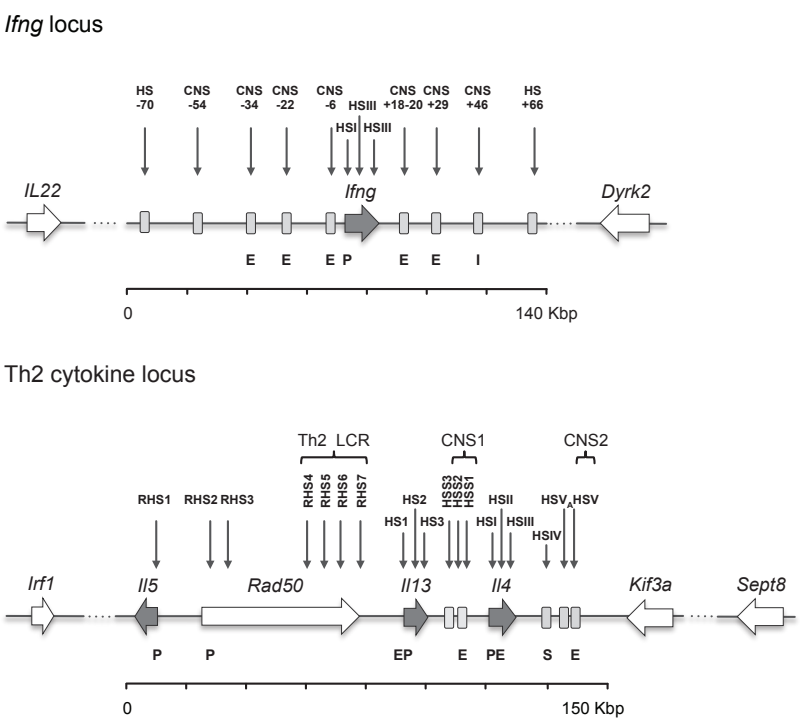

Figure 8. Schematic representation of the mouse *Ifng* and Th2 cytokine loci. The *Ifng* and Th2 cytokine loci span a 140 and 150 Kbp region, respectively. *Cis*-regulatory elements, denoted by the presence of DNaseI hypersensitive sites (HS) and conservation of non-coding sequences (CNS) are indicated by arrows. E, enhancer; P, promoter; I, insulator; LCR, locus control region; S, silencer; RHS, *Rad50* HS.

More recently, Th17 cells have been described as a distinct T helper subset that does not express T-bet or Gata3 and which differentiation is inhibited by IFN-γ or IL-4[215-219] (**Fig. 7**). These cells are characterized by the production of IL-17A, IL-17F and IL-22 as signature cytokines and contribute to host defense against extracellular pathogens particularly at mucosal surfaces. In mice, both TGF-β and IL-6 are required to drive Th17 differentiation through activation Stat3 and induction of the retinoid related orphan receptor-γt (RORγt)[220-226]. The residual IL-17 production in RORγt-deficient cells appears to be dependent on the activity of a related nuclear receptor RORα, which is also upregulated in Th17 cells[227]. Th17 cells are also good producers of IL-21 and express IL-23R as part of their differentiation process[224-226, 228]. Similarly to IL-6, IL-21 and IL-23 also signal through activation of Stat3 and in Stat3-deficient mice IL-17 production in $CD4^+$ T cells is abolished[224-226, 228-231]. IL-21 and IL-23 possibly function in a positive feedback loop to promote expansion and terminal differentiation of Th17 cells at later stages. Stat4 also mediates IL-23 signals and was also shown to direct the development of Th17 cells[230, 232].

Recent studies on T helper cell differentiation have revealed more flexibility in cytokine production than predicted by conventional models of T helper cell lineage commitment (**Fig. 7**). One example of T helper cell phenotype plasticity is that Th2 cells can acquire IL-9-producing capacity in the presence of TGF-β via Irf4 activation, being thereby reprogrammed into a "Th9" pathway[233-235]. Similarly, differentiation of T helper cells producing IL-22 ("Th22") and not IL-17 in response to IL-6 and TGF-β has recently been identified and shown to depend on the transcription factors RORγt and aryl hydrocarbon hydrocarbon receptor (AHR)[236, 237]. T follicular helper (Tfh) are yet another $CD4^+$ T cell population with a particular function, helping B cells make antibody responses to T cell-dependent antigen during a germinal center reaction (see below)[238, 239]. Tfh cells express Bcl6 as their master transcription factor and may produce Th1, Th2 or Th17 cytokines, depending on the conditions of their initial activation[240-244]. Whether Tfh cells are a distinct lineage (essentially parallel to Th1, Th2 or Th17 cells) or a particular phenotypic state of some or all of these effector lineages remains unresolved.

Regulation of lineage-specific patterns of cytokine gene expression: the Th1/Th2 paradigm

During T helper cell differentiation, fully polarized T helper cells are generated through lineage commitment and active suppression of alternative lineage fates. The mutual exclusivity among master transcription factors and Stat proteins regulated at the transcriptional levels and through suppressive protein-protein interactions appears to be one of the main mechanisms for cross-regulation during T helper differentiation[188, 191]. For

example, Gata3 suppresses Th1 development by downregulation of *Stat4*[193, 245] and T-bet restrains Th2 development by inhibiting *Gata3* expression[192] and physically interacting with Gata3 thereby repressing its function[246]. However, more precise control of gene expression during T helper differentiation is achieved through epigenetic processes, which facilitate heritable and stable programs of gene expression, while preserving the potential for these programs to be modified in response to environmental changes[247-249]. These include chromatin remodelling (induction of DNaseI hypersensitive sites by altering nucleossome positioning) through histone acetylation and/or methylation and DNA demethylation at critical *cis*-regulatory elements present in cytokine loci (**Fig. 8**). Changes to high-order chromatin structure at *Ifng* and Th2 cytokine loci, have also been implicated in the coordinate regulation of cytokine gene expression.

In naïve CD4$^+$ T cells, low-level expression of Th2 cytokines and IFN-γ can be detected upon activation by TCR ligation[250, 251], which is supported by both permissive and repressive histone modifications at both loci[247, 248]. This bivalent epigenetic state may help to "poise" the Th2 cytokine and *Ifng* loci for either expression or complete silencing during T helper differentiation. In the Th2 locus, Th2 cytokine genes promoters and the Th2 locus control region (LCR) come into close spatial proximity to form a higher-order chromatin structure, further suggesting that early expression of the Th2 cytokines in naive T cells is supported by an initial "poised" three-dimensional chromatin configuration[252, 253] (**Fig. 9**). Interchromosomal interactions between the *Ifng* and the Th2 cytokine locus have also been described in naïve CD4$^+$ T cells and are apparently

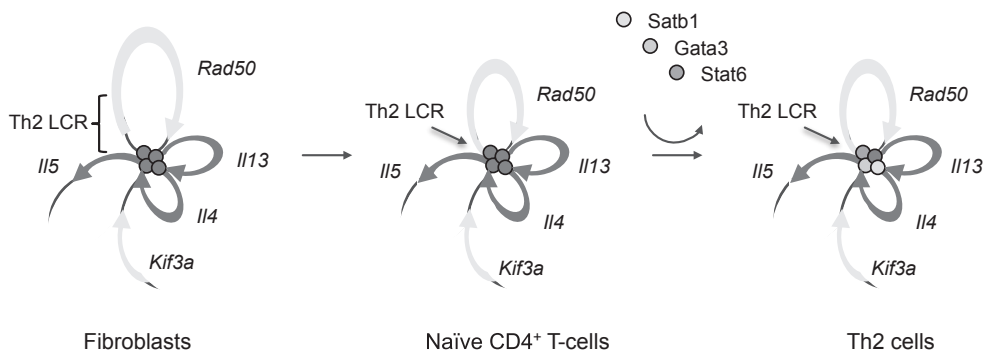

Figure 9. "Poised" chromatin configuration in the Th2 cytokine loci. An initial chromatin configuration is detected in non T-cell lineage cells, such as fibroblasts, characterized by close spatial proximity between Th2 cytokine gene promoters. In naïve CD4$^+$ T-cells, the Th2 locus control region (LCR) further contributes to this higher order chromatin conformation to generate a "poised" state. Upon Th2 differentiation, recruitment of cell-specific factors, such as Gata3, Stat6 or Satb1, to this "poised" chromatin configuration potentiates the coordinate expression of Th2 cytokine genes. Figure adapter from[253].

lost upon Th1 or Th2 cell differentiation[254]. However, unequivocal data required to prove that interchromosomal cytokine gene regulation truly represents another level of control within the nucleus is lacking[255]. Upon Th2 differentiation, a substantial increase in the transcriptional activity of *Il4*, *Il5* and *Il13* and concomitant silencing of *Ifng* are observed. The converse pattern of gene activation and silencing is present in differentiating Th1 cells. Several studies have demonstrated that cell type-specific transcription factors and Stat proteins binding to *cis*-regulatory elements of cytokine genes directs subsequent recruitment of chromatin modifying proteins, which initiate substantial and reciprocal alterations in the chromatin structure of the Th2 cytokine and *Ifng* loci[247-249]. Likewise, both Stat4 and T-bet have been implicated in permissive histone modifications (acetylation and/or methylation) at the *Ifng* locus[196, 198, 256-261]. Similarly, Stat6 and Gata3 are involved in Th2 locus permissive histone modifications and DNA demethylation[208, 257, 262-268]. By contrast, Stat6 and Gata3 binding to the *Ifng* locus when naïve $CD4^+$ T cells differentiate into Th2 cells is associated with increased acquisition of repressive histone methylation marks[269]. During Th2 differentiation, increased DNA methylation at the *Ifng* locus is also observed[269-271]. In addition, T-bet is required for the silencing of *Il4* in Th1 cells[251, 260, 272, 273]. During Th1 and Th2 differentiation, chromatin looping at *Ifng* and Th2 cytokine loci has also proven to be essential for cytokine gene expression. Indeed, both Stat6 and Gata3 are responsible for the establishment and/or maintenance of a “poised” chromatin configuration in the Th2 cytokine locus of developing Th2 cells[252, 253]. Further changes to this conformation occur through Th2-specific triggering of Satb1 (special AT-rich binding protein 1, an architectural factor) expression, which promotes the formation of additional loops and more intimate interactions between *cis*-regulatory elements and the *Il4, Il5* and *Il13* promoters[274]. In the absence of Satb1 these changes are lost and Th2 cytokine expression is compromised[274]. In Th1 cells, T-bet has also been implicated in the formation of Th1-specific *Ifng* locus architecture that brings *cis*-regulatory regions in close proximity to the *Ifng* promoter[275]. Finally, the expression of T-bet and Gata3 is not essential but does help to maintain the Th1- and Th2-specific gene expression program, respectively[199, 210, 211, 265, 276]. Although cytokine gene expression can be partially sustained through hereditable epigenetic modifications, it benefits from collaboration between lineage-specifying transcription factors and epigenetic processes.

Differentiation of cytotoxic T cells

Naïve $CD8^+$ T cells differentiate into cytotoxic T cells in response to IL-2 that drives their proliferation and differentiation[189, 190]. Cytotoxic T cells are involved in the destruction of cells infected with virus or other cytosolic pathogens and tumor cells. The main mechanism of cytotoxic T cell action is the release of specialized lytic granules upon

recognition of antigen:MHCI complexes on the surface of a target cell[277]. These granules contain three distinct classes of cytotoxic proteins required for effective target cell killing: perforin (forms transmembrane pores in target cell membranes), granzymes (induces apoptosis) and granulysin (induces apoptosis and also has antimicrobial action). Cytotoxic T cells also act by releasing cytokine such as IFN-γ, which can limit the spread of cytoxolic pathogens in a variety of ways[278, 279]. Besides being involved in the activation of macrophages, IFN-γ inhibits viral replication directly, induces the expression of MHCI molecules and other proteins involved in MHCI antigen presentation pathway, and interferes with tryptophan metabolism in target cells thus possibly killing intracellular pathogens by starvation. Two highly homologous T-box transcription factors, T-bet and Eomesodermin (Eomes) expressed in effector T cells act in concert to promote IFN-γ, granzyme and perforin production[197, 280, 281].

Other T cell subsets

Regulatory T cells

In contrast to the protective functions of T helper cells, inappropriate Th2 cell responses give rise to allergic diseases, whereas autoimmune diseases result from inappropriate Th1 and Th-17 cell responses. Regulatory T (Treg) cells restrain excessive effector T cell responses and thus account for the maintenance of immune homeostasis and prevention of immunopathology[282, 283]. Treg cells are naturally present in the immune system as a functionally distinct $CD4^+$ T cell population generated in the thymus that express CD25 and the forkhead transcription factor Foxp3, essential for their function[284-289]. In addition, Treg cells can also be induced to differentiate from naïve $CD4^+$ T cells in the periphery when activated in the presence of TGF-β, which results in the upregulation of Foxp3. The differentiation of both induced Treg (iTreg) cells and Th17 cells is developmentally related with respect to TGF-β requirement and IL-6 critically determines the balance between the two subsets by downregulating Foxp3 in differentiating and differentiated Treg cells[220-222]. Treg cells exert suppression by cell-contact mechanims (for example, killing of APCs or effector cells by means of granzyme or perforin) as well as mechanisms mediated by soluble factors (for example, secretion of immunosuppressive cytokines such as IL-10 or TGF-β or deprivation of cytokines such as IL-2 necessary for expansion and/or survival of effector cells)[290].

γδ T cells

γδ T cells represent only a minor but distinct subset of peripheral T cells present in the spleen, lymph nodes, epithelia and mucosa[291, 292]. The functional role of γδ T cells

remains obscure, although considerable insight comes from recent studies in mouse models of disease. Functional development of γδ T cells begins in the thymus, where certain subsets already exhibit differential competence for effector cytokine production[293-299]. In the periphery, most γδ T cells recirculate and temporarily reside in lymphoid tissues where they exert their functional effects as polyclonal unselected populations. Nevertheless two TCR-defined γδ T cells subsets directly colonize peripheral epithelia and mucosa, and exert relatively uniform and defined functions[300-303]. Polyclonal activation and functional induction of intrathymic committed γδ T cells in the periphery might require cytokines and also TCR-ligand interactions, although putative ligands are still unknown[299, 301, 304-308]. γδ T cells often respond faster than αβ T cells and can assume both an effector or a regulatory role in immune responses. They can also support and regulate antibody production by B cells. Recently, IL-17-producing γδ T cells have been implicated in host defense to *Mycobacterium tuberculosis* infection[309].

PART III – CTCF

During lymphocyte development and differentiation into functional effector subsets, proper spatial and temporal control of gene expression is achieved through the coordinate binding of lineage-specific transcription factors to *cis*-regulatory elements. In many developmentally regulated gene clusters, as for example the antigen receptor and T helper cytokine gene loci, gene regulation is particularly complex[165, 248]. This involves cell-type-specific interactions over large genomic distances between remote enhancers, silencers or LCRs and the promoters of particular genes. Regulation of higher-order chromatin structures is mediated by protein complexes composed of lineage-specific transcription factors and ubiquitously expressed proteins. For instance, the erythroid transcription factor Gata1 has been shown to be part of a complex composed of the hematopoietic factors Lmo-2, Tal1 and E2A and the ubiquitous nuclear protein Ldb1[310-312], involved in long-range chromatin interactions at the mouse *β-globin* locus[313, 314]. The CCCTC-binding factor (CTCF) is a leading candidate for the regulation of gene expression at complex loci during lymphocyte development and differentiation into functional effector subsets.

General introduction into the biology of CTCF: a multivalent factor

CTCF is an ubiquitously expressed and highly conserved 11-zinc finger DNA-binding protein implicated in a variety of regulatory functions including transcriptional activation/repression, enhancer blocking and/or barrier gene insulation, hormone-responsive gene silencing, genomic imprinting, X chromossome inactivation and long-range chromatin interactions[315, 316]. Recent studies have mapped genome-wide CTCF occupancy and distribution patterns in multiple divergent cell types by employing chromatin immunoprecipitation (ChIP) in combination with tilling arrays (ChIP-chip) or high-throughput sequencing (ChIP-Seq). In human fibroblasts 13,804 CTCF-binding sites were reported[317] using ChIP-chip while ChIP-Seq analysis identified 20,262 CTCF-binding sites in resting human $CD4^+$ T cells[318]. ChIP-Seq analysis revealed 39,609 CTCF-binding sites in mouse embryonic stem cells[319], as well as 19,308 and 19,572 in HeLa and Jurkat cells, respectively[320]. Importantly, these studies have enabled the identification of a ~11-15 bp core consensus sequence for CTCF-binding sites, although initially CTCF was shown to bind to highly divergent 50-60 bp sequences through the combinatorial use of different sets of its 11 zinc fingers[315]. In fact, it was proposed that “during the formation of a CTCF-DNA complex both DNA and CTCF allosterically customize their conformation to engage different zinc fingers either for making base contacts or to make a target-specific surface that determines interactions between

CTCF and other proteins"[315]. This property would be on the basis of CTCF versatility or multiple functional roles in gene regulation. In line with this, CTCF binding partners that have been identified to date are involved in multiple processes in the nucleus and include transcription factors (Yy1, Kaiso and YB-1)[321-323], chromatin modifying proteins (Sin3a, CHD8, Suz12)[324-326], the nucleolar protein nucleophosmin[327], RNA polymerase II[328] and poly(ADP-ribose) polymerase-1 (PARP1)[329].

Genome-wide CTCF binding patterns suggest a role for CTCF distinct from that of canonical transcription factors[316]. CTCF distribution strongly correlates with gene density but not with transcriptional start sites and the binding pattern of CTCF does not appear to predict cell-specific gene expression levels[319]. Domains with few or no CTCF-binding sites tend to include clusters of transcriptionally coregulated genes or developmentally related genes (for example, olfactory receptor gene clusters or keratin-associated protein gene clusters), whereby these regions are often flanked by CTCF-binding sites[317]. CTCF-rich regions contain genes displaying extensive alternative promoter usage (for example, *Tcrβ*, *Tcrα/δ* and *Igλ* L chain loci)[317]. Prior to genome-wide queries, many studies have suggested that CTCF is the archetypal vertebrate protein that binds insulator sequences. Insulators are genomic elements that affect gene expression by blocking the inappropriate communication between neighboring regulatory elements (enhancer-blocking insulators) and preventing the spread of repressive heterochromatin from adjacent sequences (barrier insulators)[330]. The first link between CTCF and enhancer-blocking gene insulation was proposed based on the discovery that CTCF binds to the 5'HS4 insulator sequence upstream of the chicken *β-globin* locus[331] and another insulator sequence at the 3'end of this domain[332]. CTCF mediated insulator activity has been predicted at several other sites including the *H19/Igf2* genes imprinted control region (ICR)[333-336], the *DM1* locus[337] and the boundaries of domains that escape X chromosome inactivation[338]. More recently, computational analyses have been employed to show that CTCF binds to highly conserved non-coding elements across 12 mammalian species, which provided evidence supporting a global role for CTCF as an insulator protein[339]. Although CTCF is generally considered to function via enhancer-blocking mechanisms, genome-wide data sets also uncovered a small proportion of CTCF-binding sites at the boundaries between active and repressive chromatin domains, which can reflect a role for CTCF in barrier insulation[318, 320].

General introduction into CTCF function in particular loci

CTCF-mediated gene insulation via chromatin loops

Enhancer-blocking CTCF-driven insulation may occur through the formation of long-

range chromatin interactions or chromatin loops. A recent study has provided the first direct evidence that loop formation can occur via contact between two CTCF-bound insulators *in vivo*[340]. This was shown, by inserting the human *β-globin* HS5 (the orthologue of the CTCF-dependent chicken HS4 insulator) between the mouse *β-globin* LCR and downstream genes, which results in the formation of a new chromatin loop with endogenous HS5 that topologically isolates the LCR and disrupt *β-globin* gene transcription. Moreover, at the imprinted *H19/Igf2* locus CTCF-mediated enhancer-blocking insulation have been demonstrated to occur via long-range chromatin interactions in the native genomic context[341, 342] (**Fig. 10**). CTCF is considered to play a critical role in the establishment and maintenance of allele-specific DNA methylation imprints and parent-of-origin *H19* and *Igf2* gene expression patterns during development.

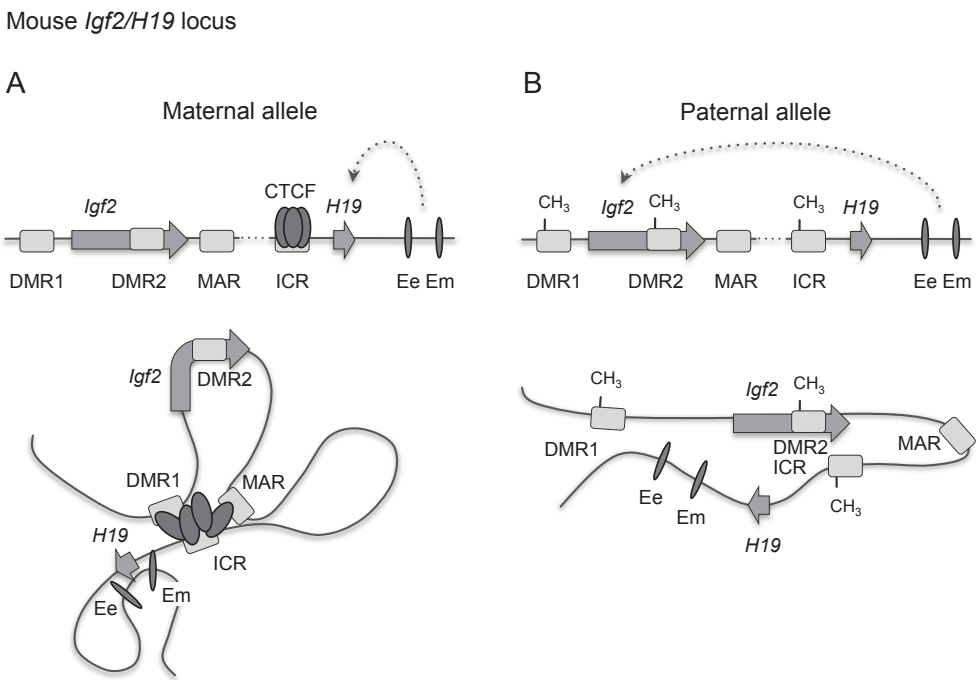

Figure 10. Schematic representation of the mouse *Igf2/H19* locus. The maternally expressed noncoding *H19* gene (**A**) is located approximately 90 Kbp downstream from the gene encoding Insulin-like growth factor 2 (*Igf2*) that is expressed exclusively from the paternal allele (**B**). The imprinting control region (ICR) ~2 Kbp upstream of *H19* contains four CTCF binding sites and is essential for the regulation of the entire locus. Differentially methylated regions (DMRs) such as DMR1 upstream of *Igf2* promoters and DMR2 within *Igf2* exon 6, act in concert to regulate reciprocal, allele-specific expression patterns from a shared set of downstream enhancers at 8 Kbp (Ee, endodermal tissue enhancer) and 25 Kbp (Em, mesodermal tissue enhancer) downstream of the H19 gene. Allele-specific patterns of CTCF binding, DNA methylation ($-CH_3$) and chromatin looping account for parent-of-origin *H19* and *Igf2* gene expression patterns during development. Figure adapter from[316]. **See Appendix for full-color figure.**

Four methylation-sensitive CTCF-binding sites are located in the ICR, immediately upstream of the *H19* gene. On the maternal allele CTCF binds to the unmethylated ICR to form a tightly coiled chromatin loop structure around the *Igf2* gene, thereby making the *Igf2* promoters inaccessible to the enhancers downstream of *H19*. By contrast, on the paternal allele the ICR is methylated, which abrogates CTCF binding and ICR-mediated insulation resulting in functional communication between the *Igf2* promoters and the enhancers in order to activate *Igf2* expression. *H19* is only expressed from the maternal allele, since loss of CTCF binding to the methylated ICR is thought to silence *H19* on the paternal allele due to promoter methylation.

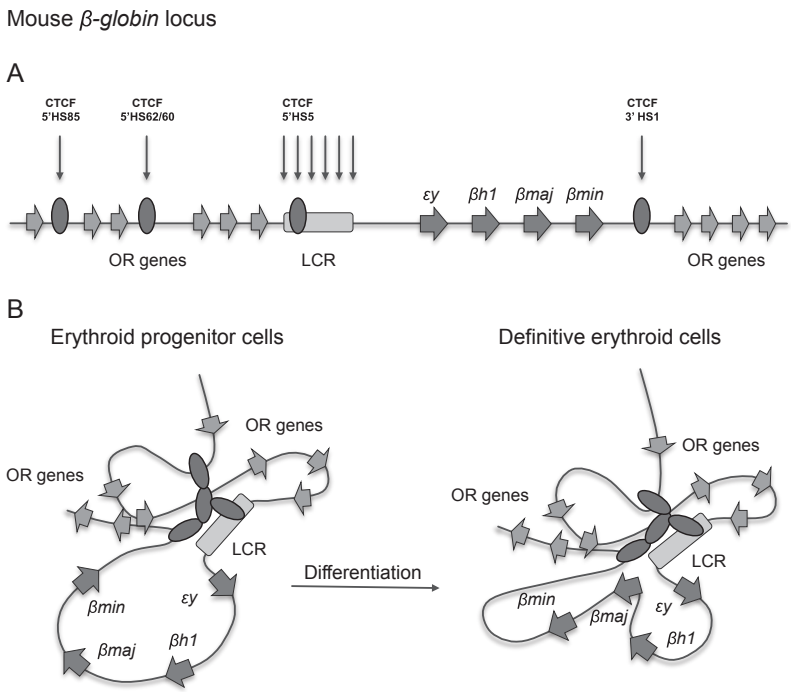

Figure 11. Schematic representation of the mouse *β-globin* locus. (**A**) Four globin genes are embedded within a larger olfactory receptor (OR) gene cluster. Developmentally regulated globin expression (*εy* and *βh1* in primitive erythroid cells; *β-major* (*βmaj*) and *β-minor* (*βmin*) in definitive erythroid cells) is regulated in part by a series of *cis*-regulatory elements surrounding the locus (indicated by arrows). An upstream locus control region (LCR) containing six DNaseI hypersensitive sites (HS) is required for high-level transcription. Three CTCF binding sites have been identified upstream (5'HS85, 5'HS62/60 and 5'HS5) and one 20 Kbp downstream (3'HS1) of *globin* genes. (**B**) Cell type-specific CTCF-mediated intrachromosomal interactions in the mouse *β-globin* locus. Although loops are illustrated by CTCF multimerization, it is not clear whether CTCF binds directly to each site or through interaction with other proteins that are not represented for simplicity. In definitive erythroid cells full expression of, for example, the *βmaj* gene depends on stable LCR-gene contacts. Figure adapter from[316]. **See Appendix for full-color figure.**

CTCF-based chromatin loops beyond gene insulation?

CTCF-based intrachromossomal interactions between distal regulatory elements have also been reported at other developmentally regulated gene clusters, such as the *β-globin* and the *MCHII* loci. CTCF binds to three sites upstream (5'HS85, 5'HS62/60 and 5'HS5 in the LCR) and one downstream (3'HS1) of the mouse *β-globin* locus in a cell-type specific manner[343-345] (**Fig. 11**). During erythroid differentiation, CTCF-bound regulatory sequences throughout the *β-globin* locus came into close spatial proximity to form an "active chromatin hub" where stage-specific *globin* genes also interact with the LCR resulting in looping-out of transcriptionally silent isoforms[345-347]. Conditional CTCF deletion or mutation of the 3'HS1 CTCF-binding site results in disruption of CTCF contacts in erythroid progenitors but surprisingly has no effects on the kinetics of levels of *globin* gene expression during erythroid differentiation[345]. One explantion could be the redundacy of chromatin contacts in the *β-globin* locus that may not be readily abrogated by mutations in one regulatory element. The functional significance of

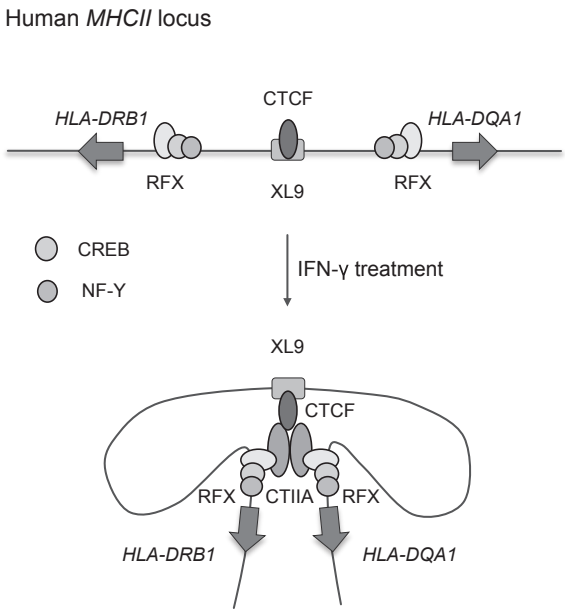

Figure 12. Schematic representation of the human *MHCII* locus.
In the human *major histocompatibility complex class II* (*MHCII*) locus, CTCF binds to the XL9 enhancer element between two coregulated genes, *HLA-DRB1* and *HLA-DQA1*, driven by divergent promoters. Transcription factors RFX, CREB and NF-Y bind to regulatory sequences within the proximal promoter of *MHCII* genes when they are transcriptionally inactive. Interferon-γ (IFN-γ) treatment induces transcription in non-expressing cell types by upregulating the non-DNA binding coactivator CIITA, which subsequently forms an heterodimer with RFX-CREB-NF-Y-bound promoters and the CTCF-bound enhancer in parallel with *HLA-DRB1* and *HLA-DQA1* gene activation. Figure adapter from[316]. **See Appendix for full-color figure.**

CTCF-mediated insulation at the 3'HS1 and 5'HS5 of the *β-globin* locus is also unclear. Genetic disruption of CTCF-binding to HS1 has no effect on the transcription of the neighbouring olfactory receptor genes[345] and deletion of 3'HS1 and 5'HS5 results in normal *globin* gene expression[348, 349].

Likewise, at the human *MHCII* locus CTCF may not function as a canonical enhancer-blocking insulator either. CTCF binds to the intergenic enhancer XL9 that coregulates the expression of the MHCII genes *HLA-DRB1* and *HLA-DQA1* and shows enhancer-blocking activity *in vitro*[350] (**Fig. 12**). In non-MHCII-expressing epithelial cells, stimulation with IFN-γ results in the expression of the transcriptional coactivator CIITA and the concurrent formation of chromatin interactions between XL9 and the two divergent promoters upstream of *HLA-DRB1* and *HLA-DQA1.* Chromatin loop formation is required for gene expression and depends on XL9-bound CTCF, the non-DNA-binding coactivator CIITA and the transcription factor RFX constitutively bound to regulatory sequences within the proximal promoter of *HLA-DRB1* and *HLA-DQA1* genes. Therefore, in the human *MHCII* locus CTCF is involved in the control of gene expression through the ability to form transcriptionally functional chromatin loops. Nevertheless, we cannot rule out the possibility that XL9-bound CTCF is blocking inappropriate regulatory elements contained outside the *HLA-DRB1* and *HLA-DQA1* region.

CTCF as a genome-wide organizer of chromatin architecture

Mechanistic insight into CTCF-mediated chromatin loop formation has recently been uncovered by genome-wide studies showing that cohesin proteins are highly enriched at most CTCF-binding sites[351-354]. CTCF is required for cohesin recruitment to these sites because depletion of CTCF leads to absence of cohesin binding. Because cohesin proteins are though to coalesce into a ring-like structure that mediates sister chromatid cohesion during mitosis, it is tempting to speculate that a similar mechanism could facilitate the stabilization of CTCF-based chromatin loops. In support of this notion, cohesin proteins are required for CTCF-mediated long-range intrachromosomal interactions at the developmentally regulated *IFNg* locus[355]. Functional interaction between cohesins and CTCF is also observed at sites where CTCF was previously shown to have enhancer-blocking insulator activity, such as the HS4 of the chicken *β-globin* locus and *H19/Igf2* locus ICR[351, 352]. The discovery that a high proportion of CTCF-binding sites are shared with cohesin provide a strong indication that cohesin and CTCF might act in concert at many genomic locations to facilitate the formation of long-range chromatin interactions. Together with unique CTCF distribution patterns genome-wide these results implicate CTCF as a genome-wide organizer of chromatin architecture, with insulation and/or downstream effects in transcription a secondary consequence of

the genomic context of the endogenous locus[316]. In line with this view, recent studies have implicated CTCF in interchromosomal interactions in the maternal (CTCF-bound) *H19/Igf2* ICR allele[356-358] or between homologous X chromosomes during the process of X chromosome inactivation[359, 360]. Although the functional significance of CTCF-mediated interchromosomal contacts remains to be fully elucidated, it has been suggested that, at least at the *H19/Igf2* ICR, these may function to control epigenetic information *in trans* [356, 358]. CTCF has also been suggested to demarcate the boundaries of "lamina-associated domains" (LADs), genomic regions localized to the nuclear periphery via interaction with the nuclear lamina protein LaminB1 that correlate with markedly decreased gene expression[361]. One example is the CTCF-binding site located 10 Kb upstream of the human *c-myc* gene that maps to a LAD border, which has been implicated in nuclear organization and locus protection from DNA methylation[362, 363]. Genome-wide CTCF-binding sites also reveal a high percentage of CTCF binding sites in exons, introns and 5' or 3' untranslated regions (UTRs), suggesting that intRagenic and/or single gene loops may also have functional roles[317-320, 339]. For example, in the human *c-Myc* gene one CTCF-binding site found immediately downstream of the major P2 promoter maps precisely within the region of RNA polymerase II pausing and release[364]. It is also plausible that intragenic and/or single gene loop formation facilitates the coordination of RNA processing or transcriptional reinitiation. Nevertheless, the possibility remains that CTCF can function in the regulation of gene expression via looping-independent mechanisms by simply recruiting proteins involved in transcriptional activation and/or repression, as for example histone modifying proteins[324, 326], chromatin remodeling factors[325] or RNA polymerase II[328].

AIM OF THE THESIS

Lymphocyte development and differentiation into functional effector subsets provide an excellent model system for the study of developmentally regulated gene expression. In particular, regulation of antigen receptor and T helper cytokine gene loci is largely determined by the coordinate binding of lineage-specific transcription factors to *cis*-regulatory elements located over considerable genomic distances. Emerging evidence suggests that higher-order chromatin structure is part of the mechanism of gene regulation at these complex loci. CTCF is a highly conserved and ubiquitously expressed DNA-binding protein implicated in genome-wide organization of chromatin architecture and non-canonical gene regulatory functions. Direct evidence for CTCF-mediated higher-order chromatin structure has been reported at several developmentally regulated gene loci, including the mouse *β-globin* and *H19/Igf2* loci and the human *MHCII* locus. Cell-type-specific regulation of chromatin looping possibly arises through cooperation between the ubiquitous architectural factor CTCF and lineage-specific transcription factors. This thesis describes studies aimed to unravel the function of CTCF throughout B- and T-lymphocyte development and T helper cell differentiation, while further exploring the role of the T-cell lineage transcription factor Gata3.

The role of CTCF in essential cellular processes as proliferation, survival and differentiation during lymphocyte development, benefits from *in vivo* analyzes. Particularly, we intended to analyze CTCF function in the regulation of antigen receptor and T helper cytokine gene loci in the *in vivo* context of cellular differentiation and function. Therefore, a conditional *Ctcf* knockout was generated for B- and T-lymphocytes using the Cre-loxP system in **Chapter 2**-**4**. As developmental arrest was expected upon *Ctcf* deletion, differentiation was rescued by Ig trangenes (B-cell lineage, **Chapter 2**) or different Cre-expressing transgenic lines (T cell lineage, **Chapter 3 and 4**). This allowed us to investigate *in vivo* function of CTCF at different stages of lymphocyte development and in the two different lymphocyte lineages, to get a comprehensive analysis of the role of CTCF. Importantly, the *Ctcf* conditional knockout mouse model provided the unique and exciting opportunity to study the consequences of CTCF deletion in the fidelity of *Igκ* locus *cis*-regulatory elements interactions genome-wide in an *in vivo* context (**Chapter 2**). Furthermore, the role of CTCF in the differentiation of naïve $CD4^+$ T-cells into different T helper cell subsets *in vitro* could be compared with *in vivo* immune responses (**Chapter 4**).

In **Chapters 5** and **6** the molecular mechanisms underlying the role of Gata3 in the T-cell lineage by enforced Gata3 expression are uncovered. **Chapter 5** describes Gata3 target genes in DP thymocytes that may underpin its role in positive selection

towards CD4 T cell lineage. **Chapter 6** shows how Gata3, the master regulator of Th2 differentiation, affects the differentiation of CD4 T cells towards the recently identified Th17 lineage. We discuss the implications of the main findings described in **Chapters 2-6** and provide directions for future research in **Chapter 7**.

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2

CTCF limits proximal V_κ recombination and restricts intronic and 3' κ enhancer interactions to the immunoglobulin κ light chain locus

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Submitted

3

CTCF regulates cell cycle progression of αβ T cells in the thymus

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ABSTRACT

The 11-zinc finger protein CCCTC-binding factor (CTCF) is a highly conserved protein, involved in imprinting, long-range chromatin interactions and transcription. To investigate its function *in vivo*, we generated mice with a conditional *Ctcf* knockout allele. Consistent with a previous report, we find that ubiquitous ablation of the *Ctcf* gene results in early embryonic lethality. Tissue-specific inactivation of CTCF in thymocytes specifically hampers the differentiation of αβ T cells and causes accumulation of late double-negative and immature single-positive cells in the thymus of mice. These cells are normally large and actively cycling, and contain elevated amounts of CTCF. In *Ctcf* knockout animals, however, these cells are small and blocked in the cell cycle due to increased expression of the cyclin-CDK inhibitors p21 and p27. Taken together, our results show that CTCF is required in a dose-dependent manner and is involved in cell cycle progression of αβ T cells in the thymus. We propose that CTCF positively regulates cell growth in rapidly dividing thymocytes so that appropriate number of cells is generated before positive and negative selection in the thymus.

INTRODUCTION

The 11-zinc finger protein CCCTC-binding factor (CTCF) is a widely expressed and highly conserved transcriptional regulator implicated in many important processes in the nucleus (for reviews, see[1, 2]). In line with this view, murine CTCF is essential for early embryonic development[3]. CTCF is the archetypal vertebrate protein that binds insulator sequences, DNA elements that have the ability to protect a gene from outside influences[4]. Its methylation-sensitive interaction with the imprinting control region of the H19/insulin-like growth factor 2 (*Igf2*) genes indeed controls enhancer access[3, 5, 6]. CTCF-mediated insulator activity has been predicted at several other sites including the *DM1* locus and boundaries of domains that escape X-chromosome inactivation[7, 8]. We have shown that CTCF mediates long-range chromatin interactions and regulates local histone modifications in the β-globin locus[9]. Evidence has furthermore been presented for a function of CTCF in inter-chromosomal interactions between *Igf2* and other loci[10]. During mitosis, CTCF remains bound to mitotic chromosomes, possibly facilitating reformation of higher-order chromatin loops after mitosis[11]. Taken together, these data suggest that CTCF is an essential organizer of imprinting, long-range chromatin interactions and transcription.

Genome-wide mapping of CTCF-binding sites revealed ~14 000 sites, whose distribution correlates with genes but not with transcriptional start sites[12]. Strikingly, the 20-bp consensus motif found in the majority of the sites is virtually identical to a consensus sequence (called LM2*), which is bound by CTCF and is found in ~15

000 conserved non-coding elements (CNEs) in the human genome[13]. Thus, CTCF-binding sites are part of CNEs, which are conserved across species and appear to have regulatory functions. High-resolution profiling of histone methylation in the human genome showed that CTCF sites mark boundaries of histone methylation domains[14] consistent with a function of CTCF as an insulator protein. Genome-wide analyses also revealed CTCF-binding sites near genes displaying extensive alternative promoter usage, including *Protocadherin* γ, the *Immunoglobulin (IG) λ light chain* and the *T cell receptor (TCR) α/δ* and *β* chain loci. In mice, CTCF-dependent insulators were found downstream of the *Tcrα/δ* and the *Ig heavy* chain loci[15, 16]. Moreover, in T cells, CTCF-binding sites overlap significantly with DNAse I hypersensitive sites (HS), suggesting that CTCF is somehow involved in global T-cell gene expression[17]. These data imply an important function of CTCF in lymphocytes, in particular in the regulation of gene transcription and recombination targeting in complex loci.

T-cell progenitors differentiate in the thymus, where early precursors (lacking the cell surface markers CD4 and CD8 and therefore termed double-negative (DN) cells) develop into mature CD4 or CD8 single-positive (SP) T cells, following a regulated differentiation programme (see **Supplementary Fig. 1** for a schematic overview; for review, see[18]). The DN precursor population is generally subdivided into four distinct developmental stages (DN1–DN4), which are defined by differential expression of the cell surface markers CD25 and CD44 (interleukin-2 receptor and phagocyte glycoprotein 1, respectively). Further differentiation of T cells depends on rearrangement of the *TCR* gene segments of four loci, that is, *α*, *β*, *γ* and *δ*. Most mature T cells express an αβ TCR on their cell surface. These are the T cells involved in the classic adaptive immune response. γδ T cells represent only a small fraction of T cells in lymphoid organs of humans and mice (1–5%) and line the epithelial layer of various organs, including the small intestine, but their function remains somewhat enigmatic (for review, see[19]). The choice of whether the *α* and *β TCR* genes are rearranged, or the *γ* and *δ* genes, is made at the DN2–DN3 stage in the thymus.

Tcrβ gene rearrangement is initiated and completed at the DN3 stage. Upon productive (in-frame) rearrangement of the *Tcrβ* gene, the TCRβ chain associates with the invariant pTα chain on the cell surface and forms the pre-TCR complex. Cells that have passed this so-called ‘β-selection’ checkpoint are termed ‘β-selected’ cells. The pre-TCR complex signals cells to proliferate and to downregulate CD25 expression. Cells subsequently acquire both CD4 and CD8 coreceptors to become double-positive (DP) cells, with CD8 usually being expressed first in most mouse strains (immature SP (ISP) cells). As a result, the late DN3, DN4 and ISP stages consist of large cycling cells (see **Supplementary Fig. 1**). The DP cells then leave the cell cycle and rearrange

their *Tcrα* gene locus. If *Tcrα* gene recombination is productive, TCRαβ is expressed on the cell surface of DP cells. TCRαβ-bearing immature cells are selected for major histocompatibility complex (MHC) recognition during the process of positive selection. TCRαβ receptors with specificity for MHC class I will develop into the CD8-positive ($CD8^+$) T-cell lineage, whereas receptors recognizing MHC class II will become CD4-positive ($CD4^+$) T cells. DP thymocytes that fail to recognize MHC class molecules die 'by neglect', whereas potential self-reactive T lymphocytes are eliminated by a process called negative selection. When combined, these selection processes result in the generation of CD4 and CD8 SP thymocytes with TCRαβ receptors that can recognize non-self-antigens presented by MHC class II and I proteins, respectively. Mature SP cells exit the thymus and circulate to the periphery as naive $CD4^+$ and $CD8^+$ T cells.

To circumvent the problem of embryonic lethality[3], we generated mice with a conditional *Ctcf* allele ($Ctcf^{f/f}$). We examined the potential function of CTCF in *Tcr* gene rearrangement and global T-cell gene expression by deleting $Ctcf^{f/f}$ in thymocytes. Here, we show that CTCF exerts an effect as a critical regulator of cell growth and proliferation following β-selection in the thymus. We demonstrate that CTCF expression varies during normal T-cell differentiation, with the highest levels occurring in subpopulations of relatively large and cycling thymocytes, including ISP cells. Interestingly, knockout of *Ctcf* results in a cell cycle arrest at the ISP cell stage, owing to highly increased amounts of the cyclin-CDK inhibitors p21 and p27. CTCF-deleted DN4 and ISP cells are also significantly smaller than normal cells. We therefore propose a global function of CTCF as a positive regulator of cell growth in αβ T cells.

RESULTS

Conditional deletion of the *Ctcf* gene in developing T lymphocytes

To understand CTCF function *in vivo*, we generated a conditional *Ctcf* allele ($Ctcf^f$) by inserting *loxP* sites upstream of exon 3 and downstream of exon 12 (**Fig. 1A**). Equivalent levels of CTCF are expressed in $Ctcf^{f/f}$ and wild-type mice (data not shown). $Ctcf^{+/f}$ mice were crossed with mice expressing Cre recombinase ubiquitously[20]. This causes removal of *Ctcf* exons 3–12 from $Ctcf^f$, yielding the $Ctcf^-$ allele, in which a *Ctcf–lacZ* fusion transcript is expressed instead of *Ctcf* (**Fig. 1A**). $Ctcf^{+/-}$ mice appear normal and are fertile, but no homozygous knockouts are born from $Ctcf^{+/-}$ crosses (**Table 1**), consistent with an essential function of CTCF in early development[3]. Surprisingly, the ratio of wild-type to $Ctcf^{+/-}$ littermates is higher than expected on a Mendelian basis in crosses among $Ctcf^{+/-}$ mice and between wild-type and $Ctcf^{+/-}$ mice (**Table 1**). These data suggest that CTCF is required in a dose-dependent manner.

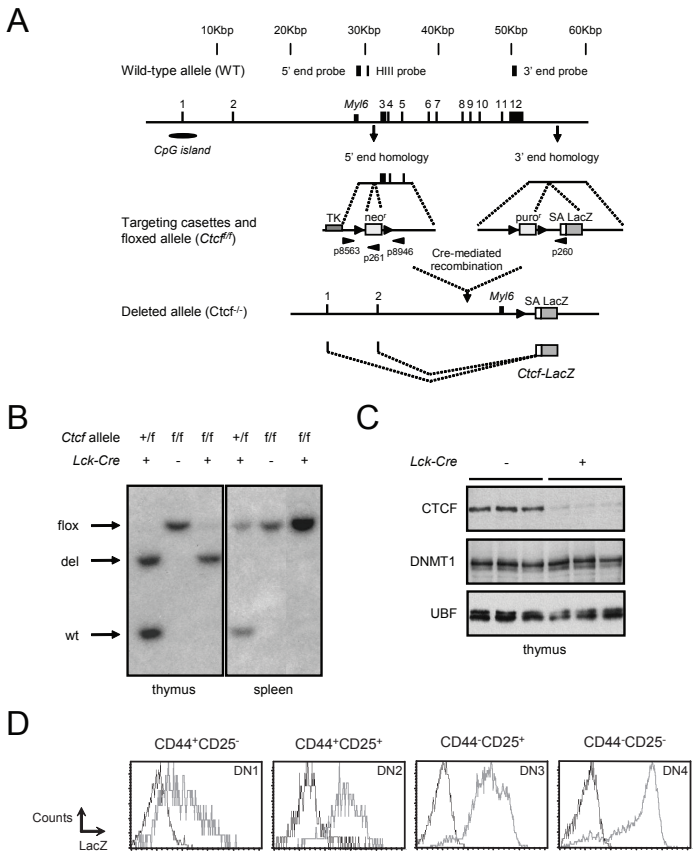

Figure 1. Conditional targeting of the mouse *Ctcf* gene. (**A**) Murine *Ctcf* locus and gene targeting constructs. Exons of the *Ctcf* gene (solid boxes) are numbered, scale is in kilobase (Kbp). Exon 1 is embedded in a CpG island. Exon 3 contains the start codon, and exon 12 contains the stop codon. Southern blot probes (5′-end and *HIII*) are shown above the *Ctcf* gene. The two targeting constructs, with loxP sites (small triangles), flanking a PMC1-neomycin cassette (neo^r) or a PGK-puromycin cassette (puro^r), are shown with homologous regions. TK, thymidine kinase gene; SA-LacZ, Splice acceptor-LacZ cassette[39]. PCR primers for genotyping (p8563, p8946, p260 and p261, large triangles) are indicated on targeting cassettes. Underneath the targeting constructs, the deleted *Ctcf* gene is shown, which is generated after complete Cre-mediated recombination at the outermost loxP sites. Owing to alternative splicing, the splice acceptor (SA) site, present at the 5′-end of the reporter LacZ cassette, is spliced on to *Ctcf* exon 1 or 2, thereby generating a hybrid *Ctcf–LacZ* transcript. (**B**) Southern blot analysis of *Lck–Cre* recombinase activity. Digested genomic DNA from thymus and spleen of mice of the indicated genotypes was analysed by hybridization with the *HIII* probe (see panel **A**). The positions of the wild-type (WT), *Ctcf$^{f/f}$* (flox) and *Ctcf$^{-/-}$* (del) alleles are indicated. (**C**) Western blot analysis of thymus. Total thymus lysates from *Lck–Cre Ctcf$^{f/f}$* and WT mice (+ indicates presence of Cre transgene; − indicates absence) were analysed for CTCF, DNMT1 and UBF protein levels. Three mice were analysed per genotype. (**D**) Flow cytometric analysis of LacZ expression in CTCF conditionally deleted mice. LacZ expression was analysed in conjunction with cell surface markers. The indicated cell populations were gated and LacZ expression data are displayed as histogram overlays of *Lck–Cre Ctcf$^{f/f}$* mice (grey) on top of background signals in wild-type mice (black).

Table 1. Genotype of *Ctcf*$^{+/-}$ × *Ctcf*$^{+/-}$ and *Ctcf*$^{+/-}$ × wild type offspring.

Age	Genotype and number		
	Wild-type	*Ctcf*$^{+/-}$	*Ctcf*$^{-/-}$
Ctcf$^{+/-}$ x *Ctcf*$^{+/-}$			
E 9.5	13	14	0
E 3.5	10	7	0
Adult	88	92	0
Ctcf$^{+/-}$ x *wild-type*			
Adult	101	74	NA

To obtain a T-cell-specific deletion of the *Ctcf* gene, we crossed *Ctcf*$^{f/f}$ mice with *Lck-Cre* transgenic mice, in which the proximal *Lck* promoter drives expression of the Cre recombinase[21, 22]. Southern blot analysis shows almost complete deletion of the *Ctcf* gene in thymus, whereas in spleen, deletion is not evident (**Fig. 1B**). These data reflect the specificity of the *Lck-Cre* transgene; they also indicate that *Ctcf* knockout T cells do not populate the spleen in large numbers. To evaluate the onset of *Ctcf*^{f} gene deletion, we analysed lacZ expression in thymocytes by flow cytometry using fluorescein-di-β-D-galactopyranoside (FDG) as a substrate in conjunction with cell surface markers that define thymocyte subpopulations. We find that deletion is almost complete from the DN2 stage onwards (**Fig. 1D**). Western blotting shows that in thymic nuclear extracts from *Lck-Cre Ctcf*$^{f/f}$ mice, CTCF protein levels are reduced to ~8% of control (**Fig. 1C**). We conclude that ablation of the *Ctcf* gene results in an efficient depletion of the protein *in vivo*.

Defective TCRαβ lineage development in *Lck-Cre Ctcf*$^{f/f}$ mice

To examine the effects of a *Ctcf* deletion, thymocyte subpopulations in 6- to 8-week-old mice were analysed by flow cytometry. This revealed that *Lck-Cre Ctcf*$^{f/f}$ mice have a reduced thymic cellularity (**Table 2**). Whereas a specific defect is observed in αβ T-cell development (**Fig. 2**), γδ T cells are not affected by *Ctcf* deletion (**Supplementary results** and **Supplementary Fig. 2**), similar to what has been reported for condtional deletion of DNMT1[21] and the RNaseIII enzyme Dicer[23]. Apparently, cell division, chromatin organization and gene regulation in γδ T cells is quite different from αβ T cells.

In αβ T cells of *Lck-Cre Ctcf*$^{f/f}$ mice, a decrease in the proportion and number of DP and SP cells, and a concomitant increase in DN3, DN4 and ISP cells, is observed (**Fig. 2AB**, **Table 2**). Thus, the effect of a complete *Ctcf* knockout is particularly prominent at the ISP-to-DP transition (**Fig. 2B**). The fact that the absolute number of *Ctcf* knockout thymocytes increases about four-fold from ISP to DP, compared with a 90-fold increase in wild-type mice (**Fig. 2C**, **Table 2**), suggests that a cell cycle block rather than increased apoptosis underlies the accumulation of ISP cells from *Lck-Cre Ctcf*$^{f/f}$ mice.

Heterozygous *Lck-Cre Ctcf*$^{+/f}$ mice also display a phenotype, which is most

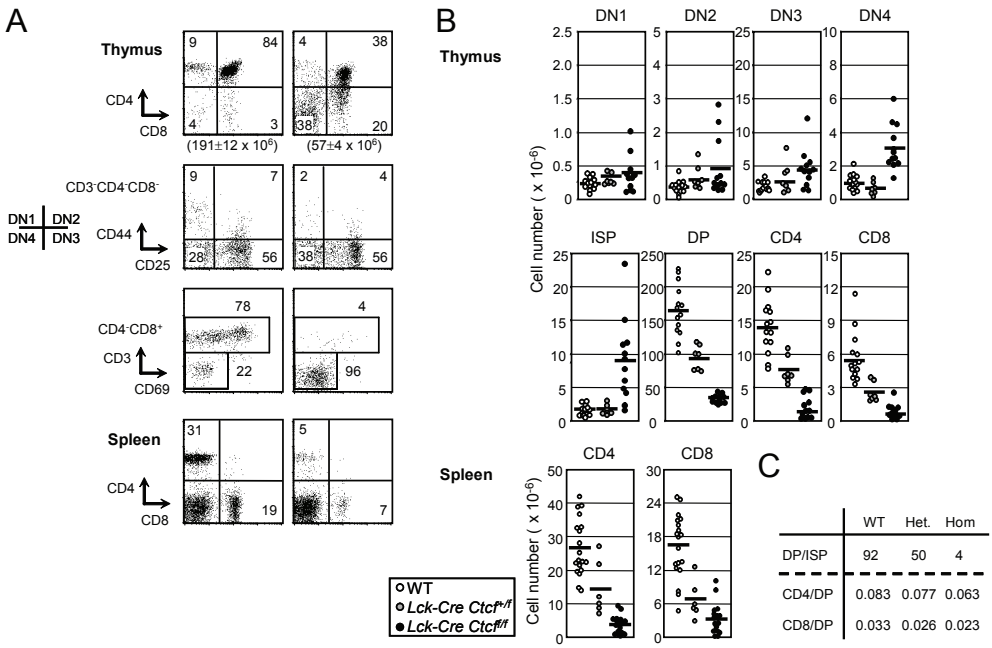

Figure 2. Defective TCRαβ lineage development in CTCF-deficient mice. (**A**) Representative flow cytometric analyses of cell populations from thymus (upper three panels) and spleen (lower panel) derived from wild-type (WT) and *Lck–Cre Ctcf*$^{f/f}$ mice. Expression profiles of surface markers (indicated on the left) are shown as dot plots, and the percentages of cells within quadrants or gates are given. The DN1–DN4 cell populations (second panel from top) were gated on the basis of CD44 and/or CD25 expression, using the CD3$^-$CD4$^-$CD8$^-$ triple-negative fraction as a basis. The third panel from the top shows gating of the ISP and CD8 SP cell populations (on the basis of low and high expression of CD3, respectively) using the fraction of CD4$^-$CD8$^+$ thymocytes (indicated in the CD4/CD8 profile of the upper panel). Note the accumulation of ISP cells in the thymus of the *Lck–Cre Ctcf*$^{f/f}$ mouse (95%) compared with wild type (20%). (**B**) Number of thymic and splenic T cell subpopulations. Each symbol represents one individual animal (n=12 for *Lck–Cre Ctcf*$^{f/f}$ mice, n=7 for *Lck–Cre Ctcf*$^{+/f}$ mice, and n=14 for wild-type (WT) mice). Note that different scales are used in the vertical axes. Horizontal lines indicate average values (see **Table 2** for actual values±SEM). *Lck–Cre Ctcf*$^{f/f}$ mice show increased numbers of DN3 (P<0.01), DN4 (P=0.0002) and ISP cells (P<0.002) compared with wild type. In *Lck–Cre Ctcf*$^{f/f}$ mice and heterozygous *Lck–Cre Ctcf*$^{f/+}$ mice DP, CD4 SP and CD8 SP subsets in the thymus were significantly reduced (P<0.0001). CD4 and CD8 T cells in the spleen were significantly reduced in *Lck–Cre Ctcf*$^{f/f}$ mice (P<0.00001) and in heterozygous *Lck–Cre Ctcf*$^{f/+}$ mice (P<0.01). (**C**) Analysis of cell numbers. Ratios were calculated (DP to ISP, CD4$^+$ to DP, and CD8$^+$ SP to DP) in *Lck–Cre Ctcf*$^{f/f}$ mice (Hom), *Lck–Cre Ctcf*$^{+/f}$ mice (Het) and wild-type (WT) mice. The DP/ISP ratio indicates the fold increase in cells and correlates with number of cell divisions. By contrast, the CD4/DP and CD8/DP ratios show a decrease in cell number and are a measure of selection.

obvious at the DP stage (**Fig. 2B**, **Table 2**). In these mice, thymic cellularity is only modestly reduced and no accumulation of ISP cells is detected (**Table 2**). These results show that normal CTCF levels are important for proper T-cell development. In

Table 2. Average numbers of thymocyte subpopulations derived from *Lck–Cre Ctcf*$^{f/f}$ mice (homozygous), *Lck–Cre Ctcf*$^{+/f}$ mice (heterozygous) and wild-type (WT) mice.

Genotype	DN1	DN2	DN3	DN4	ISP	DP	CD4 SP	CD8 SP	Total
WT (*n*=14)	0.27 ± 0.02	0.37 ± 0.05	2.09 ± 0.19	1.10 ± 0.13	1.81 ± 0.21	165 ± 10.3	14.1 ± 1.08	5.56 ± 0.58	191 ± 11.7
Heterozygous (*n*=7)	0.32 ± 0.03	0.66 ± 0.14	3.42 ± 0.93	0.73 ± 0.16	1.92 ± 0.31	95.9 ± 7.40	7.66 ± 0.81	2.48 ± 0.35	113 ± 8.77
Homozygous (*n*=12)	0.39 ± 0.08	0.91 ± 0.26	4.72 ± 0.85	3.15 ± 0.40	9.14 ± 1.82	35.4 ± 1.89	2.19 ± 0.50	0.81 ± 0.20	56.7 ± 3.76
P-value (heterozygous-WT)	NS	NS	NS	NS	NS	<0.0001	0.0001	0.0002	<0.0001
P-value (homozygous-WT)	NS	NS	0.0082	0.0002	0.0014	<0.0001	<0.0001	<0.0001	<0.0001

agreement with impaired thymic SP cell production, the number of mature CD4$^+$ and CD8$^+$ T cells in spleen and lymph nodes of *Lck-Cre Ctcf*$^{f/f}$ and *Lck-Cre Ctcf*$^{+/f}$ mice are significantly reduced (**Fig. 2AB** and H Heath, CR de Almeida, RW Hendriks and N Galjart, unpublished data). Interestingly, the ratio between mature CD4$^+$ SP and DP cells, and between CD8$^+$ SP and DP thymocytes, is similar in heterozygous *Lck-Cre Ctcf*$^{+/f}$, homozygous *Lck-Cre Ctcf*$^{f/f}$ and wild-type mice (**Fig. 2C**), indicating that differentiation from the DP to SP stage is not severely affected by a deletion of CTCF.

The accumulation of CTCF-deficient ISP cells in *Lck-Cre Ctcf*$^{f/f}$ mice could result from a developmental arrest at the ISP stage or alternatively reflect defective upregulation of CD4 expression in cells that otherwise have characteristics of DP cells, similar to thymocytes deficient for the chromatin remodeler Mi-2β[24]. To distinguish between these possibilities, we assessed expression of various cell surface markers in wild-type and *Lck-Cre Ctcf*$^{f/f}$ thymocytes. Normally, surface expression of TCRβ and its associated signalling molecule CD3 are low in ISP cells and are induced at the DP stage. CTCF-deficient ISP cells express low levels of CD3 and TCRβ (**Fig. 3A**). Furthermore, wild-type and CTCF-negative ISP and DP cells express similar amounts of the surface glycoprotein CD5, which is normally upregulated on ISP cells and somewhat further on DP cells[25]. CTCF-deficient ISP cells also express low levels of CD69, which in wild-type mice is induced in a subfraction of DP cells, reflecting TCR-mediated activation[26]. Expression of heat stable antigen (HSA, also called CD24, a cell adhesion molecule), which is normally high on DN and ISP cells and downregulated at the ISP-to-DP transition[24], is reduced in CTCF-deficient cells throughout thymocyte differentiation (**Fig. 3A**). As none of the markers tested show a 'DP-like' expression pattern in CD3$^-$CD4$^-$CD8$^+$ cells in *Lck-Cre Ctcf*$^{f/f}$ mice, we conclude that these accumulating cells truly represent ISP cells and not abberant DP cells that fail to upregulate CD4 expression. In addition to the ISP-to-DP arrest in *Lck-Cre Ctcf*$^{f/f}$ mice, we observed a significant increase in number of cells within the preceding DN3 and DN4 stages (**Table 2**), indicating that a deletion of CTCF causes a developmental arrest from DN3 to ISP.

Interestingly, the expression level of CD3 is not only reduced in ISP thymocytes from *Lck-Cre Ctcf*$^{f/f}$ mice, but also in DP cells and to a lesser extent in mature CD4$^+$ SP and CD8$^+$ SP cells (**Supplementary Fig. 3A**, note that in CD8 SP cells, the decrease

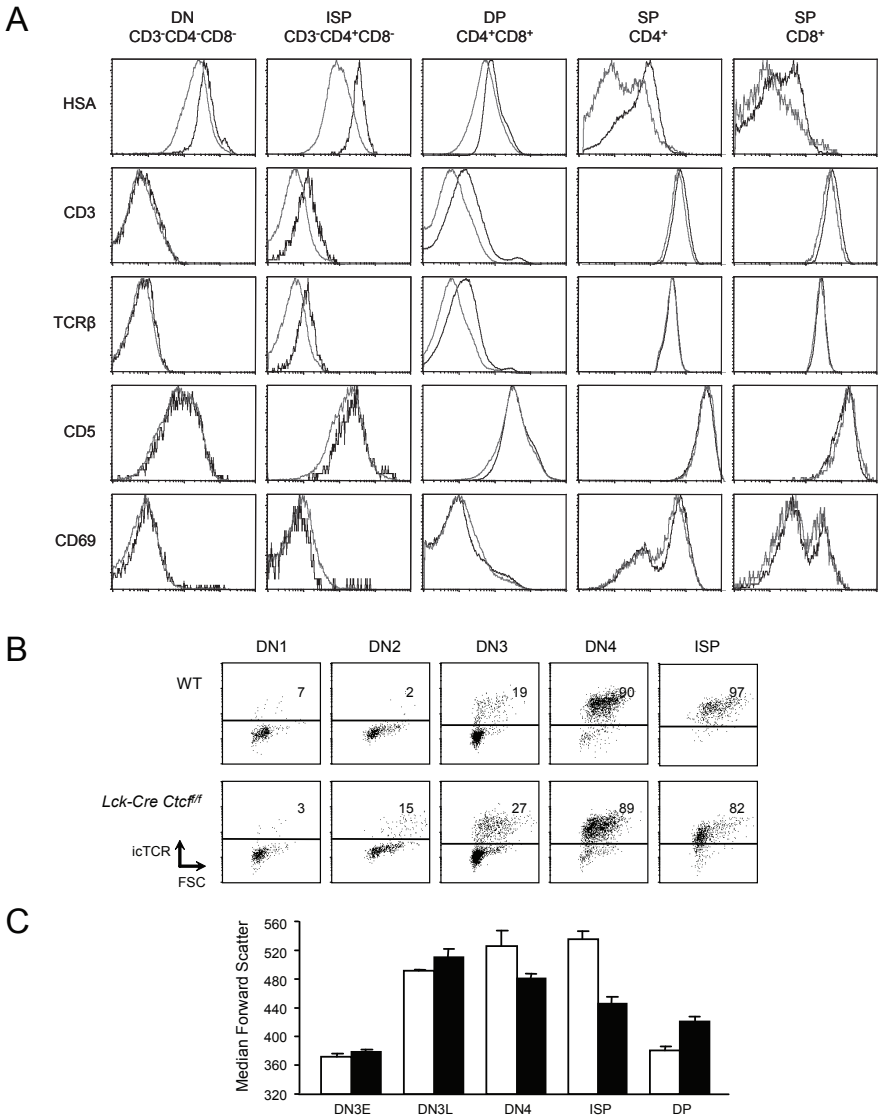

Figure 3. Characterization of CTCF-deleted thymocytes. (**A**) Expression of ISP cell markers. Flow cytometric analyses of HSA, CD3, TCR, CD5 and CD69 in thymocyte subpopulations, displayed as overlays of wild-type mice (black histograms) and *Lck–Cre Ctcf*$^{f/f}$ mice (grey histograms). Data shown are representative of 5–8 mice per group. (**B**) Intracellular TCRβ levels in wild-type and *Lck–Cre Ctcf*$^{f/f}$ mice. Representative flow cytometric analyses of intracellular TCRβ protein expression in the indicated thymic subsets from a wild-type (WT) and an *Lck–Cre Ctcf*$^{f/f}$ mouse. TCRβ/forward scatter (FSC) profiles are shown as dot plots, gating is indicated by the horizontal lines and the percentages of TCRβ$^+$ cells are shown. (**C**) Cell size in wild-type and *Lck–Cre Ctcf*$^{f/f}$ thymocytes. Quantification of median forward scatter values (which accurately reflects cell size) of the indicated thymocyte subpopulations in wild-type (white bars) and *Lck–Cre Ctcf*$^{f/f}$ (black bars) mice. Data are average values±SEM from 5 to 8 mice per group.

is not significant). In particular, CTCF-deficient DP cells do not contain the fraction of $CD3^{high}/TCR^{high}$ cells. DP cells from heterozygous *Lck-Cre Ctcf*$^{+/f}$ mice express CD3 levels that are in the range of those from WT mice (**Supplementary Fig. 3A**), showing that only a complete lack of CTCF affects CD3 expression at the DP cell stage. Although CTCF-negative DP thymocytes are affected in the expression of important molecular markers, including CD3 and TCR, they are still able to differentiate towards the mature SP stages.

Defective cell-size regulation in the TCRαβ lineage in *Lck–Cre Ctcf*$^{f/f}$ mice

As our findings indicated a specific function of CTCF at the ISP-to-DP transition, we focussed our attention on possible mechanisms underlying the hampered differentiation of these cells. We first analysed *Tcr* rearrangement, because of the many CTCF-binding sites that are found in the genes encoding the different receptors. *Tcr*β gene rearrangement is generally initiated and completed in DN3. This stage consists of early small DN3E cells that have not yet productively rearranged the *Tcrβ* locus, and more mature large proliferating DN3L cells expressing TCRβ[27]. We detect a significant increase in the population of large DN2 cells in *Lck-Cre Ctcf*$^{f/f}$ mice that contain intracellular $TCR\beta^+$ (**Fig. 3B**), 13±4% in CTCF-deficient DN2 cells (n=3), compared with 4±0.5% in wild-type controls (n=4); P<0.01). These results raise the interesting possibility that CTCF is involved in inhibiting recombination early in development. The proportion of large $TCR\beta^+$ DN3 cells appears still elevated, but the difference between the two groups (~21 and ~18% in CTCF-deficient and wild-type mice, respectively) is not significant. Thus, the *Tcrβ* locus can undergo functional V(D)J recombination in cells that have deleted the *Ctcf* gene. It should be noted that even though the *Ctcf* gene is efficiently deleted using the *Lck-Cre* transgene, residual CTCF protein might still be present at DN2 and DN3 stages, and we cannot rule out that this is sufficient for recombination.

By comparing wild-type and *Lck-Cre Ctcf*$^{f/f}$ mice, we found that CTCF-negative $TCR\beta^+$ DN3 cells have the capacity to increase their cell size at the developmental progression of TCRβ− DN3E to $TCR\beta^+$ DN3L cells (**Fig. 3BC**). However, from the DN3L-to-ISP stage, CTCF-deficient cells become much smaller than wild-type cells (**Fig. 3BC**). CTCF-deficient DP cells are again larger than wild-type cells (**Fig. 3C**, **Supplementary Fig. 3B**) and continue to be larger throughout subsequent differentiation (**Supplementary Fig. 3B**). These results indicate that CTCF is a critical regulator of cell growth of αβ T cells. Cells from heterozygous *Lck-Cre Ctcf*$^{+/f}$ mice are sized like wild types (**Supplementary Fig. 3A**), showing that only a complete lack of CTCF affects cell size.

Developmental arrest of CTCF-deficient thymocytes is not due to defective *Tcr* rearrangements

The severe reduction of DP cell numbers and low-surface CD3/TCR expression on CTCF-deficient DP cells, together with the reported presence of CTCF-binding sites in the *Tcrα* gene locus[12, 16], indicated that defective *Tcrα* V(D)J recombination could contribute to the arrest of CTCF-deficient thymocytes. We therefore crossed *Lck-Cre Ctcf$^{f/f}$* mice with transgenic mice expressing either the pre-rearranged OTII TCRαβ, which recognizes the $OVA_{323–339}$ peptide in the context of C57BL/6 MHC class II, and which positively selects thymocytes towards the CD4 lineage[28], or the MHC class I-restricted HY TCRαβ, which recognizes a male-specific HY antigen peptide in the C57BL/6 H-2^b class I female background and normally drives thymocytes into the CD8 lineage[29]. However, the impaired developmental progression of CTCF-deficient cells is not rescued (**Fig. 4**). Rather, the presence of the OTII and HY *Tcr* transgenes results in an even more severe

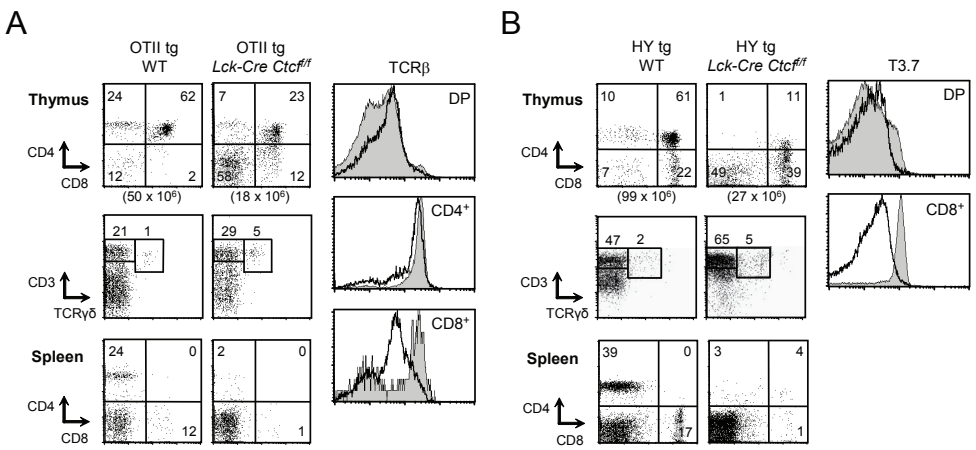

Figure 4. Arrest of CTCF-deficient thymocytes remains despite *Tcr* gene rearrangement. (**A**) Representative flow cytometric analyses of cell populations from thymus (upper two panels) and spleen (lower panel) derived from OTII transgenic mice, which are either in a wild-type (WT) or in a *Lck–Cre Ctcf$^{f/f}$* background. Expression profiles of CD4/CD8 and CD3/TCRγδ markers are shown as dot plots, and the percentages of cells within quadrants or gates are given. Thymic cellularity is shown for the individual animals underneath the first panel. In the right hand panels, data for the OTII transgenics are displayed as histogram overlays. The expression profile of total surface TCRβ within the indicated cell populations is shown for *Lck–Cre Ctcf$^{f/f}$* mice (bold lines) on top of profiles of WT littermates (grey-filled histograms). (**B**) Representative flow cytometric analyses of cell populations from thymus (upper two panels) and spleen (lower panel) derived from *HY* transgenic mice, which are either in a wild-type (WT) or in a *Lck–Cre Ctcf$^{f/f}$* background. Flow cytometric profiles of CD4/CD8 and CD3/TCRγδ are shown as dot plots; percentages of cells within quadrants or regions and total thymic cell numbers are given. The expression profile of total HY idiotype-specific T3.7 TCR within the indicated cell populations is shown on the right as a histogram overlay of *Lck–Cre Ctcf$^{f/f}$* mice (bold lines) on top of profiles of WT littermates (grey-filled histograms).

arrest of T-cell development in the thymus (**Fig. 4AB**), respectively). OTII Tg *Lck-Cre Ctcf$^{f/f}$* mice manifest a relative increase in the proportions of DN and ISP cells in the thymus, whereas the proportions of DP and CD4 SP cells are reduced. In addition, we observe an almost complete absence of mature T cells in the spleen (**Fig. 4A**). HY Tg *Lck-Cre Ctcf$^{f/f}$* mice manifest a severe block at the ISP stage and an almost complete lack of DP cells in the thymus. Those CD8$^+$ cells present in CTCF-deficient HY Tg mice are ISP cells rather than mature CD8 SP cells, because the level of HY-specific TCR expression (as detected by the T3.7 antibody) in the CD8$^+$ cell fraction is substantially lower, when compared with wild-type CD8 SP cells. In agreement with this, CD4 or CD8 cells in the spleen are strongly reduced (**Fig. 4B**). Thus, providing *Lck-Cre Ctcf$^{f/f}$* mice with a pre-rearranged TCRαβ does not correct the developmental arrest of DP cells, indicating that the developmental block in *Ctcf* knockout T cells is independent of *Tcr* gene rearrangement.

We next crossed *Lck-Cre Ctcf$^{f/f}$* mice with mice deficient for the *Recombination activating gene 2* (*Rag2*) gene. RAG2 mediates V(D)J recombination of *Tcr* and *Ig* loci. *Rag2$^{-/-}$* mice are therefore deficient in *Tcr* gene rearrangement, and they normally do not progress beyond the DN3 stage (**Fig. 5**). *In vivo* stimulation with anti-CD3ε antibodies mimics pre-TCR signalling[25] and thereby induces the formation of DP cells in the absence of rearrangement. Both in *Rag2$^{-/-}$* and in CTCF-deficient *Rag2$^{-/-}$* mice, we find that on anti-CD3ε treatment, total thymic cellularity increases from <2 × 10^6 to 13±4 and 11±3 × 10^6, respectively (n=4 in each group) and equal numbers of DN4 cells are induced (**Fig. 5**). However, the formation of DP cells is reduced in CTCF-deficient

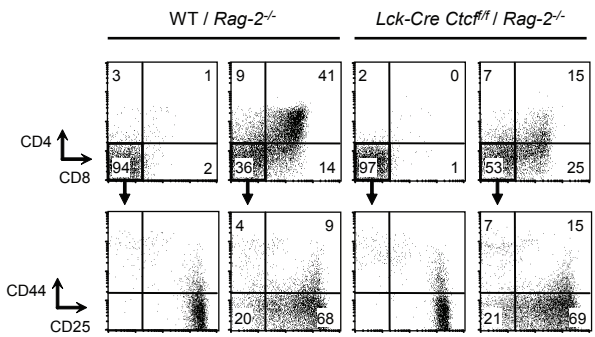

Figure 5. Arrest of CTCF-deficient thymocytes is independent of *Tcrα* gene rearrangement. Representative flow cytometric analyses of the thymus of *Rag2* knockout (*Rag2$^{-/-}$*) mice, which are either in a wild-type (WT) or in a *Lck–Cre Ctcf$^{f/f}$* background. Mice were either untreated or injected with 50 µg of rat anti-CD3 antibodies (αCD3) *in vivo*. The CD4 and CD8 expression profiles, 3 days after injection, are shown (upper part). Cell populations were gated (vertical and horizontal lines) and the CD4$^-$CD8$^-$ fraction (i.e., DN cells) was analysed for CD25 and CD44 expression (lower part). Data are shown as dot plots and the percentages of cells within the quadrants are given. The plots shown are representative for four mice in each group.

Rag2$^{-/-}$ mice, when compared with CTCF-expressing cells (**Fig. 5**).

As the *Tcrγ* and *δ* loci can also undergo functional V(D)J recombination in the absence of CTCF, we conclude that the multiple CTCF-binding sites reported to be present in *Tcr* loci[14-16] do not appear to be essential for the process of V(D)J recombination. Therefore, deficiency of CTCF results in a developmental block at the ISP-to-DP transition that is independent of *Tcr* gene rearrangement.

Cell cycle arrest during TCRαβ lineage development in *Lck-Cre Ctcf*$^{f/f}$ mice

To further examine the underlying cause of the accumulation of CTCF-negative ISP cells, we sorted wild-type and CTCF-deleted cells T cells into DN, ISP and DP fractions, and used real-time PCR to analyse mRNA expression patterns of a selected number of important T-cell factors. In wild-type cells, *Ctcf* mRNA levels increase from the DN-to-ISP stage and then decrease again in DP cells (**Fig. 6A**). These data suggest that the relatively large, actively cycling ISP cells require a higher amount of CTCF for normal functioning than DN or DP cells. In sorted cells from *Lck-Cre Ctcf*$^{f/f}$ mice, *Ctcf* expression is severely reduced in the DN fraction and is absent at later stages (**Fig. 6A**); note that residual *Ctcf* mRNA is detected in the DN pool because the *Ctcf* gene is only fully deleted from DN2 onwards).

CTCF was reported to be a negative transcriptional regulator of c-Myc[30, 31], and c-Myc is important for T-cell development[32]. In wild-type T cells, *c-Myc* is expressed in a pattern different from *Ctcf* (**Fig. 6A**). In addition, *c-Myc* expression is hardly affected by a CTCF deletion. Therefore, in T cells, CTCF does not appear to regulate expression of the *c-Myc* gene. The level of DNMT1, a maintenance methyltransferase that is essential for T-cell development[21], is also similar in wild-type and CTCF-deleted T cells (**Fig. 6A**), see also (**Fig. 1C**). Two other transcription factors are Gata3, which is critically involved in β-selection and development of CD4 SP cells[33], and Satb1, which organizes cell type-specific nuclear architecture[34]. In wild-type cells, the levels of these factors are opposite to those of *Ctcf* (**Fig. 6A**). In CTCF-deficient cells, *Gata3* and *Satb1* expression is reduced, in particular at the DP stage. We next tested two cytoplasmic factors involved in T-cell signalling. Expression of *PreTα* (that assembles with TCRβ to initiate TCR signalling) is not affected in ISP cells and is up- rather than downregulated in DP cells in the absence of CTCF (**Fig. 6A**). Finally, the expression of *Gimap4*, which is induced by pre-TCR signalling and accelerates T-cell death[35], is increased in CTCF-deficient DP T cells with a factor of ~2 (**Fig. 6A**). Nevertheless, we did not find evidence for increased apoptosis of CTCF-deficient DP cells (data not shown). Although none of the factors tested appears to be directly regulated by CTCF, changes in their expression level may contribute to the observed phenotype in CTCF-deleted cells.

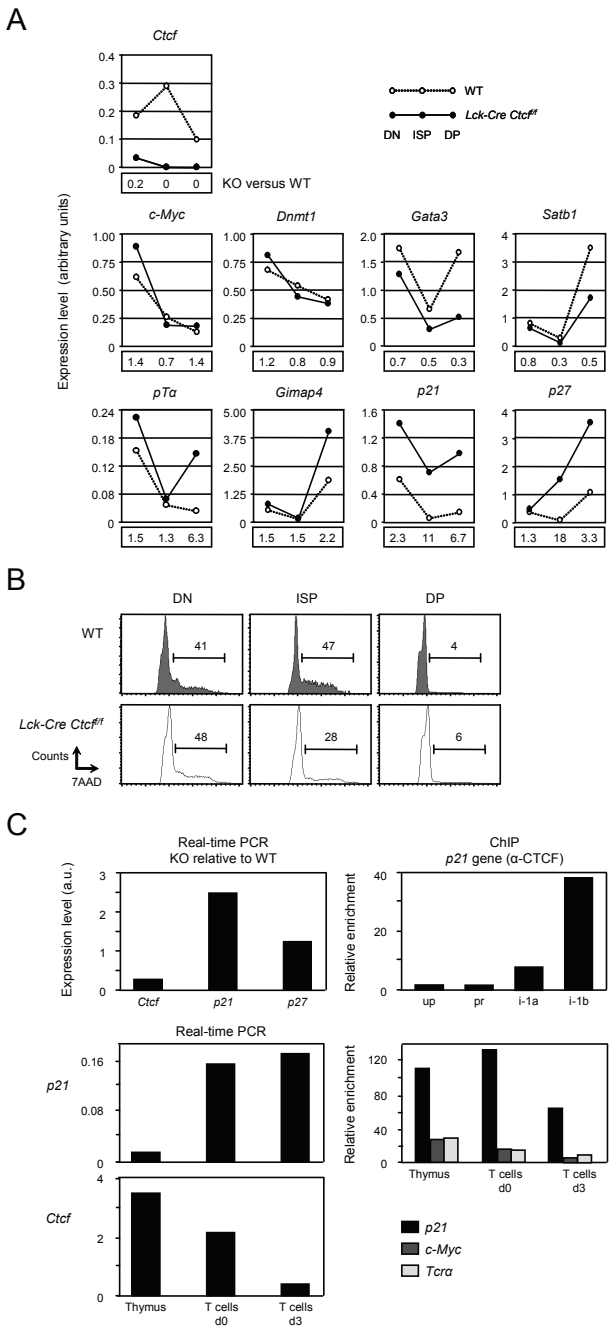

Figure 6. CTCF is important for cell cycle progression. (**A**) Quantitative RT–PCR analysis in sorted DN, ISP and DP cell fractions from wild-type (WT) and *Lck–Cre Ctcf*$^{f/f}$ mice. The DP fraction also contained CD4 SP cells. To obtain enough material, RNA was pooled from four WT and two *Lck–Cre Ctcf*$^{f/f}$ mice. (**B**) Cell cycle status of DN, ISP and DP cells isolated from WT and *Lck–Cre*

Ctcf$^{f/f}$ mice. Cell cycle was analysed using 7-AAD, which measures DNA amount. A representative analysis is shown. Numbers indicate the percentage of cells in S/G2/M. In *Lck–Cre Ctcf*$^{f/f}$ mice, this number is significantly reduced in ISP cells, showing that this population is hampered in the cell cycle. (**C**) Quantitative RT–PCR (left hand panel) and ChIP (right hand panel) analysis in WT and *Ctcf*$^{f/f}$ MEFs, after treatment with Cre recombinase. mRNA expression in Cre-treated *Ctcf*$^{f/f}$ MEFs (KO) is shown relative to WT. Although residual *Ctcf* mRNA is present in the MEFs, *p21* expression is increased. ChIP analysis was performed with anti-CTCF antibodies on four regions in the *p21* gene. Potential CTCF-binding sites in the mouse *p21* gene were chosen on the basis of a genome-wide analysis in human cells[14]. An identical binding pattern was observed in wild-type MEFs, with relatively weak CTCF binding 2.3 kb upstream of the *p21* promoter (up) and on the promoter (pr), and very strong binding on two adjacent regions within intron 1 (i-1a, i-1b). (**D**) Quantitative RT–PCR (left hand panels) and ChIP (right hand panel) analysis in WT thymocytes and T cells before (d0) and after (d3) 3 days of anti-CD3/CD28 stimulation *in vitro*. mRNA expression of *p21* and *Ctcf* is shown relative to *Hprt*. ChIP analysis was performed with anti-CTCF antibodies on intron 1 of the *p21* gene and, as positive control, on known binding sites in the *c-Myc* and *Tcrα* genes.

As the accumulation of ISP cells from *Lck-Cre Ctcf*$^{f/f}$ mice could be due to cell cycle defects, we tested the expression of two major cell cycle inhibitors, p21 and p27. In wild-type cells, the expression profile of these factors is opposite to that of *Ctcf*, whereas *Ctcf* knockout cells show significantly increased *p21* and *p27* expression (**Fig. 6A**). We subsequently analysed cell cycle profiles in wild-type and CTCF-deleted thymocytes. The CTCF-deficient ISP population from *Lck-Cre Ctcf*$^{f/f}$ mice contains approximately half the number of cycling cells compared with wild type (29%±1 cells in S/G2/M phase in *Lck-Cre Ctcf*$^{f/f}$ mice (n=3), versus 53%±8 in wild-type mice (n=3); **Fig. 6B** shows an example of analysis from individual mice). These results indicate that cell cycle progression in β-selected CTCF-deficient T cells is blocked due to the upregulation of *p21* and *p27*.

We next tested whether a deletion of CTCF always causes upregulation of the *p21* and *p27* genes, irrespective of cell type. We treated mouse embryonic fibroblasts (MEFs) from *Ctcf*$^{f/f}$ mice with Cre recombinase[9] to efficiently remove CTCF in these cells. Real-time PCR experiments show that in MEFs the expression of *p21*, but not of *p27*, is increased in the absence of CTCF (**Fig. 6C**). Thus, the combined upregulation of *p21* and *p27* seen in CTCF-deficient ISP cells is not a general regulatory mechanism.

CTCF as a critical regulator of cell growth and proliferation in T cells

Genome-wide studies in human cells have shown that CTCF-binding patterns in fibroblasts and T cells largely overlap[12, 14]. Interestingly, the human *p21* gene contains four CTCF binding sites in the vicinity of its promoter, whereas no binding sites are found near *p27*. As *p21* but not *p27* mRNA is upregulated in different cell types in the absence of CTCF, and CTCF binds near and within the human *p21* gene but not near *p27*, we hypothesized that CTCF might be a negative regulator of *p21* expression. This

idea is enforced by the observation that in wild-type DN, ISP and DP thymocytes, the *p21* mRNA expression profile is exactly opposite to that of *Ctcf* (**Fig. 6A**). To test our assumption, we first confirmed that CTCF-binding sites (which are part of CNEs) are conserved between man and mouse. Chromatin immunoprecipiations (ChIPs) in MEFs on the corresponding regions of mouse *p21* indeed reveal an identical pattern of CTCF binding, including the two adjacent strong sites in intron 1 (**Fig. 6C**).

Next, we analysed thymocytes isolated from total thymus and largely consisting of DP cells, and purified $CD4^+$ T cells, either before (day 0) or after 3 days of *in vitro* activation by anti-CD3/anti-CD28 stimulation (day 3). We reasoned that in these cells, levels of p21 must differ and that these cells therefore represent a good model to test whether CTCF binding correlates to *p21* expression in cells expressing normal amounts of CTCF. We correlated mRNA expression levels of *Ctcf* and *p21* (as tested with real-time PCR) with CTCF binding (as analysed by ChIP), using the strongest CTCF site in intron 1 of the *p21* gene as a reference. mRNA expression levels were normalized to *Hprt* (a housekeeping gene), whereas CTCF binding was normalized to the *Amylase* gene, which contains no CTCF-binding sites. The data show that CTCF binds very strongly to the *p21* intron, both in thymocytes and resting and cultured T cells (**Fig. 6D**). Although there is a correlation between *Ctcf* mRNA levels and strength of CTCF binding, there is no correlation with *p21* expression (**Fig. 6D**). Thus, despite the fact that deletion of CTCF results in increased expression of *p21* in thymocytes and MEFs, the *p21* gene does not appear to be a general target of CTCF in wild-type cells.

The mRNA expression data indicate that *Ctcf* is specifically upregulated in ISP cells (**Fig. 6A**). Furthermore, lacZ staining results show increased activity of the *Ctcf* promoter in DN2-DN4 cells (**Fig. 1D**) and in ISP cells (not shown). However, both the PCR and LacZ staining results reflect mRNA levels of CTCF. To also analyse CTCF protein levels *in vivo*, we used a $Ctcf^{gfp}$ knock-in allele in which GFP-CTCF is expressed instead of CTCF (H Heath and N Galjart, unpublished data). We used flow cytometry to identify GFP-CTCF in the different thymocyte subsets and in the spleen (**Supplementary Fig. 4**). During αβ T-cell differentiation, CTCF levels increase from the DN2 to the ISP stage and then return to DN1 values in the DP cell population (**Supplementary Fig. 4A**), consistent with the expression profile of *Ctcf* transcripts (**Fig. 6A**). In TCRαβ $CD4^+$ and $CD8^+$ T cells in the spleen, CTCF levels are identical to the levels in DN1 and SP cells in the thymus (**Supplementary Fig. 4B**). Thus, CTCF levels increase in those thymocyte populations (DN3, DN4 and ISP) that have a larger cell size (see **Fig. 3**). Interestingly, a lack of CTCF causes accumulation of these very same cells. Taken together, our data suggest that CTCF is a critical regulator of cell growth and proliferation in αβ T cells.

DISCUSSION

CTCF is an important protein involved in chromatin organization and the epigenetic regulation of gene expression. Most studies on CTCF use cultured cells as a basis, and a plethora of results regarding CTCF function have been reported. We have generated a conditional *Ctcf* knockout allele, which has allowed us to examine the *in vivo* function of CTCF. Consistent with a previous report[3], we find that deletion of *Ctcf* in early embryonic development is lethal. A novel result is that heterozygous *Ctcf* knockout mice, which are viable and fertile, are born in less than expected numbers. The development of *Ctcf*$^{+/-}$ thymocytes is also affected, although mildly. Taken together, these data suggest that CTCF is required in a dose-dependent manner.

We found no evidence for an increased tumour incidence in heterozygous *Ctcf* knockout animals, or for T lymphoid malignancies in CTCF-deficient T-cell lineages. This argues against a function of CTCF as a crucial tumour suppressor[36]. Increased expression of *p21* (and *p27*) in CTCF-negative cells would explain why loss of CTCF does not induce tumours. Still, the potential function of CTCF in cancer merits a more detailed investigation. We also do not observe a more severe phenotype in CTCF-deficient female thymocytes compared with male cells, suggesting that absence of CTCF does not cause deregulated expression of genes on the inactive X-chromosome. Moreover, the deletion of CTCF does not have an effect on the maintenance of methylation in the imprint control region of the *Igf2/H19* and ribosomal DNA (rDNA) loci (see **Supplementary results**). Unlike other studies[8, 37], our results therefore indicate that CTCF is not required to maintain X-inactivation and DNA methylation status of the *Igf2/H19* locus.

Despite the presence of multiple CTCF-binding sites near the *Tcrα* and *Tcrβ* genes, our data indicate that CTCF is not essential for recombination at these loci. CTCF also does not appear to be required for the differentiation of DP cells towards the CD4$^+$ and CD8$^+$ SP cell stage, even though substantial epigenetic and regulatory changes accompany commitment of SP cells[18]. CTCF is, however, essential for the efficient proliferation of β-selected cells, in particular for their maturation from ISP to DP cells, and for TCR upregulation at the cell surface of DP cells. As T cells were directly isolated from mice, our data provide the first *in vivo* evidence for an important function of CTCF in cell cycle progression. In line with a proliferative block, we detect strongly increased expression of two major cell cycle inhibitors, *p21* and *p27*.

Our results in T cells are completely opposite to those obtained in WEHI 231 B lymphoma cells, where conditional expression of CTCF resulted in the up- rather than the downregulation of *p21* and *p27*, whereas reduction of CTCF levels decreased rather than increased the expression of *p21* and *p27*[31]. This could be due to the fact

that the properties of the ISP thymocytes and WEHI 231 B cells are entirely different. ISP thymocytes are highly proliferating as a result of pre-TCR stimulation, whereas crosslinking of the B-cell receptor on WEHI 231 immature B cells results in cell-cycle arrest and apoptosis (thereby providing a model for self-tolerance by clonal deletion). This suggests that CTCF function is context dependent, although it should be noted that our data were obtained *in vivo*, whereas the data in the WEHI 231 B lymphoma cells were obtained with stably transfected clones selected for high expression of *CTCF* sense or antisense mRNA[31]. Using our conditional *Ctcf*$^{f/f}$ mice in combination with existing B cell-specific Cre transgenes, we will be able to examine the function of CTCF in B cells.

In *Ctcf* knockout mice, approximately four times more DP cells are present than ISP cells, suggesting that the latter cells can divide, although slowly. Moreover, whereas CTCF-negative ISP cells are smaller than their wild-type counterparts, DP cells are larger. These data argue against increased apoptosis as a cause of reduction in DP cell numbers after *Ctcf* gene deletion. This is different from the situation in *Dnmt1* knockout mice, which have similar numbers of DN thymocyte subsets as wild-type littermates, and in which increased apoptosis was shown to be a major cause of the severe reduction in DP cell number[21].

T-cell activation at the DN3 stage is accompanied by an enlargement of both cytoplasmic and nuclear volume. In the DN4 and ISP stages, growth is coupled to proliferation, presumably to sustain the rapid cell divisions that are required in these cells. When thymocytes enter the DP stage, they exit the cell cycle and become small again. CTCF levels increase when cells become bigger (from DN3 to ISP) and decrease again when cells become small. Furthermore, CTCF-negative DN4 and ISP cells are smaller than wild-type cells, whereas DP cells are larger. These data uncover a function of CTCF as a regulator of cell growth and proliferation in thymocytes. A notable enrichment of CTCF-binding sites was observed near DNAse I HSs in CD4$^+$ T cells, indicating that CTCF controls global T-cell expression[17]. Our data suggest that CTCF couples cell growth to the cell cycle. Strikingly, the ratio between DP cell number and mature CD4$^+$ SP and CD8$^+$ SP thymocytes is the same in wild-type, heterozygous *Lck-Cre Ctcf*$^{+/f}$ and homozygous *Lck-Cre Ctcf*$^{f/f}$ mice. Thus, the major function of CTCF in the thymus might be to positively regulate cell growth in rapidly dividing thymocytes so that appropriate numbers of cells are generated before positive and negative selection events at the DP stage in the thymus.

METHODS

Modified *Ctcf* alleles, mouse models and embryonic fibroblasts. Human CTCF cDNA was used to screen a 129S6/SvevTac mouse PAC library (RPCI-21)[38]. PAC clones were used to isolate 6.7 kb (for 5′-end targeting) and 8 kb (for 3′-end targeting) *Eco*RI subclones. For 5′-end targeting, the 6.7 kb *Eco*RI fragment was used to amplify 1360 bp of 5′-end homology and 5340 bp of 3′-end homology. The homologous arms were cloned into a vector containing the neomycin resistance gene flanked by loxP sites[39]. A viral thymidine kinase gene was inserted afterwards. For 3′-end targeting, we generated a *Spe*I–*Eco*RI subclone from the PAC DNA and used its unique *Bam*HI site to insert a cassette containing the puromycin resistance gene flanked by loxP sites, followed by splice acceptor sequences and the bacterial β-galactosidase (lacZ) reporter[39]. Relevant parts of the different constructs were verified by DNA sequencing. Constructs were targeted into E14 embryonic stem (ES) cells as described[39]. DNA from resistant ES cells was analysed with external radiolabelled probes by Southern blotting. Confirmation of homologous recombination was performed using different 5′-end and 3′-end probes (**Fig. 1A and B**) and a PCR-based assay for genotyping. *Ctcf*$^{f/f}$ mice were crossed back more than 10 times to the C57BL/6 background. MEFs were isolated from *Ctcf*$^{f/f}$ mice using published procedures[40]. Fibroblasts were treated with lentiviral Cre as described[9]. *Ctcf*$^{f/f}$ mice were bred to mice expressing chicken β-actin-Cre-generating *Ctcf*$^{+/-}$ animals. T-cell-specific deletion of *Ctcf* was achieved by breeding to *Lck–Cre* mice[21], which were kindly provided by Dr C Wilson (University of Washington, Seattle, WA, USA). Cre-specific primers were used for genotyping. In experiments where *Ctcf* knockout mice are compared with 'wild type' animals, the latter are littermates of the *Lck-Cre Ctcf*$^{f/f}$ animals, that is, mice that express normal ('wild type') levels of CTCF. Thus, the 'wild type' mice may be truly wild type; they may contain the *Lck–Cre* transgene or the *Ctcf*^{f} allele, but never a combination of the latter two alleles. HY/*Rag2*$^{-/-}$ (C57BL/10) mice were purchased from Taconic Europe A/S (Denmark). OT-II mice have been described[28]. Mice were bred and maintained in the Erasmus MC animal care facility under specific pathogen-free conditions and analysed at 6–10 weeks of age. For anti-CD3 treatment, Rag2-deficient mice[41] were injected i.p. with 50 μg of rat anti-CD3 antibodies (αCD3; clone *145-2C11*) as described[42]. Experimental procedures were reviewed and approved by the Erasmus University committee of animal experiments.

DNA, RNA and protein analysis. Genomic DNA was isolated, digested and blotted onto Hybond N+ membranes (Amersham) and hybridized with radiolabelled probes. *Ctcf* probes are shown in **Fig. 1**. Total RNA was prepared using RNA-Bee RNA

isolation solvent (Tel-Test Inc.). RNA (0.5–1.0 µg) was reverse-transcribed (RT) with random and oligo-dT primers, in the presence of Superscript reverse transcriptase (Invitrogen). For the experiment shown in **Fig. 6**, RNA was isolated and pooled from the thymus of four wild-type mice and from two *Lck-Cre Ctcf*$^{f/f}$ mice. Real-time RT–PCR was performed as described[9] with 100 ng of each primer and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen). Sybr-green (Sigma) was added to the reactions and PCR was performed on a DNA Engine Opticon PCR system (MJ Research Inc.) and Bio-Rad MyiQ iCycler single-colour real-time PCR detection system. To confirm the specificity of the amplification products, samples were separated by standard agarose gel electrophoresis. Threshold levels were set and further analysis was performed using the SDS v1.9 software (Applied Biosystems). The obtained C_t values were normalized to the C_t value of *Gapdh*, β-*actin* or *Hprt*. Each PCR was performed at least in triplicate, and at least two independent experiments were performed to examine the expression of individual genes. Primer sequences and PCR conditions used are available on request. For ChIP, nuclear extracts were prepared[9]. Chromatin cross-linking (2×10^7 cells treated with 1% formaldehyde for 10 min at room temperature), sonication to 300–800 base pair fragments and immunoprecipitation were as described (Upstate protocol, http://www.upstate.com). At least two independent ChIPs were carried out per experiment. Quantitative real-time PCR was performed as described above. Values were normalized to input measurements and enrichment was calculated relative to the *Amylase* gene using the comparative C_t method. PCR products were all smaller than 150 bp. Primer sequences and PCR conditions used are available on request. Western blot analysis was performed as described[39]. Primary antibody incubation was done overnight at 4°C in Tris-buffered saline, containing 5% (w/v) BSA and 0.15% (v/v) NP-40. Blots were incubated with secondary goat anti-rabbit or anti-mouse antibodies, coupled to horseradish peroxidase (GE Healthcare UK Ltd: 1:50 000). Signal detection was performed using ECL (Amersham). Anti-CTCF (N3) and anti-fibrillarin (no. 4118 and 4080) antibodies were generated as described[39] using GST-linked chicken CTCF (amino acids 2–267) and fibrillarin fusion proteins. These antisera were used in a 1:300 dilution. DNMT1 (Abcam), and UBF (Santa Cruz Biotechnology) mAbs were used at 1:100. Western blots were scanned and the levels of protein were quantified using the gel macro function in ImageJ (Rasband, WS, NIH, http://rsb.info.nih.gov/ij/). The amount of CTCF was normalized to DNMT1 in the same sample.

Flow cytometric analyses. Preparation of single-cell suspensions, FDG-loading, mAb incubations for four-color cytometry have been described[43]. All mAbs were purchased from BD Biosciences (San Diego, CA). Samples were acquired on a FACSCalibur™

flow cytometer and data was analysed using CellQuest™ software (BD Bioscience). For cell cycle profiles of thymic subsets, cells were first stained for surface markers, fixed with 0.25% paraformaldehyde and permeabilized with 0.2% Tween 20. Next, 7-AAD was added to a final concentration of 15 µg/ml in PBS. 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. FACS sorting of DN, ISP and DP cells was performed with a FACSVantage VE equipped with Diva Option and BD FACSdiva software (BD bioscience). The purity of fractions was >98%.

Statistical analysis. Statistical evaluations were performed by standard two-tailed *t*-test.

ACKNOLEDGMENTS

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SUPPLEMENTARY RESULTS

CTCF deficiency in γδ T cells

LacZ expression experiments in *Lck-Cre Ctcf$^{f/f}$* mice initially suggested that the *Ctcf* gene is expressed in γδT cells (data not shown). These results are confirmed using GFP-CTCF as a marker (**Supplementary Fig. 4B**). In ocntrast to αβ T cells, deletion of the *Ctcf* gene has no adverse effect on γδ T cell development, since the number of CD3$^+$TCRγδ$^+$ thymocytes in *Lck-Cre Ctcf$^{f/f}$* mice is ~2-fold higher than in wild type littermates (**Supplementary Fig. 2A and B**). The relative proportion of γδ T cells in the spleens of *Lck-Cre Ctcf$^{f/f}$* mice is also markedly increased (**Supplementary Fig. 2A**), due to impaired αβ T cell production. In fact, *in vitro* culture of anti-CD3/CD28 antibody-stimulated peripheral T cell fractions from *Lck-Cre Ctcf$^{f/f}$* mice results in a selective outgrowth of TCRγδ T cells (**Supplementary Fig. 2C**). We do not detect CTCF protein in a mixed population of *in vitro* activated TCRαβ and TCRγδ T cells from *Lck-Cre Ctcf$^{f/f}$* mice (**Supplementary Fig. 2D and E**). We therefore conclude that CTCF is essential for TCR-mediated activation and proliferative expansion of TCRαβ but not of TCRγδ T cells.

CTCF deficient thymocytes and the maintenance of methylation

CTCF has been proposed to be required for the maintenance of methylation at the *Igf2/H19* locus (Schoenherr *et al*, 2003). Aberrant methylation of this locus, or of other loci, might therefore cause defects in CTCF-negative T cells. As CTCF deletion is highly efficient in T cells from *Lck-Cre Ctcf$^{f/f}$* mice and expression of DNMT1, a maintenance methyltransferase with an important role in T cell development (Lee *et al*, 2001), is not significantly affected by deletion of CTCF in thymocytes (**Fig. 1C and 6A**), we used these cells to examine DNA methylation in the *Igf2/H19* locus in the absence of CTCF. We find similar methylation of a CTCF-binding site in the *Igf2/H19* imprinted locus in wild type and CTCF-deleted thymocytes (**Supplementary Fig. 5A**). We also examined the methylation status of the ribosomal DNA (rDNA) repeats in *Ctcf* knockout cells, as these repeats have been shown to be heavily methylated (Bird *et al*, 1981), and CTCF binds to a region of the rDNA repeat upstream of the transcription start site (Van de Nobelen *et al*, manuscript in preparation). We do not detect differences in rDNA methylation in the thymus (where CTCF is virtually absent) and spleen (where CTCF is not deleted) from *Lck-Cre Ctcf$^{f/f}$* mice (**Supplementary Fig. 5B**). We conclude that a deletion of CTCF does not lead to aberrant methylation patterns. The paralogue of CTCF, named CTCF-L or BORIS, can bind the same DNA sequences as CTCF (Loukinov *et al*, 2002). However, we do not detect *Ctcf-l* mRNA in T cells in the presence or absence of CTCF

(data not shown). Thus, CTCF-L neither acts in a dominant-negative fashion in T cells lacking CTCF, not does it compensate for loss of CTCF.

CTCF deficient thymocytes and nuclear organization

Given the important role of CTCF in chromatin organization of the rDNA repeat (Van e Nobelen *et al*, manuscript in preparation) we examined whether lack of CTCF affects nucleolar organization in T cells. We visualized the rDNA repeats in wild type nd CTCF-deficient T cells by fluorescent in situ hybridization (FISH) with an rDNA probe (Akhmanova *et al*, 2000) (**Supplementary Fig. 6**). Based on the FISH signals we counted the number of rDNA dots in $CD4^+$ and $CD8^+$ T cells, derived from *Ctcf* knockout (KO) and wild-type (WT) mice (**Supplementary Table 1**). We found a very small shift towards a lower number of dots in *Ctcf* knockout cells. Combined our data indicate that in naive resting T cells nuclear and nucleolar organization are not dramatically perturbed. The effects that we observe are therefore mainly established upon T cell activation, explaining the phenotype observed in ISP cells.

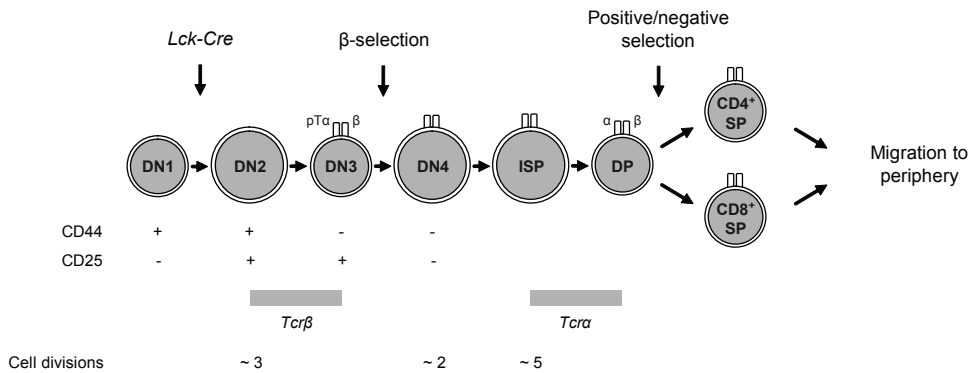
SUPPLEMENTARY METHODS

In vitro T cell cultures. For *in vitro* T cell cultures from wild-type or *lck-Cre Ctcf$^{f/f}$* mice, either total T cell fractions (**Supplementary Fig. 2C**) were purified from lymph node/ spleen by MACS depletion (using anti-B220, anti-NK1.1, anti-Ter119, anti-CD11b and anti-Gr-1 antibodies), TCRαβ−enriched T cell fractions (**Supplementary Fig. 2D**) were purified by MACS depletion using the same antibody mix supplemented with anti-TCRγδ antibodies, or $CD4^+$ T fractions (**Fig. 6D**) were purified by MACS depletion using the same antibody mix supplemented with anti-CD8 antibodies. Purity of obtained fractions was >98%, but the TCRαβ−enriched T cell fractions still contained TCRγδ low T cells (<2% in wild-type and ~30% in CTCF-deficient mice). T cells were cultured at a concentration of 1 x 10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) (Bio Whittaker, Walkersville, MD) containing 10% heat-inactivated FCS, $5x10^{-5}$ M β-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. Stimulation was with plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) mAbs (coated at 10 µg/ml each) at 4 °C overnight for 7 days.

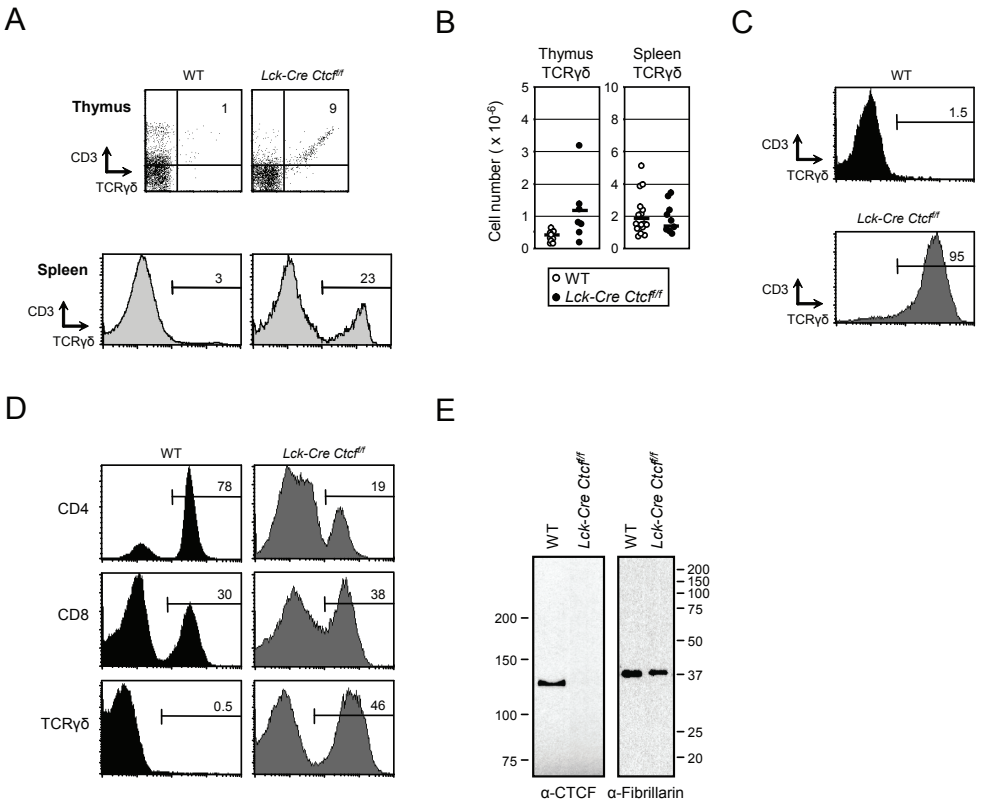
Fluorescent *in situ* hybridization (FISH). FACS sorted naïve $CD62L^+$ $CD4^+$ T cells from *cd4-Cre Ctcf$^{f/f}$* mice were briefly cultured (Ribeiro de Almeida *et al*, manuscript in preparation) and allowed to attach to glass slides for 30 min. Cells were fixed for 10 min with 4% PFA/PBS. Slides were stored in 70% EtOH until further use. For DNA-FISH

slides were pre-treated by two PBS wash-steps followed by a permeabilization step (4 min incubation in 0,1% pepsin in 0,01M HCl at 37 °C). Slides were washed once in PBS on ice and fixed again for 5 min in 4% PFA/PBS. Slides were washed twice in PBS and dehydrated. Denaturation was done for 2 min at 80 °C in denaturing solution (70% formamide; 2xSSC; 10 mM phosphate buffer, pH 7), after which the slides were cooled in 70% EtOH, dehydrated and hybridised as described (Gribnau *et al*, 2005). The rDNA probe (an 11.8 kb *Sal*I fragment of a murine rDNA cosmid which contains non transcribed rDNA only (Akhmanova *et al*, 2000) was DIG labelled by nick translation (Roche). We used a Zeiss Axioplan 2 microscope for image acquisition and cell counting. Cells were counted without knowledge of the genotype, statistical significance was tested with the chi-square tool (Excel), with a *P*-value of 0.005.

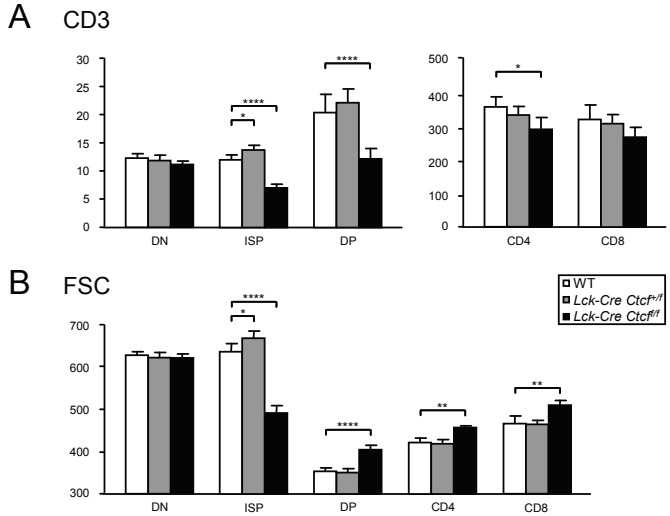
SUPPLEMENTARY FIGURES AND TABLE



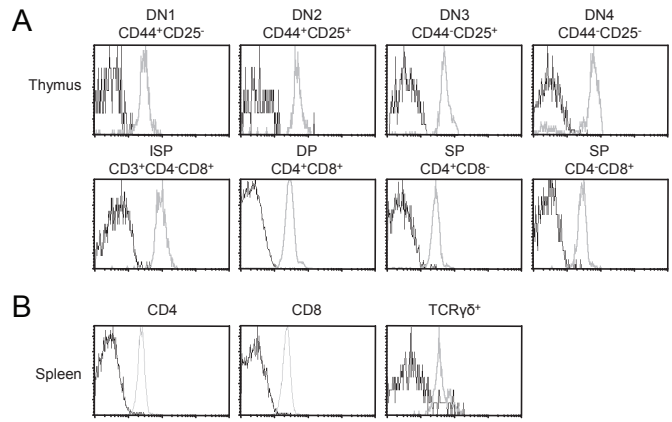
Supplementary Figure 1. Schematic overview of T cell differentiation in the thymus. T cell progenitors that enter the thymus are called double-negative (DN) cells. These cells differentiate as outlined in the figure in a series of stages towards mature thymocytes, which leave the thymus and migrate to the periphery. The DN1-DN4 early T cell stages are characterized by the differential expression of the CD25/CD44 markers. At DN2 a choice is made whether to rearrange the αβ or the γδ *Tcr* genes. The γδ T cell differentiation pathway is not depicted here. In the case of αβ T cells, the *Tcrβ* gene is rearranged first (the approximate time frame of rearrangement is indicated below the cells). Upon productive recombination (mediated by the RAG proteins, see main text) a functional β receptor is expressed at the cell surface, where it associates with pTα, a "surrogate" TCRα receptor, required for signalling. Cells subsequently start to grow, and they divide rapidly. At the double-positive (DP) stage cells cease division and start rearranging their *Tcr*α genes (approximate time frame is indicated below the cells). Productive rearrangement results in the expression of the αβ TCR. Positive and negative selection result in the formation of a pool of competent CD4 and CD8 single-positive (SP) cells. It should be realized that more than 90% of the DP cell population dies during the processes of selection. The approximate number of cell divisions in the different stages are indicated on the bottom of the scheme. The Lck-promoter turns on Cre expression in DN1-DN2 (indicated by downward arrow). This T cell developmental scheme is based on (Laurent *et al*, 2004; Lucas *et al*, 1993).



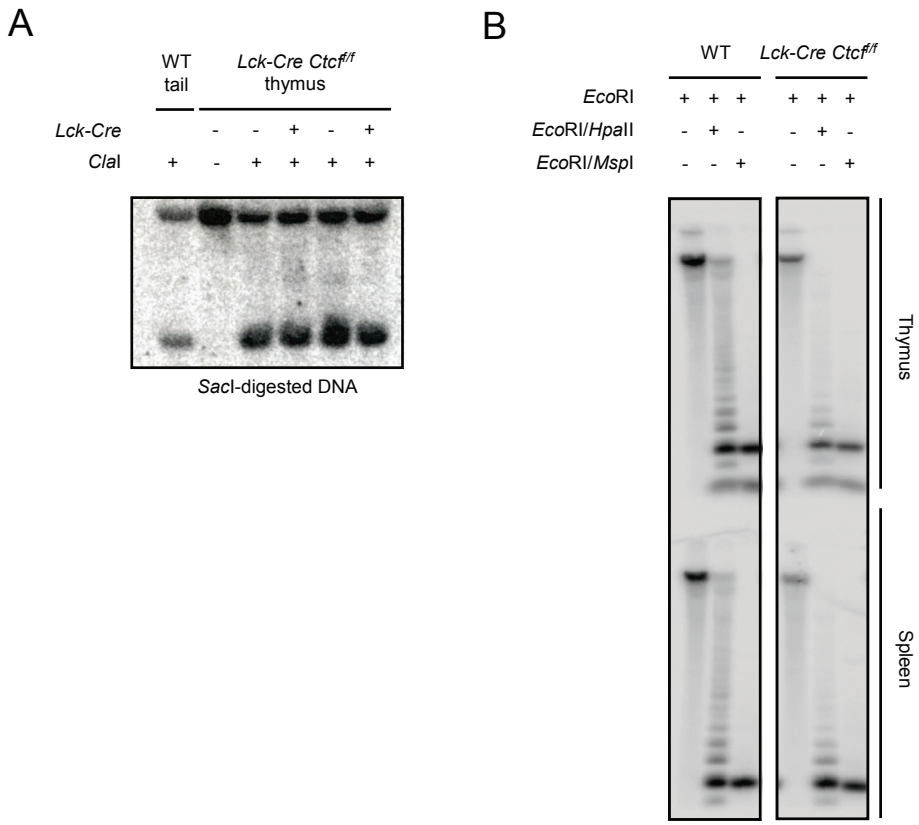
Supplementary Figure 2. Characterization of TCRγδ cells in *Lck-Cre Ctcf*$^{f/f}$ mice. (**A**) Flow cytometric analyses of total thymocytes and CD3$^+$ splenocytes derived from wild type (WT) and *Lck-Cre Ctcf*$^{f/f}$ mice. For the thymus, expression profiles of CD3 and TCRγδ surface markers are shown as dot plots. Gating is indicated by the horizontal and vertical lines in the graph. The percentages of CD3$^+$TCRγδ$^+$ cells are shown. For the spleen, data are displayed as histograms and the percentages represent the fractions of CD3$^+$ cells that are TCRγδ$^+$. Data shown are representative of all animals. (**B**) Absolute numbers of TCRγδ$^+$ T cells in thymus and spleen of WT and *Lck-Cre Ctcf*$^{f/f}$ mice. Each symbol represents a measurement in one animal (9 or more tested per group). Horizontal lines indicate averages. TCRγδ$^+$ T cells are significantly increased in the thymus of *Lck-Cre Ctcf*$^{f/f}$ mice (P<0.05). (**C**) Flow cytometric analysis of TCRγδ expression in T cell cultures from WT and *Lck-Cre Ctcf*$^{f/f}$ mice. Lymph node fractions were stimulated by anti-CD3/CD28 and cultured for 7 days. The percentages represent the fractions of TCRγδ$^+$ T cells. The proportions of γδ$^+$ T cells in the T-cell enriched cell suspensions before culture was <2% in WT and ~30% in *Lck-Cre Ctcf*$^{f/f}$ mice (see also panel **A**). (**D**) Flow cytometric analysis of CD4, CD8 and TCRγδ expression in mixed T cell cultures from WT and *Lck-Cre Ctcf*$^{f/f}$ mice. Lymph node cell fractions were enriched for CD4 and CD8 cells and depleted for TCRγδ$^+$ T cells, stimulated by anti-CD3/CD28, and cultured for 7 days. The percentages represent the fractions of different T cells. (**E**) Western blot, showing the absence of CTCF protein in mixed T cell cultures from WT and *Lck-Cre Ctcf*$^{f/f}$ mice. Fibrillarin was used as a loading control. Molecular weight markers are indicated in kDa.



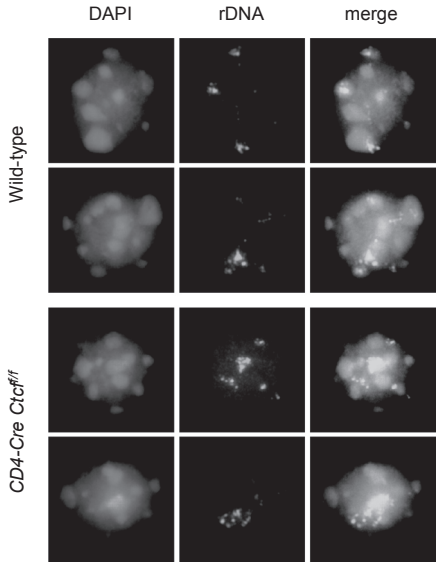
Supplementary Figure 3. Comparison of CD3 expression and cell size in wild type and CTCF-deficient thymocytes. Quantification of CD3 levels (CD3, **A**) and forward scatter values (FSC, **B**) in thymocyte subpopulations of wild type (WT, white bars), heterozygous *Lck-Cre Ctcf*$^{+/f}$ (grey bars) and homozygous *Lck-Cre Ctcf*$^{f/f}$ (black bars) mice. FSC is a measure of cell size. Plotted are average values ± SD from 5 WT, 4 heterozygous and 3 homozygous mice. Statistically significant differences (*t*-test) are indicated (*P* <0.005: *, *P* <0.01: **, *P* <0.001: ***, *P* <0.0001: ****). DN: double-negative, ISP: immature single-positive, DP: double-positive, CD4: CD4 single-positive, CD8: CD8 single positive (see also **Supplementary Figure 1**).



Supplementary Figure 4. Flow cytometric analysis of GPF-CTCF protein expression. GPF-CTCF protein was analyzed, in conjunction with cell surface markers, in cell suspensions from thymus (**A**) and spleen (**B**) from mice carrying a *GFP-Ctcf* knock-in allele (*CtcfGFP*, H.H. *et al.,* manuscript in preparation, for targeting strategy see (Akhmanova *et al*, 2005). The indicated cell populations were gated and expression data are displayed as histogram overlays of GFP-CTCF (grey) on top of background signals in wild type mice (black). Note that CTCF is also expressed in γδ T cells.



Supplementary Figure 5. DNA methylation in T cells. (**A**) DNA methylation analysis in the Imprinting Control Region (ICR) of the *Igf2/H19* locus. DNA was isolated from the thymus of *Ctcf*$^{f/f}$ mice, which were either not crossed (-, lanes 2, 3 and 5) or crossed (+, lanes 4 and 6) with *Lck-Cre* transgenics. Samples were digested with *Sac*I only (lane 2) or with both *Sac*I and *Cla*I (other lanes). *Cla*I cuts within CTCF binding site 1 of the *Igf2/H19* ICR (Schoenherr et al, 2003). Normal tail DNA (lane 1) is shown as control. (**B**) Methylation status of ribosomal DNA (rDNA) repeats. Southern blot analysis of genomic DNA from thymus and spleen of the indicated mice. DNA was digested with *Eco*RI (*lanes 1*), *Eco*RI and *Hpa*II (*lanes 2*), or (3) *Eco*RI and *Msp*I (*lanes 3*). Blots were hybridized with the unstable 5' external transcribed spacer probe (Akhmanova *et al*, 2000).



Supplementary Figure 6. CTCF deletion does not affect nucleolar organization in resting T cells. FISH analysis of nucleolar organization in FACS-sorted naive CD62L+ peripheral T cells from the indicated mice. Slides were hybridized with a DIG-labeled rDNA probe containing non transcribed rDNA (Akhmanova *et al*, 2000). Cells were counterstained with DAPI. **See Appendix for full-color figure.**

Supplementary Table 1. Number of nucleoli in wild-type (WT) and CTCF-negative (KO) T cells.

CD4$^+$	**number of rDNA dots (%)**								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total**
WT	0	2	17	20	32	22	5	1	100 (*n*=358)
KO	0	3	16	29	26	18	6	1	100 (*n*=368)

CD8$^+$	**number of rDNA dots (%)**								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total**
WT	1	5	9	27	26	21	7	3	100 (*n*=351)
KO	0	6	12	27	28	18	8	1	100 (*n*=354)

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Critical role for the transcription regulator CCCTC-binding factor in the control of Th2 cytokine expression

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ABSTRACT

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 signalling 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.

INTRODUCTION

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[1, 2]) Th1 cells, which produce IFN-γ, are predominantly involved in cellular immunity against intracellular pathogens. Two major signalling 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[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[17, 18]) Indeed, both Stat6 and Gata3 are responsible for the establishment and/or maintenance of the chromatin conformation changes in the Th2 cytokine locus of developing Th2 cells[16, 19]. Particularly, because Gata3 can induce chromatin-remodeling activity[20], it may facilitate the interaction between the Th2 locus control region and the cytokine gene promoters and thereby coregulate IL-4, IL-5. and IL-13 expression. In addition, Th2 cytokine expression requires special AT-rich binding protein 1 (Satb1), which mediates the formation of a densely looped, transcriptionally active chromatin structure at the Th2 locus containing Gata3, Stat6, c-Maf, the chromatin-remodeling enzyme Brg1, and RNA polymerase II[21].

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[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 insulation[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 λL 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.

RESULTS

CTCF binding sites in the Th2 cytokine locus

CTCF binding sites in the human Th2 locus have recently been identified in $CD4^+$ 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 *Il5*, *Rad50*, *Il13*, *Il4*, and *Kif3a* 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[35]. 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+ 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 *Kif3a* 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[36, 37]. Our data indicate that the CTCF protein binds to CBS-1, CBS-3, CBS-6, and CBS-7, irrespectively, of Th1 or Th2 polarization.

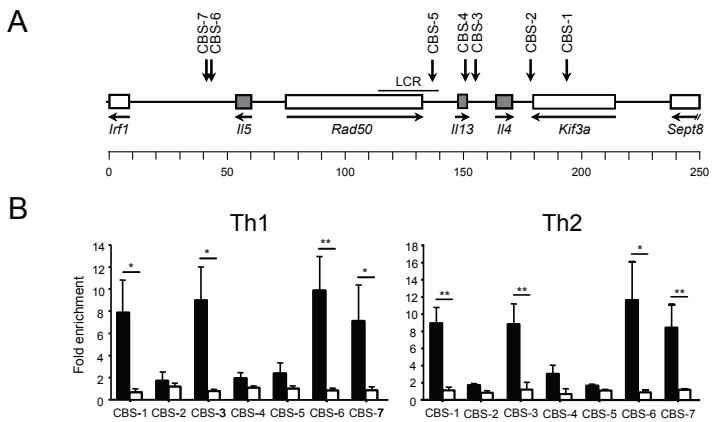

Figure 1. CTCF binding sites in the murine Th2 cytokine locus. (**A**) Schematic representation of the Th2 cytokine locus and CTCF binding sites (CBS-1 to CBS-7). CBS were identified by a bioinformatics approach in combination with ChIP assays. (**B**) ChIP analysis in Th1 and Th2 cells. Analysis was performed with CTCF (black) or IgG (white) Abs in 7-day polarized Th1 or Th2 cells. Mean values and SD are given for two ChIP experiments; *, $P<0.05$ and **, $P<0.01$.

Conditional deletion of the *Ctcf* gene in T lymphocytes

To study CTCF function in vivo, we generated a conditional *Ctcf* allele (*Ctcf*^{f}) 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 *Ctcf*$^{f/f}$ mice to mice carrying a Cre-encoding transgene under the control of the CD4 promoter[38]. Southern blotting showed almost complete deletion of the *Ctcf* gene in the thymus of CD4-Cre *Ctcf*$^{f/f}$ mice, whereas in the spleen *Ctcf* deletion was only partial, reflecting the presence of many non-T lineage cells (**Fig. 2B**)

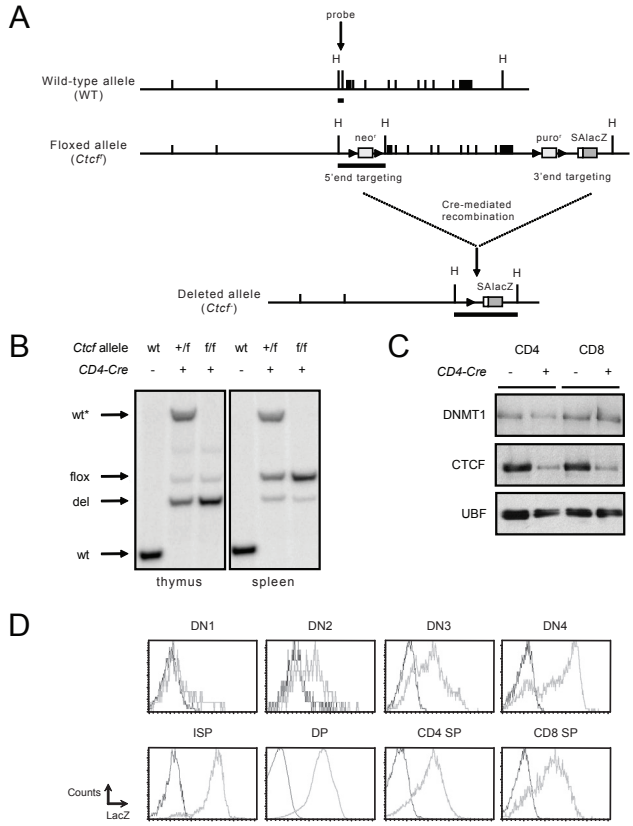

Figure 2. Conditional targeting of the mouse *Ctcf* gene. (**A**) Simplified map of the wild-type (WT) murine *Ctcf* locus and modified alleles. *Top line*, WT *Ctcf* allele, with exons shown as solid boxes, and position of *Hin*dIII sites (H) and the Southern blot probe (arrow) indicated. *Middle line*, Floxed *Ctcf* allele (*Ctcf*^{f}). Two targeting cassettes were inserted into the *Ctcf* gene via consecutive rounds of homologous recombination. The first cassette contained LoxP sites (black arrows) flanking a PMC1-driven neomycin resistance gene (neo^{r}), the second cassette contained LoxP sites flanking a PGK-driven puromycin resistance gene (puro^{r}), followed by a splice acceptor-lacZ cassette (SA-LacZ). *Bottom line*, deleted Ctcf gene (*Ctcf*$^{-}$) generated after complete Cre-mediated recombination at the outermost loxP sites. For details on constructs and targeting, see[34]. The small horizontal lines underneath each allele represent *Hin*dIII fragments recognized by the probe in the Southern blot analysis of **B** below. (**B**) Southern blot analysis of *CD4-Cre* recombinase activity. *Hin*dIII-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*$^{f/f}$ (flox), and *Ctcf*$^{-/-}$ (del) alleles are indicated (asterisk indicates a polymorphic WT allele from the FVB background). (**C**) Western blot analysis of sorted naive CD62L^{+}CD4^{+} and CD8^{+} T cell fractions from nontransgenic (–) or *CD4-Cre*-transgenic (+) *Ctcf*$^{f/f}$ 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*$^{f/f}$ mice (grey) on top of the background signals in WT mice (black). ISP, Immature SP.

To evaluate the onset of *Ctcf* gene deletion, we analyzed thymocyte subpopulations for expression of the Ctcf-*lacZ* fusion transcript, using fluorescein-di-β-D-galactopyranoside as a substrate in conjunction with cell surface markers. Consistent with the reported CD4-Cre activity at the double-negative (DN) stage[39], *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$^+$CD4$^+$ and CD8$^+$ 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 *Ctcf*$^{f/f}$ mice and wild-type (WT) littermates were analyzed by flow cytometry. CD4-Cre *Ctcf*$^{f/f}$ mice displayed low thymic cellularity, with reduced numbers of double-positive (DP), CD4, and CD8 single-positive (SP)

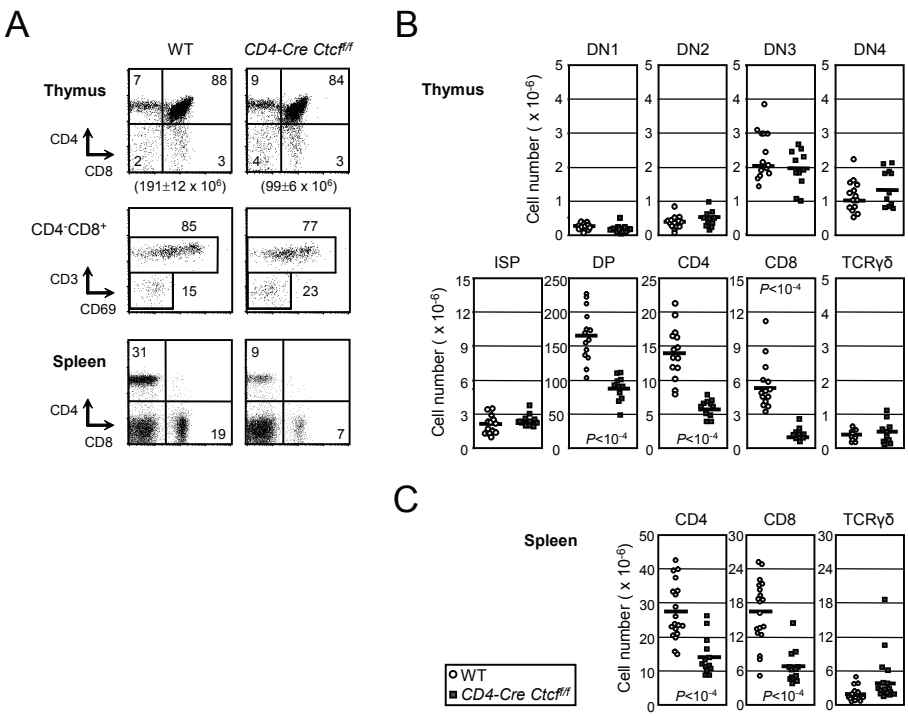

Figure 3. Defective TCRαβ lineage development in CTCF-deficient mice. (**A**) Flow cytometric analyses of the indicated cell populations in thymus or spleen from wild-type (WT) and *CD4-Cre Ctcf*$^{f/f}$ mice. Expression profiles of surface markers are shown as dot plots and the percentages of cells 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 *P* of significant differences between WT and *CD4-Cre Ctcf*$^{f/f}$ mice are indicated.

cells, when compared with WT controls (**Fig. 3A,B**). The $CD4^{-}CD8^{+}$ thymocyte fraction had a relative increase of $CD3^{low}CD69^{low}$ ISP cells and a decrease of $CD3^{+}CD8^{+}$ SP cells. Consistent with impaired thymic SP cell production, the numbers of mature $CD4^{+}$ and $CD8^{+}$ T cells in spleen and lymph nodes of CD4-Cre *Ctcf*$^{f/f}$ mice were significantly reduced (**Fig. 3A,C** and *data not shown*). Furthermore, the numbers of γδ T cells in the spleens of CD4-Cre *Ctcf*$^{f/f}$ mice were increased (**Fig. 3C**).

In summary, in CD4-Cre *Ctcf*$^{f/f}$ mice, αβ T cell development is partially arrested at the DP stage, resulting in a significant reduction of the numbers of peripheral $CD4^{+}$ and $CD8^{+}$ T cells.

Defective TCR/CD3-mediated proliferation of CTCF-deficient $CD4^{+}$ and $CD8^{+}$ T cells

To investigate cellular activation of CTCF-deficient T cells, we performed in vitro stimulation experiments with highly purified naive $CD62L^{+}CD4^{+}$ and $CD8^{+}$ 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 $CD4^{+}$ 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 $CD4^{+}$ T cells to proliferate upon a more physiological, Ag-specific stimulation was investigated by crossing CD4-Cre-*Ctcf*$^{f/f}$ mice with OT-II-transgenic mice, which harbor a TCR specific for OVA peptide. Upon activation by OVA peptide$_{323–339}$-pulsed APC, survival of nondividing (WT and CTCF-deficient OT-II-transgenic $CD4^{+}$ T cells was similar, but we noticed a severe proliferation defect in the absence of CTCF (**Fig. 4A**). Although anti-CD3/CD28-activated 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 signalling events and directly activates protein kinase C signalling[40]. 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-G_2-M phase of the cell cycle (**Fig. 4B**). Although CTCF-deficient PMA/ionomycin-stimulated $CD4^{+}$ or $CD8^{+}$ T cell cultures

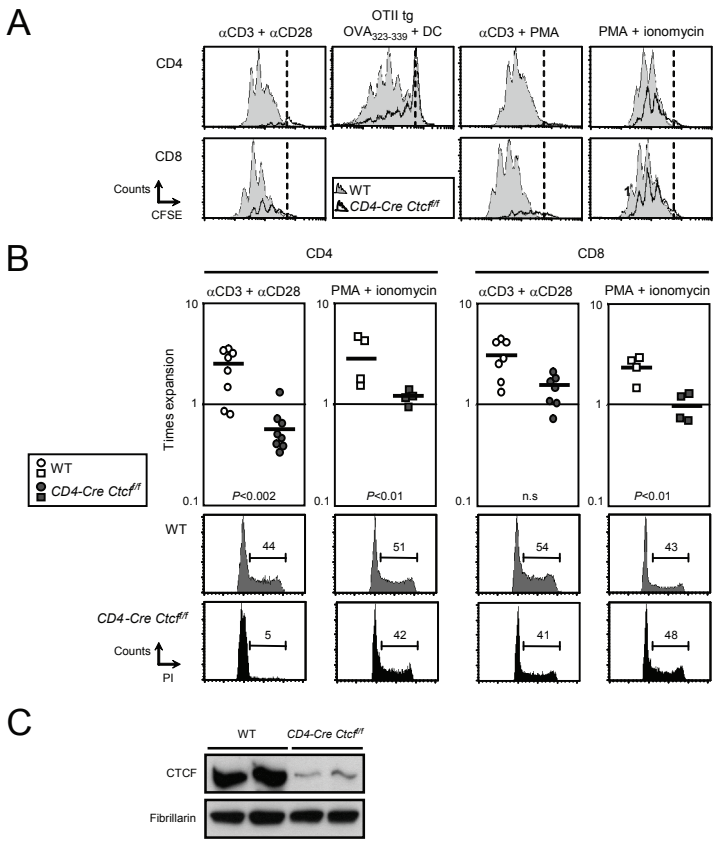

Figure 4. Impaired anti-CD3ε-mediated proliferation of CTCF-deficient T cells. (**A**) CFSE profiles of T cell cultures of sorted CD62L$^+$CD4$^+$ and CD8$^+$ T cell populations from wild-type (WT) mice (gray histograms) in comparison to cultures from *CD4-Cre Ctcf$^{f/f}$* mice (black line). Cells were activated by the indicated stimuli and cultured for 3 days (or 4 days for *OTII* CD4 T cells). Dotted lines indicate the fluorescence intensity of unstimulated cells. (**B**) Cellular expansion in 3-day cultures upon anti-CD3/CD28 or PMA/ionomycin stimulation (upper part). Symbols represent the expansion values of cultures from individual mice, whereby cell numbers at the start of the culture were set to one; lines indicate average values. Values of *P* of significant differences between WT and *CD4-Cre Ctcf$^{f/f}$* mice are indicated; n.s., not significant. The *lower part* shows the cell cycle status of the indicated cultures, whereby DNA content was examined by propidium iodide (PI) staining. The percentages of cycling cells (S-G_2-M phase) are shown. (**C**) Western blotting analysis of naive CD62L$^+$CD8$^+$ T cells from WT or *CD4-Cre*-transgenic *Ctcf$^{f/f}$* mice cultured for 5 days and examined for CTCF levels. Fibrillarin was used as a loading control.

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 *Ctcf$^{f/f}$* 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 signalling 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 signalling 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[41]. 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$^+$ or CD8$^+$ 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$^+$ and CD8$^+$ T cells was severely impaired, when activated by plate-bound anti-CD3/CD28 (**Fig. 5B**). When activated by PMA/ionomycin, CTCF-deficient CD4$^+$ T cells displayed a partial defect in CD25 up-regulation, while in CD8$^+$ T cells CD25 induction was normal.

Expression of CD69, an ~30-kDa glycoprotein induced in activated T cells, was hampered in CTCF-deficient CD4$^+$ T cells upon TCR stimulation, but was normal in CD8$^+$ 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$^+$ cells were more affected than CD8$^+$ 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$^+$ T cells are somewhat more affected than CD8$^+$ 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 Ctcf$^{f/f}$* mice have severely reduced serum levels of IgG1 and IgE**

Concentrations of Ig subclasses in the serum of CD4-Cre *Ctcf$^{f/f}$* mice were reduced, except for IgM and IgG3, which are T cell independent and IgA which requires TGF-β expression (**Fig. 6A**). This does not necessarily mean that CTCF-deficient CD4$^+$ T cells

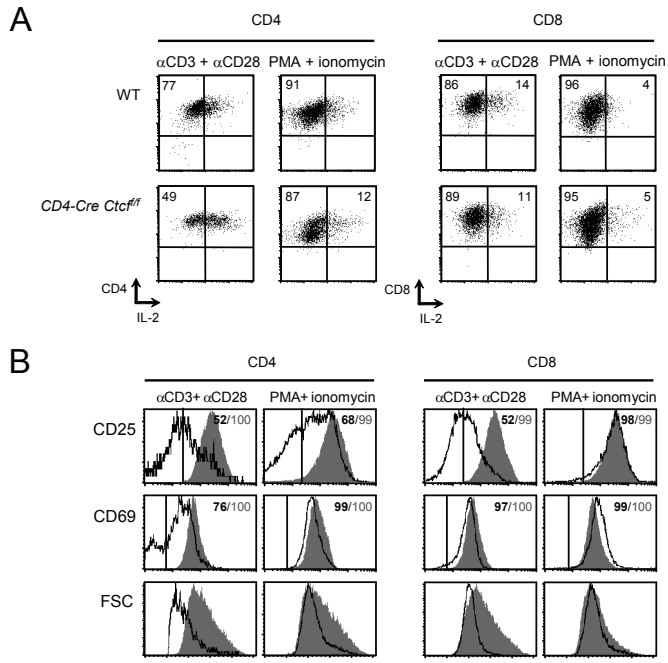

Figure 5. Impaired cellular activation of CTCF-deficient T cells. (**A**) Analysis of IL-2 expression in anti-CD3/CD28 and PMA/ionomycin-stimulated cultures of sorted CD62L$^+$CD4$^+$ and CD8$^+$ T cell fractions from wild-type (WT) and *CD4-Cre Ctcf$^{f/f}$* mice. At day 3, cells were restimulated for 4h before intracellular flow cytometric analysis. Total living cells were gated and CD4/IL-2 and CD8/IL-2 profiles are displayed as dot plots and the percentages of cells within the quadrants are given. Data shown are representative of four mice per group. (**A**) Phenotypic characteristics of anti-CD3/CD28 or PMA/ionomycin-stimulated CD4$^+$ and CD8$^+$ T cells. CD25, CD69, and forward scatter profiles are displayed as histogram overlays of WT (gray histograms) and *CD4-Cre Ctcf$^{f/f}$* cultures (bold lines). The percentages shown represent the fractions of the cells within the indicated marker in WT (grey) or *CD4-Cre Ctcf$^{f/f}$* (black, bold) cultures. Data shown are representative of four to six mice per group.

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 *Ctcf$^{f/f}$* 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 *Ctcf$^{f/f}$* 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.

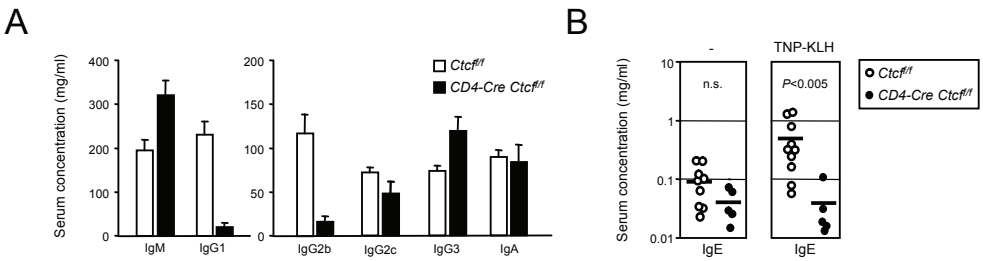

Figure 6. Serum Ig analysis in CTCF-deficient mice. (**A**) Serum concentrations of Ig isotypes displayed as average values plus SD. Mice were 2 mo of age. (**B**) Total IgE serum concentrations in nonimmunized mice (left) and in immunized mice 7 days after i.p. injection with 10 μg TNP-KLH (right).

Th2 cytokine defect in *CD4-Cre Ctcf$^{f/f}$* 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 in 7 days (**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 cell cultures IL-5 production was

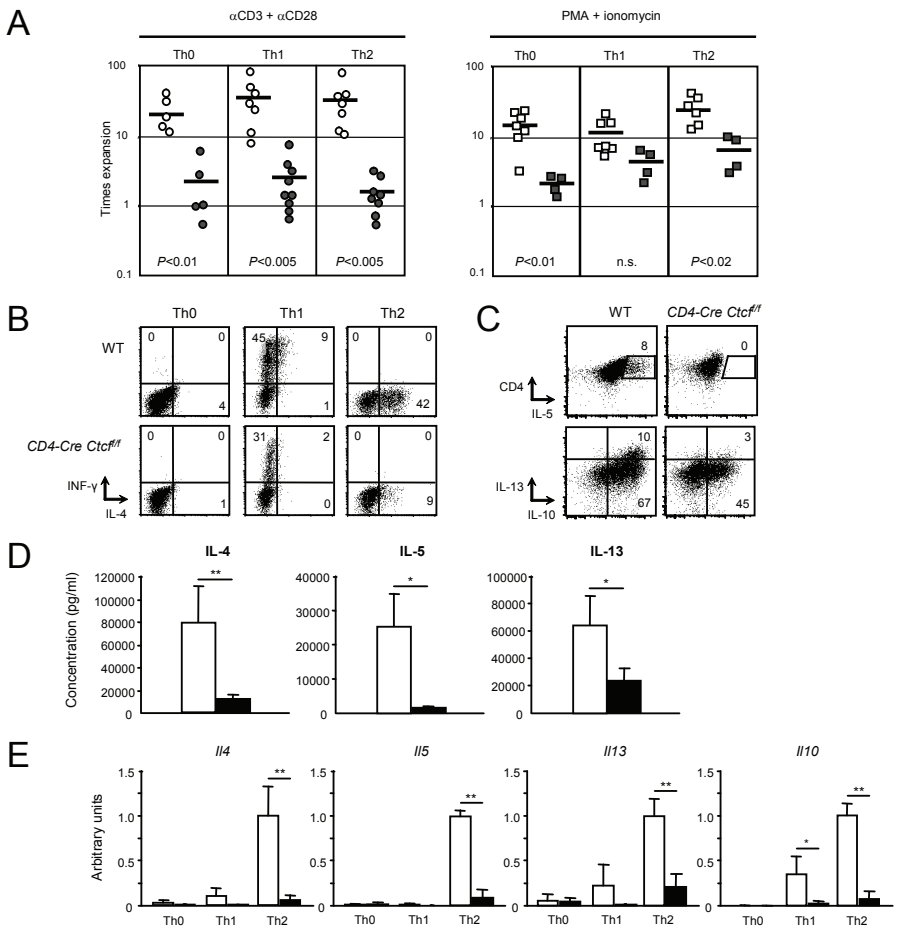

Figure 7. CTCF regulates Th2 cytokine expression. (**A**) Expansion of Th0, Th1, and Th2 cultures 7 days after stimulation with anti-CD3/CD28 or PMA/ionomycin. Symbols represent the expansion values of cultures from individual mice, whereby cell numbers at the start of the culture were set to 1. Lines indicate average values. Values of *P* of significant differences between wild-type (WT, white) and *CD4-Cre Ctcf*$^{f/f}$ (black) 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. Mean fluorescence values for IL-4 were 138 and 75 for WT and *CD4-Cre Ctcf*$^{f/f}$ IL-4$^+$ Th2 cells, respectively. (**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 (white) and *CD4-Cre Ctcf*$^{f/f}$ (black) 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, white; *CD4-Cre Ctcf*$^{f/f}$, black). 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.

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 Ctcf$^{f/f}$* 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%, n = 3; **Fig. 8C**) expressed Foxp3, the transcription factor associated with Treg differentiation[42]. Such Foxp3$^+$ cells were also present in Treg cultures of CTCF-deficient T cells, albeit that the proportions were slightly lower (62 ± 2%; n = 3). Furthermore, the proportions of naturally occurring CD25$^+$Foxp3$^+$CD4$^+$ Treg cells in spleen and lymph nodes in vivo were not different between WT and CTCF-deficient mice (**Fig. 8D**).

As a control, we also evaluated IFN-γ and granzyme B expression in day 7 cultures of anti-CD3/CD28- or PMA/ionomycin-stimulated CD62L$^+$CD8$^+$ T cells. We found that production of IFN-γ and granzyme B was only moderately affected (data not shown).

Collectively, these findings show that differentiation of CTCF-deficient T cells into

IL-17-producing Th17 cells is apparently normal and lack of CTCF has limited effects on differentiation of Treg and $CD8^+$ T cells. We therefore conclude that the absence of CTCF does not result in a global defect in effector T cell differentiation.

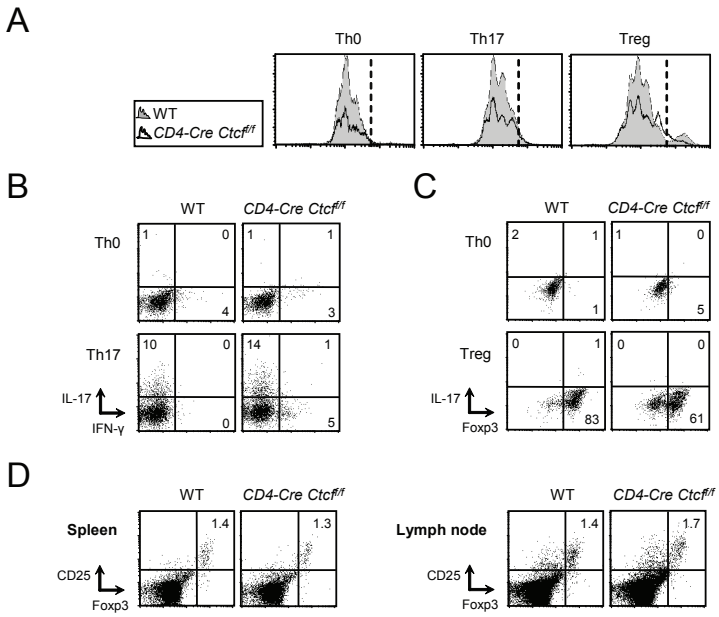

Figure 8. CTCF is not essential for Th17 or Treg differentiation. (**A**) CFSE profiles of Th0, Th17, and Treg cultures of sorted $CD62L^+CD4^+$ T cell populations from wild-type (WT) mice (gray histogram) in comparison to cultures from *CD4-Cre Ctcf$^{f/f}$* mice (black line). Cells were activated with PMA/ionomycin and cultured for 3 days. Dotted lines indicate the fluorescence intensity of unstimulated cells. (**B**) Flow cytometric analysis for intracellular expression of IFN-γ and IL-17 in Th0 and Th17 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 Foxp3 and IL-17 expression in PMA/ionomycin-stimulated Treg cultures. $CD4^+$ T cells were gated and expression profiles are displayed as dot plots. (**D**) Flow cytometric analysis for intracellular Foxp3 and membrane CD25 with quantification of $CD25^+Foxp3^+$ Treg cells in spleen and mesenteric lymph nodes *in vivo*. Data shown are representative of four to six mice per group.

Gata3 and Satb1 expression in *CD4-Cre Ctcf$^{f/f}$* Th2 cells

Because Th2 cytokine production depends on the transcription factor Gata3[6-10] we evaluated its expression in the T cell cultures. As determined by intracellular flow cytometry, Gata3 expression appeared unaffected in CTCF-deficient Th2 cultures (**Fig. 9A**), excluding the possibility that Th2 cytokine production was impaired due to defective Gata3 induction. Furthermore, the CTCF-deficient Th2 cultures displayed clear features of Th2- polarized cells, including low mRNA levels of T-bet and Stat4 (**Fig. 9B**). Since Gata3 has the capacity to inhibit Stat4 transcription[43], the finding of low Stat4 expression

levels suggest that in the absence of CTCF Gata3 still operated as a negative regulator of Stat4. In CTCF-deficient Th1 cultures, T-bet expression was reduced when compared with WT (**Fig. 9B**), which is consistent with the observed reduction in IFN-γ expression. Next to Gata3, Satb1 has also been implicated in Th2 locus expression[21]. Satb1 was specifically induced, both in WT and CTCF-deficient Th2 cultures (**Fig. 9C**).

Taken together, these data indicate that differentiating CTCF-deficient Th2 cells show impaired expression of Th2 cytokines IL-4, IL-5, and IL-13, but nevertheless up-regulate the Th2-specific factors Gata3 and Satb1 and down-regulate T-bet and Stat4.

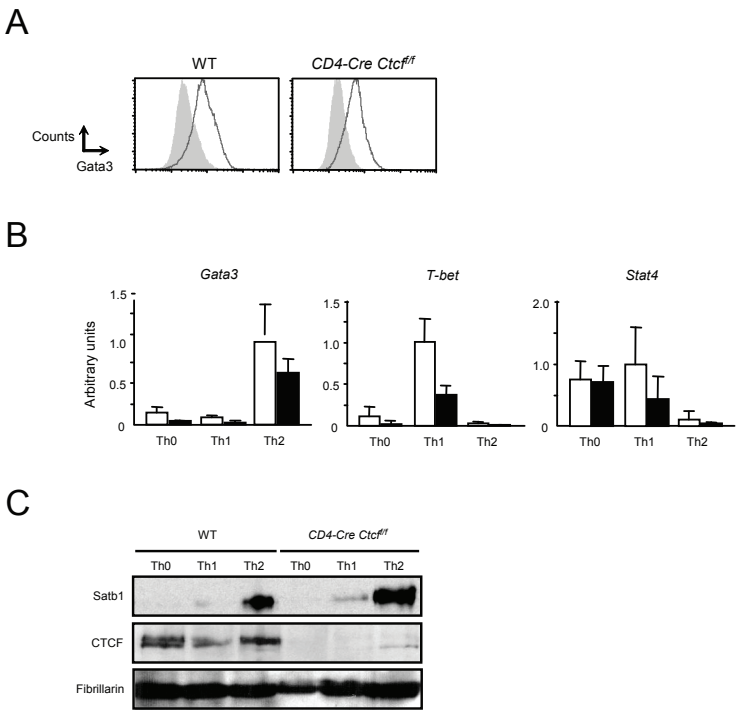

Figure 9. Gata3 and Satb1 are induced in CTCF-deficient Th2 cultures. (**A**) Flow cytometric analysis for intracellular Gata3 protein expression in PMA/ionomycin-stimulated T cell cultures. $CD4^+$ T cells were gated and for the indicated mice expression profiles are displayed as histograms overlays 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 (white) or *CD4-Cre Ctcf$^{f/f}$* mice (black). 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 signalling 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[44, 45]. The finding that PMA/ionomycin stimulation bypassed the CD3/CD28-signalling defect suggests that the presence of CTCF is important for signalling 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 signalling 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 Ribeiro 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 production in CTCF-deficient Th1 cells does not necessarily implicate CTCF in transcriptional regulation of the *Ifng* locus, as decreased IFN-γ production may well result form reduced expression of essential transcription factors such as T-bet and Stat4 (**Fig. 9B**).

Our analysis of CTCF binding in the Th2 cytokine locus revealed four CBS: three sites flanking the Th2 locus (CBS-6 and CBS-7 upstream of the *Il-5* gene, CBS-1 downstream of *Il-4*, within the *Kif3a* gene) and one site in the intergenic region between *Il-13* and *Il-4* (CBS-3), irrespective of Th1 or Th2 polarization. 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[36, 37]. 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[36]. 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[46]. Moreover, Satb1 was originally identified as a matrix attachment region DNA-binding protein[47], which possibly contributes to chromatin loop organization[48]. 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, 46, 49, 50]. Additionally, CTCF has also been shown to interact with the large subunit of RNA polymerase II[51], 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.

METHODS

Mice. T cell-specific deletion of *Ctcf* was achieved by breeding *Ctcf*$^{f/f}$ mice[34], which were crossed on the C57BL/6 background for >10 generations to CD4-Cre mice[38], which were provided by Dr. C. Wilson (University of Washington, Seattle, WA). OT-II-transgenic mice have been described previously[52]. 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[53]. Abs specific for CTCF (N3) and fibrillarin have been previously described[34]. Alternatively, anti-CTCF antiserum was purchased from Millipore. Anti-Dnmt1 was from Abcam and anti-Ubf, 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 I11 erythroid cells was performed using a Solexa 1G Genome Analyser[54]. ChIP analysis of CTCF binding in the Th2 cytokine locus was performed as described in the ChIP Assay Kit protocol (UpState Biotechnology) using the anti-CTCF Abs listed above or 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'-GGTCTTAGCAGGTTCCCAA-3'; CBS-1R, 5'-CGTTCGGTAAGACAAGCAC-3'; CBS-2F, 5'-CACTCAGCACCTTACCTG-3'; CBS-2R, 5'-CCTGGGCTAAATGAATCAGT-3'; CBS-3F, 5'-AGGCACAGTGTAGAAGTGT-3'; CBS-3R, 5'-GTCTCTCTTCCAGTCCAGTT-3'; CBS-4F, 5'-GGCACTTGTAACGCTCTAA-3'; CBS-4R, 5'-CCCTGACCAACATCTCCAA-3'; CBS-5F, 5'-ATTGTGGAGGCTGGCAAG-3'; CBS-5R, 5'-GGTGACAGCCCAAATAAGT-3'; CBS-6F, 5'-CCACATCCACCTGTCACTT-3'; CBS-6R, 5'-CTGTTTCACATCCATCGCA-3'; CBS-7F, 5'-CAGGCTTGTATCATCACCA-3' and CBS-7R, 5'-TTCTTGAGGGACAGCACT-3'.

Flow cytometric analyses. Preparation of single-cell suspensions and mAb incubations for four-color cytometry has been previously described[55]. 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 Pharmingen) for 4 h. Cells were harvested and stained extracellularly, followed by standard intracellular staining using paraformaldehyde 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[56]. Samples were acquired on a FACSCalibur 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, naive $CD62L^+CD4^+$ or $CD8^+$ T cells were purified by cell sorting using a FACSVantage VE equipped with Diva Option and BD FACSDiva software (BD Biosciences). The purity of obtained fractions was >98%. For ChIP experiments, $CD4^+$ T cells from C57BL/6 mice were obtained through incubation with biotinylated mAbs (BD Pharmingen) specific for CD11b (M1/70), Gr-1 (RB6-8C5), Ter119 (Ly-76), TCR$\gamma\delta$ (GL3), B220 (RA3-6B2), NK1.1 (PK136), and CD8 (53-6.7), followed by streptavidin-conjugated microbeads and autoMACS purification according to the manufacturer's instruction (Miltenyi Biotec). The purity of $CD4^+$ T cell fractions was confirmed by FACS to be >95%. T cell fractions were cultured at a concentration of 1×10^6 cells/ml in IMDM (BioWhittaker) containing 10% heat-inactivated FCS, 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin. Stimulation was with plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) mAbs (coated at 10 µg/ml each at 4°C overnight) or PMA (50 ng/ml) plus ionomycin (300 ng/ml). For Th1-polarizing conditions, anti-IL-4 (10 µg/ml; 11B11) and IL-12 (10 ng/ml) were added to the medium. Th2-polarizing cultures included anti-IFN-γ (5 µg/ml; R4-6A2,), anti-IL-12 (5 µg/ml; C17.8), and IL-4 (10 ng/ml). Treg- and Th17-polarizing conditions included TGF-β (3 ng/ml), anti-IL-4, and anti-IFN-γ. Th17-polarizing conditions additionally contained IL-6 (20 ng/ml). For Th0 conditions, no cytokines or mAbs were added. For differentiation of $CD8^+$ effector T cells, 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 $CD4^+$ 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 x 10^6 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 x 10^6 CFSE-labeled OT-II-transgenic naive $CD4^+$ T cells were cocultured with 0.2 x 10^6 BM-DC previously pulsed with OVA peptide$_{323–339}$ (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 x 10^6 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[57].

Statistical evaluations. All Statistical evaluations were done with Student's *t* test.

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5

Gene expression profiling in mice with enforced Gata3 expression reveals putative targets of Gata3 in double positive thymocytes

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ABSTRACT

The zinc-finger transcription factors Gata3 and ThPOK have both been implicated in positive selection of double positive (DP) thymocytes towards the CD4 lineage. As in the absence of Gata3, expression of ThPOK is lacking, Gata3 may directly regulate ThPOK expression. As ThPOK failed to promote $CD4^+$ lineage differentiation of Gata3-defcient cells, ThPOK cannot be the only Gata3 target gene essential for the induction of the $CD4^+$ lineage program. Therefore, it is conceivable that Gata3 is essential for selected DP T cells to reach the developmental stage at which ThPOK expression is induced. Here, we show that Gata3 overexpression does not affect ThPOK expression levels in DP or $CD4^+$ thymocytes, providing evidence that Gata3 does not directly regulate ThPOK. To identify additional target genes that clarify Gata3 function at the DP thymocyte stage, we performed gene expression profiling assays in wild-type mice and transgenice mice with enforced expression of Gata3, in the presence or absence of the MHC class II-restricted DO11.10 TCR. We found that Gata3 expression in DP cells undergoing positive selection was associated with downregulation of the V(D)J-recombination machinery genes *Rag1*, *Rag2* and *Dntt*. Moreover, Gata3 overexpression was associated with downregulation of many signalling molecules and the induction of modulators of TCR signalling, including Ctla-4 and thrombospondin 2. Together with our previous finding that Gata3 reduces expression of CD5, a negative regulator of TCR signalling, and upregulates TCR expression, these findings indicate that Gata3 in DP cells mainly functions to (i) terminate *Tcrα* gene rearrangement, and (ii) regulate TCR signal intensity or duration in cells undergoing positive selection towards the CD4 lineage.

INTRODUCTION

Gata3 is a member of a family of transcription factors that bind to a consensus GATA site through a highly conserved zinc-finger domain[1, 2]. Within the hematopoetic system Gata3 is essential for T cell development from the earliest stages and is involved in NK and invariant NKT cell differentiation[3, 4]. In addition, Gata3 acts as master regulator of Th2 differentiation in mature $CD4^+$ T cells, where it is crucial for transcription of the T helper 2 cytokines IL-4, IL-5 and IL-13, as well as for the inhibition of Th1 and regulatory T cells[5-7]. Gata3 is also expressed in non-hematopoietic tissues, including kidney, central nervous system, skin and mammary gland[8]. *Gata3*-deficient mice die at day 11 of gestation due to noradrenaline deficiency of the sympathetic nervous system[9, 10].

The essential function of Gata3 in T cell development was demonstrated by the inability of *Gata3*-deficient ES cells to give rise to early $CD4^-CD8^-$ double negative (DN) T cell progenitors in the thymus[11, 12]. In this context, Gata3 function for T cell specification is dependent on Notch signalling[13]. In DN thymocytes gene segments

encoding the T cell receptor β (TCRβ) chain undergo V(D)J recombination and only cells with a functional *Tcrβ* rearrangement are selected for further maturation. This process, termed β-selection, is associated with an increase in cell size and induction of proliferation[14]. Analysis of *Gata3*-lacZ reporter mice showed that Gata3 expression is upregulated in cells that have undergone β-selection[11]. Furthermore, conditional deletion experiments demonstrated that *Gata3*-deficient DN cells failed to undergo β-selection, indicating the essential role for Gata3 in this process[15]. After β-selection, cells upregulate expression of CD4 and CD8 and *Tcrα* gene segments are recombined, resulting in surface expression of TCRαβ at the $CD4^+CD8^+$ double positive (DP) stage. Upon engagement by self major histocompatibility complex (MHC) peptide complexes, low- to intermediate-avidity interactions rescue DP thymocytes from death by neglect, resulting in positive selection to either CD8 single positive (SP) cells in the context of MHC class I or CD4 SP cells in the context of MHC class II[16, 17]. In DP thymocytes TCR stimulation in the context of MHC class II results in the induction of Gata3 expression, which is maintained in CD4 SP thymocytes and downreglated in CD8 SP cells[11, 18, 19]. An absolute requirement for Gata3 for CD4 positive selection became evident from the finding that conditional deletion of the *Gata3* gene at the DP stage resulted in a failure of CD4 SP generation[15]. We recently found that during CD4 positive selection Gata3 has the capacity to downregulate expression of CD5, which is a negative regulator of TCR signalling, and to upregulate TCR expression[19]. Based on the observed induction of Gata3 by TCR stimulation in the context of MHC class II[18], we concluded that Gata3 probably acts in a positive feedback loop to upregulate TCR expression in developing CD4 SP thymocytes[19].

Accurate regulation of Gata3 expression levels is essential for correct T cell development. Early overexpression of Gata3 rapidly induced respecification of early T cell precursors to the mast cell lineage[20]. CD2-Gata3 transgenic mice, with enforced expression of Gata3 driven by the CD2 promoter, display impaired CD8 T cell maturation and develop thymic lymphoma[21]. These lymphomas appear to arise at the DP cell stage, where Gata3 overexpression induces a dramatic cell size increase and increased expression of the c-Myc oncogene[22].

The CD4/CD8-cell fate choice of double positive (DP) thymocytes is mainly determined through differences in TCR signal strength and duration[23]. Both Gata3 and the zinc-finger transcription factor ThPOK have been implicated in positive selection of DP thymocytes towards the CD4 lineage. The finding that forced expression of ThPOK in thymocytes can redirect cells that should become CD8 cells into the CD4 lineage, indicated that ThPOK is a master regulator that is necessary and sufficient for the CD4 T cell fate[24, 25]. Because ThPOK is not expressed in the absence of Gata3 and Gata3-

binding sites are present in the ThPOK locus, it has been hypothesized that Gata3 directly regulates ThPOK expression[26]. However, transgenic expression of ThPOK failed to promote $CD4^+$ lineage differentiation of Gata3-defcient cells[26]. Therefore, Gata3 acts via additional targets to initiate the $CD4^+$ lineage program and thus Gata3 expression may be required to reach a developmental stage at which ThPOK expression is induced.

In this report, we aimed to identify putative Gata3 targets in DP thymocytes. We compared gene expression profiles of sorted DP thymocytes from wild-type mice and from CD2-Gata3 transgenic mice. To specifically identify Gata3 target genes in the context of positive selection towards the CD4 lineage, we also investigated wild-type and CD2-Gata3 transgenic DP cells harboring the DO11.10 transgenic MHC class II-restricted TCRαβ specific for ovalbumine[27]. We found that Gata3 overexpression did not affect ThPOK expression levels. Instead, our findings implicate Gata3 in the termination of *Tcrα* gene rearrangement and in the regulation of TCR signal strength.

RESULTS AND DISCUSSION

Enforced Gata3 expression results in increased occupancy of Gata3-binding sites in the *Tcrα* enhancer region

To be able to identify Gata3 target genes in DP thymocytes in the context of positive selection towards the CD4 lineage, we took advantage of our CD2-Gata3 transgenic mouse model, which we crossed with DO11.10 transgenic mice carrying a transgene encoding an ovalbumin peptide-specific TCRαβ that is MHC class II restricted[27]. As previously shown[11, 18, 19], the presence of the CD2-Gata3 transgene did not affect DO11.10 CD4 lineage restriction (**Fig. 1A**). The presence of the DO11.10 transgene is associated with an upregulation of TCR expression levels, which was clearly more pronounced in CD2-Gata3 DO11.10 double transgenic mice (**Fig. 1B**). Gata3 has been shown to bind to GATA sites present in the *Tcrα* transcriptional enhancer located just 3′ to the *Tcr* Cα gene segment[28]. ChIP experiments on total thymocyte fractions from wild-type and CD2-Gata3 transgenic mice showed that transgenic overexpression of Gata3 resulted in increased binding of Gata3 protein to its recognition sites in the *Tcrα* enhancer region (**Fig. 1C**).

Thus, overexpression of Gata3 leads to enhanced TCR expression and increased occupancy of Gata3-binding sites in the *Tcrα* enhancer region. In this context, Gata3 may indirectly specify the CD4 lineage, because divergence between CD4 and CD8 cell fates is thought to be mainly determined by a greater intensity or a longer duration of TCR signalling in MHC class II-restricted than in MHC class I-restricted thymocytes[23].

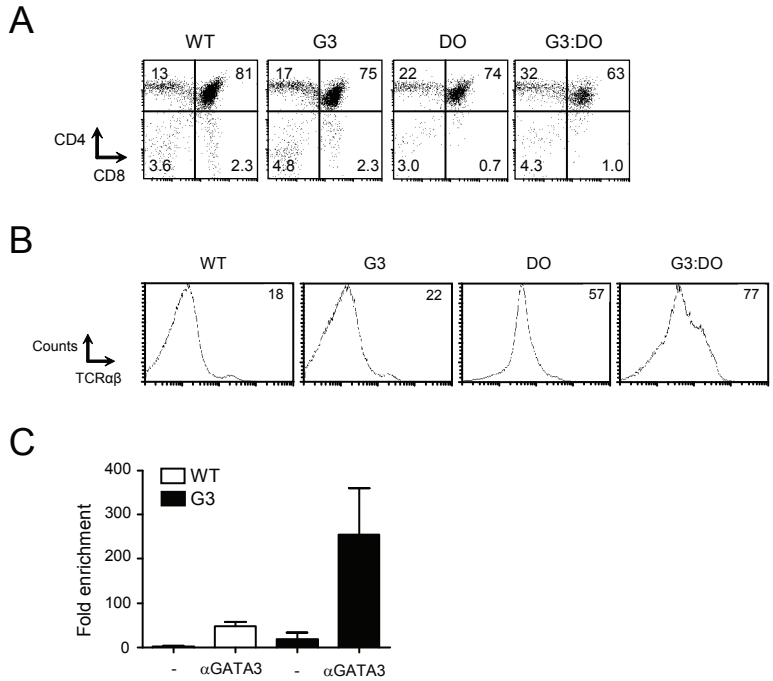

Figure 1. Enforced Gata3 expression results in increased Gata3-binding in the *Tcrα* enhancer region. (**A**) The presence of the CD2-Gata3 transgene does not affect DO11.10 CD4 lineage restriction. Flow cytometric CD4/CD8 dot plots of total thymocytes of from wild-type (WT), CD2-Gata3 transgenic (G3), DO11.10 TCR transgenic (DO) and CD2-Gata3: DO11.10 double transgenic (G3:DO) mice. Numbers indicate the proportions of cells in each quadrant. The plots shown are representative for 6–10 mice per group. (**B**) Surface TCRβ chain expression on gated $CD4^+CD8^+$ DP thymocytes from the indicated mouse groups. Numbers indicate mean fluorescence intensities. Histograms shown are representative for 6–10 mice per group. (**C**) ChIP analysis in total thymocyte fractions in WT and CD2-Gata3 transgenic thymocytes, performed with (+) or without (−) Gata3-specific antibodies. Mean values and SEM are given for two RT-PCR runs on one ChIP experiment.

Furthermore, this finding indicates that enforced expression of Gata3 is an informative tool and does not lead to unphysiological levels of Gata3 reaching a functionally saturated point. Analyses of Gata3 protein, both by Western blotting and intracellular flow cytometry, previously showed that Gata3 expression in DP cells was increased to levels that were only slightly higher than those in CD4 SP cells[21]. In agreement with this, we have reported that the presence of the CD2-Gata3 transgene resulted in reduced expression of CD5 and increased TCR expression in DP cells and observed the converse in a conditional Gata3 knock-out model: increased CD5 and reduced TCR expression[19]. Taken together, this finding indicates that enforced expression of Gata3 is a reliable tool to identify genes controlled by Gata3.

Enforced Gata3 expression does not enhance ThPOK expression in DP or CD4 SP thymocytes

To investigate whether enforced expression of Gata3 was associated with increased ThPOK expression, we performed quantitative RT-PCR analyses. Whereas in DP cells the presence of the CD2-Gata3 transgene, either on the wild-type or the DO11.10 background, resulted in a ~4-fold increase in Gata3 transcription, we did not detect any effect on ThPOK transcription (**Fig. 2A**). We found that in CD4 SP thymocytes enforced Gata3 expression was even associated with reduced ThPOK transcription (**Fig. 2B**). Finally, we analyzed ThPOK transcription in our previously reported complex crosses of CD2-Gata3 transgenic mice with conditionally deleted *CD4-Cre Gata3$^{f/f}$* mice. In these mice, the endogenous floxed *Gata3* gene is deleted and developing CD4 SP and CD8 SP thymocytes express the same level of Gata3 transcription, contributed exclusively by the CD2-Gata3 transgene, irrespective of their developmental choice[11, 18, 19].Quantitative RT-PCR experiments showed that in the CD2-Gata3 transgenic *CD4-Cre Gata3$^{f/f}$* mice ThPOK was exclusively expressed in CD4 cells and not in CD8 cells (**Fig. 2C**). Therefore, Gata3 expression in CD8 SP cells is not sufficient to induce the expression of ThPOK.

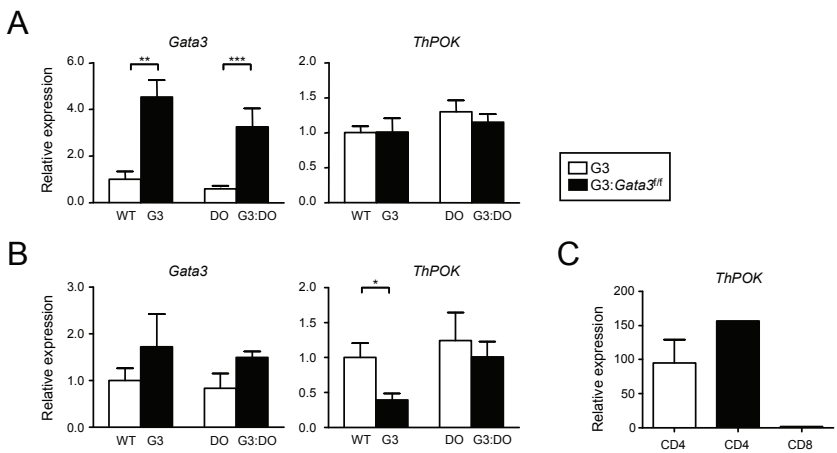

Figure 2. Enforced Gata3 expression does not enhance ThPOK expression in DP or CD4 SP thymocytes. (**A**) and (**B**) Quantitative RT-PCR analysis of *Gata3* and *ThPOK* expression in sorted DP thymocytes (**A**) or CD4 SP thymocytes (**B**) from wild-type (WT, white bars), CD2-Gata3 transgenic (G3, black bars), DO11.10 TCR transgenic (DO, white bars) and CD2-Gata3: DO11.10 double transgenic (G3:DO, black bars) mice. Expression was normalized to *Gapdh* and expression levels of *Gata3* or *ThPOK* in wild-type DP (**A**) or CD4 SP (**B**) thymocytes were set to one. Average values and SEM are given for 4–10 mice per group (*P<0.05; **P<0.01; ***P<0.001; *t*-test). (**C**) Quantitative RT-PCR analysis of *ThPOK* expression in the indicated sorted CD4 SP or CD8 SP thymocyte subpopulations from CD2-Gata3 transgenic (G3, white bar) and CD2-Gata3 transgenic *Lck-Cre Gata3$^{f/f}$* mice (G3: Gata3$^{-/-}$). Expression was normalized to *Gapdh* and *ThPOK* expression levels in WT DP were set to one.

Taken together, these findings show that Gata3 overexpression did not affect ThPOK expression levels in DP, CD4 SP or CD8 SP cells. Therefore, Gata3 does not directly act as a CD4 specification factor by promoting the expression of ThPOK. Instead we conclude that, in the absence of Gata3, DP thymocytes do not reach the developmental stage at which ThPOK expression is induced.

Identification of differentially expressed genes as a result of enforced Gata3 expression

The DP compartment in the thymus is heterogeneous and contains (i) cells that are positively selected towards the CD4 or (ii) the CD8 lineage, (iii) cells that are negatively selected, and (iv) a major proportion of cells that are not selected and die by neglect. Because Gata3 expression is specifically induced in DP cells by TCR signalling in the context of MHC class II and maintained during positive selection towards the CD4 SP cell lineage, we considered two different expression profile comparisons. First, we compared wild-type and CD2-Gata3 transgenic DP thymocytes, allowing the identification of genes induced or repressed by Gata3 in a heterogeneous DP cell population containing many cells in which Gata3 is normally not induced. We also compared DP thymocytes from DO11.10 single transgenic and DO11.10:CD2-Gata3 double transgenic mice. Hereby, we investigated the effects of premature and enhanced Gata3 expression in DP cells in which Gata3 would normally be induced because DO11.10 transgenic DP cells are efficiently positively selected towards the CD4 lineage.

FACS-sorted DP thymocyte fractions were obtained from three individual 11-week-old mice from the following four groups: wild-type, CD2-Gata3 transgenic, DO11.10 transgenic and CD2-Gata3:DO11.10 double transgenic mice. Oligonucleotide arrays were co-hybridized with RNA from these DP cell fractions and RNA from a control pool of sorted DP T cells from 12 wild-type mice. After performing a non-supervised hierarchical clustering of individual mice and genes, we were able to identify differential gene expression profiles for the four different groups of mice. The gene expression profiles of the three individual mice present in each group clustered together, indicating that enforced expression of Gata3 and the presence of the DO11.10 TCR transgene resulted in unique gene expression signatures (**Fig. 3A**). The almost complete absence of differentially expressed genes in the individual wild-type mice, when compared to the control pool, provided a good quality control indicating that the observed differential gene expression profiles represent specific effects of enforced Gata3 expression.

To be able to identify genes, which are affected by enforced Gata3 expression at the DP T cell stage, the expression profiles of the individual mice were grouped per genotype, and a non-supervised clustering of all four groups and genes was performed

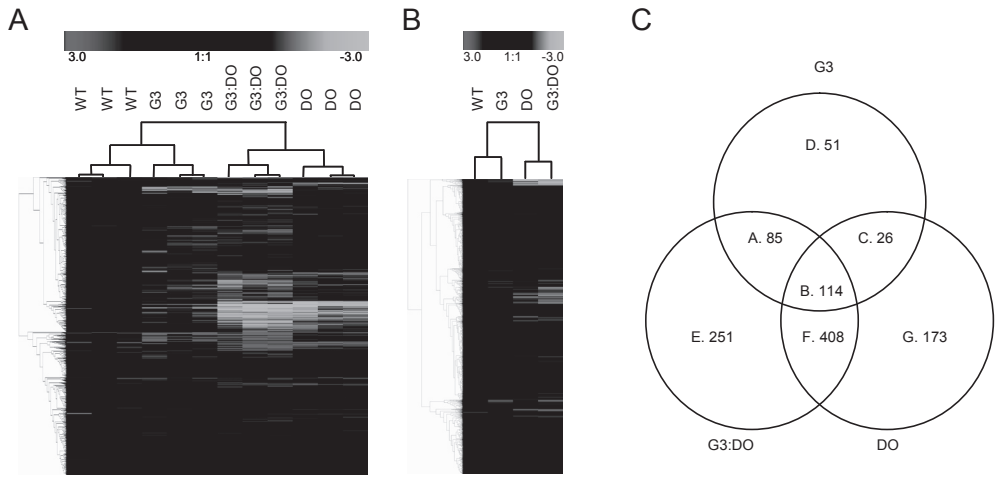

Figure 3. Identification of specific gene expression signatures in DP cells as a result of enforced Gata3 expression. Gene expression profiles were generated from FACS-sorted DP cells from wild-type (WT), CD2-Gata3 transgenic (G3), DO11.10 TCR transgenic (DO) and CD2-Gata3:DO11.10 double transgenic (G3:DO) mice. (**A**) Non-supervised hierarchical clustering of both genes (rows) and individual mice (columns). Gene expression profiles were analyzed for 3 individual mice per group. (**B**) Non-supervised hierarchical clustering of both genes (rows) and grouped (*n*=3) mice of the indicated genotype. Dendograms above and on the left side of the matrixes indicate the average linkage clustering of respectively individual mice and genes. Green and red colors indicate the level of downregulation or upregulation of genes, when compared with a pool of DP cells obtained from WT mice (*n*=6). The color scale above the matrix correlates with gene expression and the given value numbers represent ^{2}log values. (**C**) Differentially expressed genes in DP thymocytes from CD2-Gata3 and DO11.10 single transgenic and CD2-Gata3:DO11.10 double transgenic mice, displayed in a Venn diagram. Genes are either up- or downregulated 1.4-fold (*P*<0.0001) and are not differentially expressed in WT mice. Numbers of differentially expressed genes per cluster are given. **See Appendix for full-color figure.**

(**Fig. 3B**). In this analysis 3751 differently expressed genes were identified within the four groups of mice. To further identify the most relevant differentially expressed genes, the following selection criteria were used: (i) only genes which were >1.4-fold upregulated or <1.4-fold down-regulated with a *P* value <0.0001 were allowed, and (ii) genes which were differently expressed in wild-type DP T cells or genes with unknown function were excluded. This resulted in a total number of 1126 genes that were differentially expressed in CD2-Gata3 or DO11.10 single transgenic or CD2-Gata3:DO11.10 double transgenic DP thymocytes.

Clustering of differentially expressed genes into three groups of genes

Next, a Venn diagram was composed, allowing for the identification of differentially expressed genes that are common or unique for the four DP genotypes. Our expression profiling revealed 276 (85 + 114 + 26 + 51, clusters A–D, **Fig. 3C**) differentially expressed

genes in CD2-Gata3 transgenic DP thymocytes, when compared with wild-type DP cells. The comparison of DO11.10 single transgenic and wild-type DP thymocytes revealed differential expression of many more genes: 711 genes (114 + 26 + 173 + 408, clusters B, C, F and G), suggesting that the introduction of the DO11.10 TCR transgene, which induces positive selection towards the CD4 lineage, has a larger impact on gene expression in DP cells than the presence of the CD2-Gata3 transgene.

276 genes of clusters A–D represent genes that were induced or repressed by Gata3 in total DP cells, apparently irrespective of their cell fate (positive selection, negative selection, or death by neglect). From these 276 genes 136 genes (clusters A and D) were not differentially expressed in DO11.10 DP cells. The A/D cluster therefore represents genes that were upregulated or downregulated by transgenic Gata3 expression but were not involved in positive selection towards the CD4 lineage. Likewise, the 140 genes in the B/C cluster represent genes that were not only upregulated or downregulated by transgenic Gata3 expression in total DP cells but also in DO11.10 transgenic DP cells undergoing positive selection towards the CD4 lineage.

Finally, we found that enforced Gata3 expression in DO11.10 transgenic DP cells undergoing positive selection towards the CD4 lineage was associated with differential expression of 336 genes (85 + 251, clusters A and E), when compared with DO11.10 single transgenic DP cells. Importantly, only cluster E contained genes that were upregulated or downregulated by transgenic Gata3 expression in DP cells exclusively in the context of positive selection towards the CD4 lineage, but not in the heterogeneous total DP cell fractions. Therefore, cluster E is expected to contain putative Gata3 target genes associated with the crucial function of Gata3 in CD4 SP cell development.

Taken together, we have now identified three gene clusters of putative Gata3 targets: target genes in total DP cells that are (cluster B/C) or are not implicated in positive selection (cluster A/D), and target genes that are differentially expressed exclusively in the presence of a MHC class II-restricted positively selecting TCR signal (cluster E).

Putative Gata3 target genes that are not specifically implicated in positive selection

First, we focused on cluster A/D, which containss 136 genes that were induced or repressed by Gata3 in DP cells, irrespective of their cell fate, but were not implicated in positive selection (**Table 1** and **Supplementary Table 1**). We found a striking ~18.5-fold upregulation of *very low density lipoprotein receptor* (*Vldl-R*), which is like *apolipoprotein E* (~2.7-fold upregulated) involved in lipoprotein metabolism[29]. Together with the upregulation of multiple genes associated with structural cellular organization, including

Table 1. Selection of putative GATA3 target genes.

Upregulated genes	G3	DO	G3:DO	Downregulated genes	G3	DO	G3:DO
*Cluster A and D:**							
Very low-density lipoprotein receptor (VldlR)	18.5	0.5	9.0	Matrix metalloproteinase 14	2.3	1.1	1.8
Thrombospondin 2 (Thbs2)	13.9	0.8	8.6	Sodium/nucleoside cotransproter 2	2.3	0.9	2.1
Alpha-actinin 2	10.8	2.3	7.7	Calcitonin receptor-like	2.1	1.6	2.3
Collagen alpha 2(I) chain	6.1	1.6	6.6	Lymphoid enhancere binding factor 1 (Lef1)	2.0	1.5	1.4
Interleukin 18 receptor 1	5.0	1.6	9.2	T-cell surface glycoprotein CD5	2.0	0.7	1.2
Cytotoxic T-lymphocte protein 4 (Ctla-4)	4.7	0.9	2.8	T-cell specific transcription factor (Tcf1)	1.9	1.6	2.0
Transferrin	4.1	0.8	3.7	T cell transduction molecule SAP	1.8	1.2	1.7
MYC proto-oncogene (c-Myc)	3.8	1.1	3.9	C-X-C chemokine receptor type 4 (Cxcr4)	1.7	1.3	2.2
Trans acting T-cell specific transcription factor GATA3	3.6	1.0	3.1	C-ETS-2 protein	1.7	1.4	3.2
Myosin IF	2.8	1.0	1.9	Interleukin 16 (IL-16)	1.7	1.3	1.6
Apolipoprotein E (Apo-E)	2.7	1.6	6.1				
Cyclin D2	2.6	0.8	3.3				
Spermidine synthase	2.0	0.9	2.1				
*Clusters B and C:**							
Granzyme A	5.0	1.6	5.2	Death associated protein kinase 1	2.5	2.6	6.9
Nuclear receptor Ror-alpha	4.8	1.7	6.6	Interleukin-10 receptor alpha chain	2.0	1.6	1.6
FLT3 ligand	4.4	2.4	6.8	Transcription factor Sp3	1.9	1.8	1.9
CD44	3.3	2.6	3.9	Neurogenic locus notch homolog protein 1 (Notch1)	1.7	1.4	1.5
LIM domian transcription factor Lmo4	1.8	2.4	2.9	DNA-binding protein inhibiter Id3	1.6	0.6	1.3
Signal and transducer and activator of transcription 4	1.8	3.0	3.0				
Interleukin-12 recepeptor beta-1 chain	1.5	1.9	2.3				
*Cluster E:***							
Max-interacting transcriptional repressor Mad4	3.8	1.3	8.9	Secreted modular calcium-binding protein 1	1.9	2.2	9.1
T-lymphocyte activated protein	2.1	1.6	2.2	H-2 class I histocompatibility antigen (H-2K(B))	1.8	2.4	3.4
L-selectin (CD62L)	1.9	1.3	2.9	DNA nucleotidylexotransferase (Tdt)	1.8	1.4	3.2
Thy1 membrane glycoprotein	1.4	1.7	2.1	Tyrosine protein kinase Tec	1.6	1.4	3.1
Guanine nucleotide-binding protein, aplha-13 (Galpha13)	1.3	2.3	2.8	V(D)J recombination activating protein 2 (Rag2)	1.4	1.7	2.9
Cop9 signalosome complex subunit 2	1.1	2.5	2.9	T cell surface glycoprotein CD4	1.6	2.0	2.5
				V(D)J recombination activating protein 1 (Rag1)	1.4	2.2	2.3
				Mitogen activated protein kinase4-2 (Mapk4-2)	2.2	1.7	2.3
				Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	1.9	1.6	2.2
				Signal transducer and activator of transcription (Stat5b)	1.6	1.5	2.0
				Phosphatidylinositol-4-phosphate 5-kinase type1 (PI4P5K-I)	0.6	1.6	2.0
				Ccr4-not transcription complex, subunit 7 (Caf1)	1.3	1.4	1.8
				B Cell translocation gene 1 (Btg1)	1.5	1.4	1.8
				Interleukin 21 receptor (IL-21R)	0.9	1.4	1.8
				Calpain L3	1.4	1.8	2.1
				Nuclear factor of activated T cells, cytoplasmic 1 (Nfat-c1)	1.1	1.4	1.8
				Tyrosine protein kinase Itk/Tsk	1.2	1.3	1.8
				Nuclear factor of activated T cells 5 (NFAT5)	1.3	1.5	1.7
				MHC class II transactivator CIITA	1.2	1.3	1.7
				Mitogen activated protein kinase 6 (Erk3)	1.7	1.5	1.7
				P300/Cbp associated factor	1.3	1.3	1.6

G3 = CD2-GATA3, DO = DO11.10, G3:DO = CD2-GATA3:DO11.10. [a] Genes sorted by G3, [b] Genes sorted by G3:DO.

α-actinin 2, *collagen alpha 2 (I) chain* and *myosin IF*, this might be linked to the observed increased cell size of CD2-Gata3 DP thymocytes. We also found increased expression of *c-Myc* and its target *cyclin D2*, which we recently described as a putative Gata3 target in lymphomagenesis and cell size regulation[22]. In addition, we found induction of *Ctla-4*, which is a negative regulator of TCR-proximal signalling proteins, and the *IL-18 receptor*. Gata3 also induced significant expression of *thrombospondin-2* (*Thbs2*),

a member of the thrombospondin family of extracellular matrix proteins that regulate cell adhesion, growth, survival, differentiation and motility. Although Thbs receptors can elicit co-stimulating signals, Thbs1 has been shown to inhibit TCR signalling and CD69 induction[30]. Thbs receptor ligation on human T cells induced the expression of *Ctla-4*, *OX40*, *GITR*, and *Foxp3*, associated with regulatory T cell function[31]. Thbs receptors include various integrins, CD36, CD47, low density lipoprotein receptor-related protein and heparan sulfate proteoglycans, some of which are also expressed on DP cells. Therefore, the induction of *Ctla-4* and *TSP2* on DP thymocytes would allow the formation of cell-autonomous regulatory loops that dampen cellular activation of DP thymocytes by TCR-mediated signalling. As quantitative RT-PCR showed that enforced Gata3 expression is also associated with high *Thbs2* transcript levels in DO11.10 Tg mice (**Fig. 4D**), we conclude that *Thbs2* is also induced during CD4 lineage differentiation.

Interestingly, we noticed a ~2-fold down-regulation of the transcription factors *Tcf1* and *Lef1* (**Table 1** and **Supplementary Table 1**), both of which are regulated by the Wnt/β-catenin signalling pathway and have been implicated in a subset of peripheral T cell lymphomas[32, 33]. A ~2-fold down-regulation was also observed for *Cd5*, a negative regulator of TCR signalling, which we recently reported as a putative direct or indirect Gata3 target gene[19]. Furthermore, Gata3 expression downregulated ~1.8-fold the *SLAM-associated protein SAP*, which is involved in TCR signalling.

Taken together, this cluster of putative Gata3 targets mainly consists of genes implicated in lipoprotein metabolism, cell size regulation and lymphomagenesis, including *Vldl-R*, *c-Myc* and *cyclin D2*. In addition, we identified target genes that affect TCR-mediated signalling, including *Ctla-4*, *Thbs2*, *Cd5* and *SAP*. RT-PCR analyses for *Thbs2* and flow cytometric studies for CD5[19] showed that Gata3 regulates expression of these genes also in thymocytes that are positively selected for CD4 lineage differentiation.

Putative Gata3 target genes that are implicated in positive selection

Cluster B/C contained 140 genes that were differentially expressed in DP cells in the presence of CD2-Gata3 transgene, irrespective of their cell fate, that were also induced or repressed in DO11.10 TCR transgenic DP cells (**Table 1** and **Supplementary Table 2**). In this group, we surprisingly found upregulation of *granzyme A*, which is expressed in $CD8^+$ T cells and NK cells and *Rorα*, which was recently implicated in Th17 cell differentiation[34]. Differential expression of *granzyme A* and *Rorα* was confirmed by semi-quantitative PCR, by which also upregulation of the cluster A/D genes *Vldl-R* and *IL-18R* could be verified (**Fig. 4A and B**). The relevance of *Rorα* upregulation is not clear, because lymphocyte-specific *Rorα*-deficient mice do not display defects in thymocyte development[35].

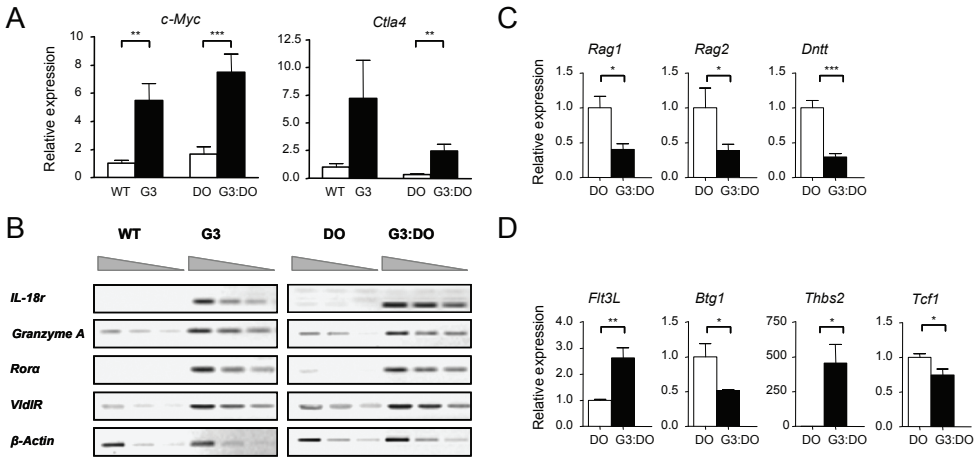

Figure 4. Expression analysis of genes upregulated as a result of enforced Gata3 expression in DP thymocytes. (**A**) Quantitative RT-PCR analysis of *Gata3*, *c-Myc* and *Ctla-4* expression in sorted DP cells from wild-type (WT), CD2-Gata3 (G3), DO11.10 (DO) and CD2-Gata3:DO11.10 double transgenic (G3:DO) mice. Expression was normalized to *Gapdh* and expression levels of *c-Myc* and *Ctla-4* in WT DP thymocytes were set to one. For statistical analysis a *t*-test was performed (***P*<0.01 and ****P*<0.001; *t*-test). Data are from 4 to 10 mice per group. (**B**) Semi-quantitative PCR analysis for *IL-18R*, *Granzyme A*, *Rorα*, *VldlR* and *β-actin* on serial 3-fold cDNA dilutions prepared from sorted DP cells of the indicated mice. Data are representative for 4 mice analyzed per group. (**C**) and (**D**) Quantitative RT-PCR analysis of the indicated transcripts in sorted DP cells from DO11.10 (DO) and CD2-Gata3:DO11.10 (G3:DO) transgenic mice. Expression was normalized to *Gapdh* and expression levels in WT DP thymocytes were set to one. Average values ± SEM are displayed for 5–8 mice per group (**P*<0.05; ***P*<0.01; ****P*<0.001; *t*-test).

In addition, *IL-12Rβ1* and *Stat4* involved in cytokine signalling, and *Cd44*, *Lmo4*, and *Flt3-ligand*, were induced (**Table 1** and **Supplementary Table 2**). Using quantitative RT-PCR, we could confirm that *Flt3-ligand* expression is induced in DP cells overexpressing Gata3 (**Fig. 4D**). Transcriptional regulators, such as *Sp3* and *Id3* were ~2-fold down-regulated (**Table 1** and **Supplementary Table 2**). The transcription factor Id3 has been shown to be an important regulator for thymocyte selection, via the regulation of E2A[36]. This in combination with our finding in CD2-Gata3 transgenic DP cells of increased expression of E2A and reduced expression of CD5[11, 18, 19], might indicate a role of Gata3 in downregulating CD5 via Id3 during thymocyte selection. Finally, we observed that Gata3 overexpression was associated with downregulation of *Notch1* transcription. This would suggest a negative feedback mechanism regulating *Notch1* expression, given the fact that Gata3 was recently shown to be a direct target of Notch1 during T helper cell differentiation[37, 38].

We conclude that the B/C cluster consists of a very diverse set of genes most of which cannot obviously be linked to induction of positive selection towards the CD4

lineage, except for *Id3* or *Notch1*. These findings may be consistent with the notion that Gata3 expression is insufficient to induce positive selection towards the CD4 cell lineage[15, 21].

Putative Gata3 target genes in the presence of a MHC class II-restricted TCR signal

Cluster E contained genes that were upregulated or downregulated by transgenic Gata3 expression in DP cells exclusively in the context of positive selection towards the CD4 lineage, but not in total DP cell fractions. Importantly, transgenic Gata3 expression induces enhanced differentiation of DP cells, as evidenced by high surface TCR and CD69 expression levels of CD2-Gata3 transgenic DP cells, which were close to those normally reached only at the CD4 SP stage[19]. Therefore, cluster E was expected to contain natural Gata3 targets associated with its crucial function in CD4 SP cell development.

Within cluster E, 118 genes were upregulated and 133 were downregulated as a result of enforced Gata3 expression (**Table 1** and **Supplementary Table 3**). Among the upregulated genes, *Mad4* a suppressor of c-Myc[39], *G protein G-alpha13* and *Cd62L* were present. Upregulation of *Cd62L* transcription would be in agreement with the previously reported increased surface expression of CD62L on CD2-Gata3 transgenic CD4 SP cells[40].

Enforced Gata3 expression was associated with reduced activity of the V(D)J recombination machinery, as *recombination activating gene-1* (*Rag1*), *Rag2* and *terminal deoxynucleotidyltransferase* (*Dntt*) were ~2–3-fold downregulated (**Table 1** and **Supplementary Table 3**). Reduced gene expression of *Rag1*, *Rag2* and *Dntt* was verified by quantitative RT-PCR analysis (**Fig. 4C**). In the promoter of the *Rag2* gene a Gata3 binding site has been identified, mutation of which resulted in the reduction of promoter activity in T cells, indicating a direct regulation of V(D)J recombination by Gata3[41]. Thus, our findings indicate that the induction of Gata3 upon TCR signalling in DP cells results in termination of V(D)J recombination activity, precluding further *Tcrα* gene rearrangement. Interestingly, overexpression of Gata3 in early DN fetal thymocytes also caused downregulation of *Rag1* and *Rag2*[42]. Because Gata3 expression is upregulated in DN3 cells that have undergone β-selection[11], in the context of downregulation of the V(D)J recombinase system Gata3 has parallel functions in DN3 cells where it is induced by pre-TCR signalling and in DP cells where it is induced by TCR signalling.

Enforced Gata3 expression resulted in reduced expression of *B cell translocation gene 1* (*Btg1*) and *CCR4-associated factor 1* (*Caf1*). Btg1 and Caf1 have been reported to act together in negative regulation of the gene for estrogen receptor α (ERα)[43]. Using

quantitative RT-PCR, we could confirm that *Btg1* expression is reduced in DP cells overexpressing Gata3 (**Fig. 4D**). This is particularly interesting, since Gata3 has been shown to positively regulate the expression of estrogen receptor alpha in breast cancer, by binding to the *ERα* gene itself[44]. These data suggest that Gata3 can regulate *ERα* in a direct manner, but as well indirectly via Btg1.

Gata3 expression resulted in downregulation of genes involved in cellular signalling pathways such as, *mitogen activated protein kinase4-2* (*Mapk4-2*), *signal transducer and activator of transcription 5b* (*Stat5b*), *phosphatidylinositol-4-phospate 5-kinase* (*PI4P5K-I*), and *extracellular signal regulated kinase-3* (*Erk3*). In addition, the tyrosine protein kinases *Tec* and *Itk*, which are involved in proximal TCR signalling, were ~2–3-fold downregulated. Also *secreted modular calcium-binding protein-1*, *sarcoplasmic/endoplasmic reticulum calcium ATPase-3* and *calpain L3*, which are related to calcium signalling, were reduced. *Secreted modular calcium-binding protein-1* was even reduced by a factor ~9.

Enforced Gata3 expression also resulted in downregulated expression of genes encoding for cell surface molecules, such as *MHC class I*, *IL-21R* and *Cd4*. Reduction of CD4 expression is of particular interest, because DP cells downregulate the expression of the CD4 and CD8 to become “double dull” thymocytes co-receptors when positive selection is initiated[45]. Although we did not detect a significant reduction in cell surface protein expression of CD4 by flow cytometric analysis of Gata3 transgenic DP cells, we did observe reduced CD4 expression in *in vitro* cultured CD2-Gata3 transgenic T helper-2 (Th2) cells, when compared to wild-type Th2 cells (data not shown). At the level of transcription, enforced Gata3 expression resulted in reduced expression of the *nucleus class II transactivator* (*CIITA*), *p300/CBP* and *nuclear factor of activated T cells cytoplasmic 1* (*Nfat-c1*).

Overlapping gene expression signature of CD2-Gata3 DP cells and lymphomas

When comparing the gene expression profiles of non-transformed CD2-Gata3 transgenic DP cells with those from thymic lymphoma samples obtained from CD2-Gata3 transgenic mice[22], we noticed striking parallels. Also in the DP T cell lymphomas we observed downregulation of *Rag1* and *Rag2*, the cell surface molecules *Cd4*, *Cd5* and *IL-21R*, TCR signalling molecules *Itk*, *Tec* and *Sap*, as well as the transcription regulators *Stat5b* and *Nfat5*. Moreover *Ets2*, *Tcf1*, *IL-16*, *Cxcr4*, were found to be downregulated in both non-malignant DP thymocytes as in Gata3-induced lymphoma cells. Among the upregulated genes a similar expression profile in non-malignant DP cells and lymphoma cells was found of *c-Myc*, *cyclin D2*, *transferrin*, *Apo-E*, *spermidine synthase* and *granzyme A*.

CONCLUSIONS

In this report, we have shown that Gata3 overexpression does not affect ThPOK expression levels in DP or $CD4^+$ thymocytes, providing evidence that Gata3 does not directly regulate ThPOK. Therefore, we propose that Gata3 is required during $CD4^+$ lineage specification to reach the developmental stage at which expression of the CD4 commitment factor ThPOK is induced. Our gene expression profiling studies allowed the identification of novel putative Gata3 target genes in DP thymocytes undergoing positive selection towards the CD4 lineage: Gata3 expression was associated with downregulation of the V(D)J-recombination machinery genes *Rag1*, *Rag2* and *Dntt*, as well as various signalling molecules implicated in the transmission of TCR-mediated signals in DP thymocytes, including *Tec*, *Itk*, *Sap*, *PI4P5K-I*, *Mapk4-2*, *Erk3* and various molecules involved in calcium signalling. Gata3 overexpression was also associated with the induction of *Ctla-4* and *thrombospondin-2*, which may act as modulators of TCR signalling in DP thymocytes. Together with our previous finding that Gata3 reduces expression of CD5, a negative regulator of TCR signalling, and upregulates TCR expression in a positive feedback loop[19], these findings indicate that Gata3 in DP cells mainly functions to (i) terminate *Tcrα* gene rearrangement and (ii) regulate TCR signal intensity or duration in cells undergoing positive selection towards the CD4 lineage. Validation of the identified targets in combination with functional experiments will provide improved insight into the essential physiological function of Gata3 during the developmental progression of DP cells that have received TCR-mediated positive selection signals into CD4 SP cells.

METHODS

Mice. The generation and genotyping of CD2-Gata3 (FVB)[21], CD2-Gata3:DO11.10 (BALB/c)[19] and Lck-Cre Tg $Gata3^{f/f}$ [19] have been described. Mice were kept under pathogen-free conditions in the Erasmus MC animal care facility. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

Flow cytometry, antibodies and cell sorting. The generation of single-cell suspensions and cell labeling for flow cytometry have been described previously[21]. Antibodies were purchased from BD Bioscience (San Diego, CA). FACS sorting of $CD4^+CD8^+7AAD^-$ thymocytes was performed with a FACSVantage VE, equipped with Diva Option and BD FACSdiva software (BD, San Diego, CA). Purity of fractions was >99%.

Chromatin immunoprecipitation (ChIP). ChIP analysis of Gata3 binding in the TCRα

enhancer[28] was performed as described previously[46] using anti-Gata3 antibodies (HG3-31 AC, Santa Cruz, CA). Quantitative real-time PCR on immunoprecipitated DNA was performed using SYBR Green qPCR Master Mix (Fermentas). Enrichment was calculated relative to Necdin (Necdin-F: GGTCCTGCTCTGATCCGAAG and Necdin-R: GGGTCGCTCAGGTCCTTACTT) and values were normalized to input measurements.

Preparation of probes, microarray hybridization and data analysis. Oligonucleotide arrays printed with the Operon Mouse Genome Oligo Set version 3.0 (32K mouse) were obtained from The Netherlands Cancer Institute central microarray facility (NKI-CMF, Amsterdam, The Netherlands). Protocols for sample preparation and array hybridization were supplied by NKI-CMF (http://microarrays.nki.nl). In brief, total RNA was isolated using the GeneElute mammalian total RNA miniprep system (Sigma–Aldrich, St. Louis, MO). The quantity and quality of RNA were determined using a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE). Samples with a 260/280 nm optical density ratio >1.8 were used. Two micrograms of total RNA was used for amplification using T7 MEGAscript Kit (Ambion, Austin, TX), whereby aminoallyl-UTP (Ambion) was incorporated into amplified RNA (aRNA). Subsequently, Cy5- or Cy3-dye (Amersham, GE Healthcare, Piscataway, NJ) was coupled to the aminoallyl-modified aRNA. Labeled aRNA was purified and concentrated using Microcon YM30 columns (Millipore, Billerica, MA). Oligonucleotide arrays were co-hybridized with purified probes derived from DP thymocytes from individual mice and control probes derived from pooled wild-type DP thymocytes thymocytes (n = 6) and scanned with a Scanarray Express HT scanner (PerkinElmer, Boston, MA). Data were extracted using Imagene software 6.0 (Biodiscovery, CA, USA). Each experiment consisted of 2 oligonucleotide arrays, whereby dyes were reversed between aRNA from sorted DP cells of CD2-Gata3, CD2-Gata3:DO11.10 or DO11.10 transgenic mice and the control wild-type DP T cells pool. Obtained array data were uploaded in the NKI-CMF database, analyzed using NKI-CMF software (http://dexter.nki.nl) and normalized per subarray using the Lowess normalization method. Normalized data from dye-reversed hybridizations were combined by means of a weighted average. Experiments were combined and data were filtered for genes that were differentially expressed in experiments. Hierarchical clustering of genes and experiments was performed using Genesis 1.5.0[47].

Semi-quantitative and quantitative PCR analysis. Total RNA was extracted using the GeneElute mammalian total RNA miniprep system (Sigma). One microgram of total RNA was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers. Semi-quantitative PCR analysis

was performed on samples serially diluted 3-fold before amplification. Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). PCR primers and probes are listed in **Supplementary Table 4**. To confirm the specificity of amplified products, samples were analyzed by standard agarose gel electrophoresis. Threshold levels were set and analysis was performed using the SDS v1.9 software (Applied Biosystems). Obtained C_t values of the genes of interest were normalized to the C_t value of glycereraldehyde-3-phosphate dehydrogenase (Gapdh).

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Supplementary Table 1. GATA3 target genes differentially expressed in clusters A and D

Genes up-regulated ≥ 1.4 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥ 1.4 fold	Source*	G3	DO	G3: DO
Very low-density lipoprotein receptor (VldlR)	P98156	18.5	0.5	9.0	Matrix metalloproteinase 14	P53690	2.3	1.1	1.8
Thrombospondin 2 (Thbs2)	Q03350	13.9	0.8	8.6	Sodium/nucleoside cotransproter 2	O88627	2.3	0.9	2.1
Alpha-actinin 2	Q9JI91	10.8	2.3	7.7	Epithelial membrane protein-1	P47801	2.2	1.4	2.3
Protein tyrosine phosphatase, non receptor type 13	NM_011204	9.9	0.7	6.3	5'-TG-3' interacting factor	P70284	2.1	1.3	1.6
Neuroendocrine convertase 1	P21662	9.8	1.7	6.5	Calcitonin receptor-like	NM_018782	2.1	1.6	2.3
ODD OZ/TEN-M homolog 4	NM_011858	7.7	0.8	4.3	Protein tyrosine phospatase 4A3	NM_008975	2.1	1.2	1.7
Collagen alpha 2(I) chain	Q01149	6.1	1.6	6.6	GTP-binding protein GEM	P55041	2.0	2.3	3.3
Reduced expression 3	NM_009052	6.1	0.7	3.6	Lymphoid enhancere binding factor 1 (Lef1)	P27782	2.0	1.5	1.4
Interleukin 18 receptor 1	Q61098	5.0	1.6	9.2	Solute carrier family 37	NM_020258	2.0	1.4	3.9
Cytotoxic T-lymphocte protein 4 (Ctla-4)	P09793	4.7	0.9	2.8	T-cell surface glycoprotein CD5	P13379	2.0	0.7	1.2
Revesrion induce LIM gene	NM_019417	4.5	2.0	6.5	Tristetraproline	P22893	1.9	1.2	1.9
Sam domain and HD domain-containing protein 1	Q60710	4.1	0.7	3.2	Sodium/potasium-transporting atpase beta-1 chain	P14094	1.9	1.1	2.4
Transferrin	Q921I1	4.1	0.8	3.7	T-cell specific transcription factor (Tcf1)	Q00417	1.9	1.6	2.0
Interferon consensus sequence binding protein	P23611	3.9	1.3	3.4	Similar to circadian oscillatory protein	Q8QZU8	1.9	1.3	1.4
MYC proto-oncogene (c-Myc)	P01108	3.8	1.1	3.9	Immune associated nucleotide family member 6	NM_153175	1.8	0.7	1.6
Similar to DKFZP564O0823 protein	NM_145562	3.8	0.7	2.4	T cell transduction molecule SAP	O88890	1.8	1.2	1.7
Trans acting T-cell specific transcription factor GATA3	P23772	3.6	1.0	3.1	Brain-muscle-ARNT-like protein2	NM_172309	1.8	0.3	0.7
ERO1-like	NM_015774	3.0	1.2	2.7	Phosphoribosyl pyrophosphate synthetase 2	NM_026662	1.8	1.4	2.5
Cerebellar degeneration-related 2	NM_007672	2.8	1.2	2.1	Similar to diaglycerol kinase	Q91YS0	1.8	1.2	1.7
Myosin IF	P70248	2.8	1.0	1.9	Dead ringer homolog 2	NM_019689	1.8	1.4	1.7
Glucosamine-fructose-6-phospahte aminotransferase	P47856	2.8	1.3	1.7	Inducible 6-phosphofructo-2-kinase	NM_172976	1.7	1.4	1.7
Apolipoprotein E (Apo-E)	P08226	2.7	1.6	6.1	NDRG3 Protein	Q9QYF9	1.7	1.0	1.9
Cyclin D2	P30280	2.6	0.8	3.3	C-X-C chemokine receptor type 4 (Cxcr4)		1.7	1.3	2.2
Glucocorticoid receptor	P06537	2.3	1.3	2.2	EH-domain containing protein 3	Q9QXY6	1.7	1.3	2.6
Probable G protein-coupled receptor GPR72	P30731	2.3	0.8	2.2	C-ETS-2 protein	P15037	1.7	1.4	3.2
Phosphatidylinositol-4-phospahte 5-kinase	NM_008846	2.2	0.8	0.6	Makorin 1	Q9QXP6	1.7	1.3	1.7
Calcium/calmodulin-dependent protein kinase II	NM_023813	2.2	0.9	2.1	EMU1 protein	NM_080595	1.7	1.2	1.7
Insig-1 membrane protein	NM_153526	2.2	0.9	1.6	ELAV-like protein 4	P70372	1.7	1.6	1.5
Similar to LD47277P	Q8K2Q6	2.2	0.7	2.0	Cyclin I	Q9Z2V9	1.7	1.2	1.2
Cytosol aminopeptidase	Q9CPY7	2.2	1.0	3.1	D15WSU75E protein	NM_134095	1.7	1.4	2.1
Splicing factor U2AF 65 kda subunit	P26369	2.1	1.7	2.3	Interleukin 16 (IL-16)	NM_010551	1.7	1.3	1.6
Matrilin-2	O08746	2.1	0.8	1.9	Solute carrier family 12 member 6	Q924N4	1.6	1.7	2.1
Thrombin receptor	P30558	2.0	0.8	1.6	G protein kinase 2	Q99MK8	1.6	1.3	1.5
Spermidine synthase	Q64674	2.0	0.9	2.1	Lymphocye specific formin related protein	NM_019679	1.6	1.3	1.5
Acetyl-coa carboxylase 265	Q925C4	1.9	0.8	1.8	Putative phospatase	NM_008916	1.6	1.1	1.4
Annexin A11	P97384	1.9	0.8	1.6	Glucosamine-6-phosphate isomerase	O88958	1.6	1.4	1.4
SH3 domain-binding protein 5	Q9Z131	1.9	1.7	2.1	HASH	NM_026470	1.6	1.1	1.7
TOB1 protein	Q61471	1.9	1.4	2.1	RAS-related protein RAB-33B	O35963	1.6	1.3	1.5
CDNA sequence BC019755	NM_145395	1.8	1.1	2.5	Inosine triphosphatase	NM_025922	1.6	1.4	1.5

Supplementary Table 1. GATA3 target genes differentially expressed in clusters A and D (continued)

Genes up-regulated ≥ 1.4 fold	Source*	G3	DO	G3:DO	Genes down-regulated ≥ 1.4 fold	Source*	G3	DO	G3:DO
ND1-S	NM_028582	1.8	1.8	2.0	Microsoamal glutathione S-transferase 3	NM_025569	1.6	1.1	1.8
Mitochondrial import inner membrane translocase	Q9WV98	1.8	1.3	1.9	Myosin regulatory light chain 2	P97457	1.6	1.2	1.6
RAC/CDC42 guanine nucleotide exchange factor	NM_152801	1.8	1.5	1.7	P150 target of rapamycin (Tor)	Q8K4Q0	1.6	1.2	1.6
Galectin-3	P16110	1.7	1.3	2.3	RHO GTPase activating protein 4	NM_138630	1.5	0.9	1.3
Arginine/serine-rich splicing factor 10	Q15815	1.7	1.7	1.7	RAS-related protein RAL-A	P05810	1.5	1.3	1.4
Centrin2	Q9R1K9	1.6	1.4	1.7	Thyroid hormone receptor associated protein		1.5	1.4	1.3
Splicing factor, arginine/serine-rich 1	NM_173374	1.6	1.5	1.7	Mitxantrone resistance protein 1	NM_011920	1.5	1.1	1.7
Phosphoserine aminotransferase	Q99K85	1.6	1.0	2.0	Glycerol-3-phosphate dehydrogenase (NAD+)	P13707	1.5	1.3	1.1
Cyclin-dependent kinase inhibitor 1B (P27KIP1)	P46414	1.6	1.5	1.5	Leukocyte common antigen precursor (CD45)	P06800	1.5	1.4	1.7
WW domain binding protein 5	Q9DD24	1.6	0.8	1.2	Splicing factor 3b subunit	Q99NB9	1.5	1.5	2.1
40S ribosomal protein S11	P04643	1.6	1.4	1.7	Copine 1	NM_170588	1.5	1.4	1.7
Similar to ribosomal protein S20	Q921M2	1.6	1.4	1.6	Ubiquitin-like 5	NM_025401	1.5	1.2	1.4
Similar to dendritic cell protein	NM_145380	1.5	1.3	1.2	ADP-ribosylation-like factor 6 interacting protein 2	NM_019717	1.5	1.2	2.5
14-3-3 protein zeta/delta	P35215	1.5	1.4	1.5	Cystinosin	P57757	1.5	1.3	1.6
Guanine nucleotide binding protein	NM_025277	1.5	1.2	1.5	F-actin capping protein alpha-1 subunit	P47753	1.5	1.4	1.4
Protein kinase C and casein kinase substrate 2	Q9WVE8	1.5	1.0	1.7	Kinesin-associated proetein 3	P70188	1.5	1.3	1.2
ADP-ribosylation factor 1	P32889	1.5	1.6	1.6	ATP-dependent DNA helicase II, 70 kda subunit	P23475	1.4	1.4	2.1
Ubiquitin carboxyl-terminal hyydrolase isozyme L5	Q9WUP7	1.5	1.4	1.6	Ornithine decarboxylase antizyme	P54369	1.4	1.1	1.4
Transmembrane protein 4	NM_019953	1.5	1.3	1.6	Histone 1	NM_023422	1.4	1.4	1.5
ADP,ATP carrier protein, isoform T1	P48962	1.5	1.2	1.3	SH2-B PH domain containing signaling mediator 1	NM_011363	1.4	1.1	1.5
Fyn binding protein	O35601	1.4	1.2	1.4	Similar to histone H2A.F/Z variant	Q8R029	1.4	1.1	1.3
Similar to ubiquitin-conjugating enzyme E2 variant 1	Q8VEB5	1.4	1.3	1.6	Axin 1 up-regulated gene 1	P59054	1.4	1.5	1.8
Bone morphogenetic protein 7	P23359	1.4	0.6	1.6	Fibulin-2	P37889	1.4	1.4	2.2
Cytochrome B-245 light chain		1.4	1.3	1.4	Protein translation factor SUI1 homolog	P48024	1.4	1.1	1.5
Hyaluronidase 2		1.4	1.1	1.6	Enhancer of zeste homolog 2	Q61188	1.4	1.3	1.5
LUC7 homolog	NM_025881	1.4	1.7	1.5	Lysosomal-associated multitrasnmembrane protein	Q61168	1.4	1.2	1.5
Complement component 1	O35658	1.4	1.0	1.5	ACYL-COA-bindin protein	P31786	1.4	1.3	1.5
Mitochondrial import inner membrane translocase	Q9WVA2	1.4	1.0	1.5	Similar to programmed cell death 6 interacting protein	Q8K2Q7	1.3	1.3	1.7
					Intercellular adhesion molecule-2 (ICAM-2)	P35330	1.3	0.9	1.5
					ACYL-COA dehydrogenease	P45952	1.3	1.5	1.8

* RefSec, Swissprot or Sptrembl.

G3 = CD2-GATA3, DO = DO11.10, G3:DO = CD2-GATA3:DO11.10

Genes sorted by G3

Supplementary Table 2. GATA3 target genes differentially expressed in clusters B and C

Genes up-regulated ≥ 1.4 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥ 1.4 fold	Source*	G3	DO	G3: DO
Granzyme A	P11032	5.0	1.6	5.2	Ephrin type-A receptor 2	Q03145	3.1	6.8	8.4
Nuclear receptor Ror-alpha	P51448	4.8	1.7	6.6	Pleckstrin homology-like domain, family A, member 1	NM_009344	3.1	2.2	2.7
FLT3 ligand	Q9JIP3	4.4	2.4	6.8	M-phase inducer phospatase	P30306	2.7	1.8	1.7
Tumor necrosis factor ligand superfamily member 14	Q9QYH9	3.8	1.9	2.3	Death associated protein kinase 1	NM_029653	2.5	2.6	6.9
CD44	P15379	3.3	2.6	3.9	Protein-tyrosine phosphatase epsilon	P49446	2.5	2.0	4.1
RNA-binding protein FUS	P56959	2.9	4.6	2.8	Lymphocyte antigen 108	NM_030710	2.4	1.4	3.6
Alpha-2,8-sialyltransferase	Q64687	2.8	1.7	2.3	Solute carrier family 15 (H+/peptide transporter)	NM_021301	2.4	41.3	36.7
Elav-like protein 4	Q61701	2.4	0.2	0.3	Ziinc ring finger protein 1	NM_133206	2.3	2.1	3.4
Natural resistance-associated mecrophage protein 2	P49282	2.4	0.6	1.1	Nuclear protein 95	NM_010931	2.3	2.3	2.0
Putative 40-2-3 protein	NM_027226	2.4	2.1	2.8	Retinal short-chain dehydrogease/reductase	NM_011303	2.3	4.1	5.2
Prolin-rich protein 7	O35327	2.4	2.4	3.3	Dual specificity protein phosphatase	O09112	2.1	3.7	2.9
Type II 65kd keratin	Q99M73	2.3	2.1	2.9	Glypican	NM_016696	2.0	2.7	3.0
Protein kinase C, zeta type	Q02956	2.2	1.6	2.1	Interleukin-10 receptor alpha chain	Q61727	2.0	1.6	1.6
Phosphatidylinositol transfer protein beta isoform	P53811	2.2	1.7	1.8	F-box and WD-40 domain protein 7	NM_080428	2.0	2.0	2.2
Camp-dependent protein kinase	P05206	2.2	2.1	2.1	Channel-interacting PDZ domain protein	NM_007704]	1.9	2.4	3.2
Voltage-dependent anion-selective channel protein 3	Q60931	2.1	1.8	2.5	Endothelial monocyte-activating peptide	P50543	1.9	0.7	1.0
Embigin	P21995	2.0	2.3	2.8	Protein 4.1	P48193	1.9	2.2	3.0
Prostaglandin E2 receptor EP4 subtype	P32240	2.0	2.0	2.4	DNA methyl transferase 3A	O88508	1.9	1.5	1.5
Egl nine homolog 3	NM_028133	2.0	1.5	1.9	Transcription factor Sp3	O70494	1.9	1.8	1.9
Surfeit locus protein	P09926	1.9	1.6	1.9	Diaglycerol kinase gamma	Q91WG7	1.8	2.7	3.5
5'-methylthioadenosine phosphorylase	Q9CQ65	1.9	1.8	2.7	Ribinucleoside-diphosphate reductase	P11157	1.8	1.7	1.8
SAM-domain protein SANSN-1	P57725	1.9	2.2	2.3	Lymphoid-restricted membrane protein	NM_008511	1.8	1.6	1.8
Adrenodoxin	P46656	1.9	1.6	1.9	H-2 class I histocompatibility antigen	P01898	1.8	3.5	5.1
LIM domian transcription factor Lmo4	O00158	1.8	2.4	2.9	G2/mittotic-specific cyclin B1	P24860	1.8	1.5	1.4
Trans-acting transcription factor Sp4	NM_009239	1.8	2.5	2.0	Sphingolipid delta 4 desaturase/C-4 hydroxylase	NM_027299	1.8	2.3	2.3
Cell cycle related kinase	NM_053180	1.8	0.6	0.9	Similar to REV/REX activstion domain binding protein	NM_145566	1.8	1.9	2.5
Bifunctional aminoacyl-tRNA synthase	Q9CRF9	1.8	2.1	2.9	Transgelin 2	Q9WVA4	1.8	1.4	1.3
Signal and transducer and activator of transcription 4	P42228	1.8	3.0	3.0	Death associated protein kinase 1	NM_029653	1.8	2.0	3.9
Regulatory protein Tsc22	Q00992	1.7	2.5	2.1	Lipin 1	Q91ZP3	1.8	1.5	1.7
X-linked lymphocyte-regulated 4	NM_021365	1.7	1.7	1.2	Coiled-coil transcriptional coactivator	NM_026192	1.8	2.0	2.7
Exosome complex exonuclease Rrp42	Q9D0M0	1.7	1.3	1.6	Caspase 2	P29594	1.8	2.0	2.1
BCR downstream signalling 1	NM_019992	1.7	1.7	2.1	Transient receptor potential cation channel	NM_011706	1.7	1.3	1.8
Pre-mRNA splicing factor Srp55	Q9CSJ3	1.7	1.6	1.6	Camp regulated phosphoprotein 21	Q9DCB4	1.7	1.7	2.6
Cyclin ANIA-6A	NM_019937	1.7	1.8	1.5	Placental protein 11 related	NM_008902	1.7	2.0	2.8
Signal recognition particle 54 kda protein	P14576	1.6	2.4	2.1	Neurogenic locus notch homolog protein 1 (Notch1)	Q01705	1.7	1.4	1.5
Metal response element binding transcription factor 2	Q02395	1.6	1.7	1.8	RNA-binding protein EWS	Q61545	1.7	1.4	1.5
Calcineurin B subunit isoform 1	Q63810	1.6	2.1	1.8	Retinitis pigmentosa gtpase regulator interacting protein 1	NM_023879	1.7	3.3	3.3
EURL protein homolog	Q9D7G4	1.6	2.0	1.4	Histone H1.2	P15864	1.7	1.8	2.3
Hematopoietic cell signal transducer	NM_011827	1.6	1.5	2.0	Interferon-activatable protein 204	P15092	1.7	2.9	4.1

Supplementary Table 2. GATA3 target genes differentially expressed in clusters B and C (continued)

Genes up-regulated ≥ 1.4 fold	Source*	G3	DO	G3:DO	Genes down-regulated ≥ 1.4 fold	Source*	G3	DO	G3:DO
Dual specificity protein phosphatase 2	Q05922	1.6	1.9	1.7	High mobility group nucleosomal binding domain 3 isoform	NM_026122	1.7	2.8	4.2
Regulator of g-protein signalling 10	Q9CQE5	1.6	2.0	2.3	Cytidine monophospho-N-acetylneuraminic acid hydroxylase	NM_007717	1.7	2.2	3.4
28S ribosomal protein S18B	Q99N84	1.6	1.6	1.9	Similar to kinesin light chain KLCT	NM_146182	1.7	1.8	3.4
AU RNA-binding enoyl-coenzyme A hydratase	NM_016709	1.6	1.5	1.5	G protein-coupled receptor 69	NM_021295	1.7	1.8	2.7
Nucleolysin TIA-1	P52912	1.6	1.8	1.8	Treacle	NM_011552	1.7	2.0	1.9
TRIF	NM_019706	1.5	1.5	1.8	Pericentiolar material 1	NM_023662	1.6	1.8	1.7
Interleukin-12 recepeptor beta-1 chain	Q60837	1.5	1.9	2.3	SWI/SNF related regulator of chromatin	NM_053124	1.6	1.6	1.7
Membrane spanning 4-domains, subfamily A, 6B	NM_027209	1.5	2.8	3.3	Cytochrome B5	P56395	1.6	1.5	2.0
Protein-tyrosine phosphatase, non-receptor type 2	Q06180	1.5	1.5	2.0	DNA-binding protein inhibiter Id3	P41133	1.6	0.6	1.3
Pyruvate kinase, M2 isoenzyme	P52480	1.5	2.0	2.0	Polyhomeotic-like 2	NM_018774	1.6	1.5	1.5
Tumor susceptibility gene 101 protein	Q61187	1.5	1.8	1.6	Pituitary tumor transforming 1	NM_013917	1.6	1.4	1.7
Putative SP100-related protein	Q99388	1.5	2.0	1.9	BCL2/adenovirus E1B 19-kda	O54940	1.6	1.3	1.6
Nucleoporin 50kda	Q9JIH2	1.5	1.6	1.7	Similar to retinoblastoma binding protein 8	Q8VE67	1.6	1.4	1.4
Lysosomal-associated protein transmembrane 4B	NM_033521	1.5	0.6	0.8	DNAJ homolog subfamily B, member 9	Q9QYI6	1.6	1.6	2.2
Methionine aminopeptidase	O08663	1.5	1.3	1.5	Alpha-1 catenin	P26231	1.6	3.2	3.5
Dihydrolipoamide dehydrogenase	O08749	1.5	1.6	1.5	Etoposide-induced protein 2.4	Q61070	1.6	1.9	1.6
TFIIH basal transcription factor complex P52 subunit	O70422	1.4	2.7	2.1	Similar to neurofilament heavy polypeptide 200kd	Q8R3A0	1.5	1.6	1.9
Actin related protein 2/3 complex, subunit 5	NM_026369	1.4	1.6	1.4	Similar to proline-rich protein 48	Q8R5A3	1.5	1.4	1.8
Zinc finger protein 265	Q9R020	1.4	1.3	1.4	Zinc finger protein 147	Q61510	1.5	1.6	2.0
H-2 class I histocompatibility antigen	P14432	1.4	2.7	2.1	Solute carrier family 23, member 1	Q9EPR4	1.5	1.6	1.9
Zinc finger protein 313	Q9ET26	1.4	1.7	1.4	Protein tyrosine phosphatase alpha	P18052	1.5	1.9	2.0
Cell division protein kinase 4	P30285	1.4	1.6	1.9	Pptassium voltage-gated channel subfamily H member 2	O35219	1.5	1.6	2.7
Solute carrier family 25	NM_172436	1.4	2.2	2.7	Caspase 8 associated protein 2	NM_011997	1.4	1.6	1.6
SWI/SNF related regulator of chromatin, subfamily B1	Q9Z0H3	1.4	1.8	1.7	TOB2 protein	Q9JM55	1.4	1.5	1.8
60S acidic ribosomal protein P0	P14869	1.4	1.4	1.7	BH3 interacting domain death agonist	P70444	1.4	1.3	1.3
Alsin	NM_028717	1.4	0.4	0.6	TBC1 domain family protein C22ORF4 homolog	Q8R5A6	1.4	1.8	2.1
					Similar to microsomal glutathionine S-transferase 2	Q8R032	1.4	0.6	0.8
					Similar to spermatogenesis associated 2	NM_170756	1.4	1.5	1.6
					Er degradation enhancing alpha mannosidase-like	NM_138677	1.4	1.5	1.8

* RefSec, Swissprot or Sptrembl.

G3 = CD2-GATA3, DO = DO11.10, G3:DO = CD2-GATA3:DO11.10

Genes sorted by G3

Supplementary Table 3. GATA3 target genes differentially expressed in cluster E

Genes up-regulated ≥ 1.6 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥ 1.5 fold	Source*	G3	DO	G3: DO
Max-interacting transcriptional repressor Mad4	Q60948	3.8	1.3	8.9	Secreted modular calcium-binding protein 1	NM_022316	1.9	2.2	9.1
Trb2	NM_144551	1.9	0.9	4.7	H-2 class I histocompatibility antigen (H-2K(B))	P01901	1.8	2.4	3.4
Ubiquitin carboxyl-terminal hydrolase 14	Q9JMA1	2.6	1.1	4.0	DNA nucleotidylexotransferase (Tdt)	P09838	1.8	1.4	3.2
3110009E13RIK protein	Q9CXT1	1.6	1.4	3.9	Glutathionine S-transferase 5.7	P24472	1.8	1.6	3.2
Melanin concentrating hormone receptor interacting	NM_026021	3.3	1.3	3.7	Tyrosine protein kinase Tec	P24604	1.6	1.4	3.1
3'-5' Exoribobuclease Csl4 homolog	Q9DAA6	2.4	1.5	3.6	L-lactate dehydrogenase B chain	P16125	1.5	1.8	3.0
TRNA-nucleotidyltransferase	Q920N6	1.6	1.3	3.2	V(D)J recombination activating protein 2 (Rag2)	P21784	1.4	1.7	2.9
Cornichon homolog	O35372	2.0	1.6	3.0	Camello-like 2	NM_053096	1.1	1.8	2.9
Similar to GTP binding protein 5	Q8R3J9	1.3	2.2	3.0	Transcription factor Mafb	P54841	1.6	1.0	2.7
L-selectin (CD62L)	P18337	1.9	1.3	2.9	Oligodendrocyte transcription factor 3	NM_053008	1.3	2.0	2.7
Cop9 signalosome complex subunit 2	Q15647	1.1	2.5	2.9	Voltage dependent l-type calcium channel alpha-1C	Q01815	1.3	2.4	2.6
Guanine nucleotide-binding protein, aplha-13 (Galpha13)	P27601	1.3	2.3	2.8	T cell surface glycoprotein CD4	P06332	1.6	2.0	2.5
Vesicle transport v-snare protein VTI1-like	O88384	1.5	2.3	2.5	Vomeronasal 1 receptor I5	NM_134222	1.2	1.6	2.5
Ethanol induced gene product EIG180	NM_133237	1.7	0.9	2.5	Synaptic vesicle membrane protein Vat1 homolog	Q62465	2.1	2.0	2.4
Thioredoxin dependent peroxide reductase	P20108	1.8	0.6	2.5	Single stranded DNA-binding protein 2	Q9CYZ8	1.2	1.5	2.4
Cell growth regulating nucleolar protein	Q08288	1.0	1.7	2.5	Stress induced protein	NM_021897	1.7	1.5	2.4
Porphobilinogen deaminase	P22907	1.7	0.9	2.5	Protein 4.1	P48193	1.8	1.7	2.3
Eukaryotic translation initiating factor 3 subunit 11	Q9DBZ5	0.9	2.4	2.4	V(D)J recombination activating protein 1 (Rag1)	P15919	1.4	2.2	2.3
Similar to autoantigen Ngp1	NM_145552	0.8	2.2	2.4	Mitogen activated protein kinase4-2 (Mapk4-2)	NM_009006	2.2	1.7	2.3
Coatomer zeta1 subunit	Q9Y3C3	1.6	1.5	2.4	Zinc finger protein 39	Q02525	0.9	1.8	2.3
Huntigton's disease protein homolog	P42859	1.2	2.7	2.4	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	Q64518	1.9	1.6	2.2
U6 snRNA-associated sm-like protein Lsm4	Q9QXA5	0.9	2.4	2.3	Amyloid-like protein 2	Q06335	1.2	2.0	2.1
T-lymphocyte activated protein	P17950	2.1	1.6	2.2	Expressed sequence	NM_134133	1.2	1.5	2.1
Zinc finger protein BC027407	NM_153063	1.2	1.5	2.2	Lim/homeobox protein Lhx2	Q9Z0S2	1.6	2.4	2.1
Adipocyte derived leucine aminopeptidase	Q9EQH2	1.9	2.3	2.2	Tripartite motif protein Trim11	Q99PQ2	1.0	1.6	2.1
Ring finger protein 8 Q8VC56	Q8VC56	1.3	2.0	2.2	Calpain L3	Q64691	1.4	1.8	2.1
Peptidylprolyl isomerase like 3	NM_027351	1.6	2.1	2.2	F-box and leucine-rich repeat protein 12	NM_013911	1.2	1.3	2.0
POU domain, class 2, transcription factor 1	P25425	1.6	1.5	2.2	Signal transducer and activator of transcription (Stat5b)	P42232	1.6	1.5	2.0
Thy1 membrane glycoprotein	P01831	1.4	1.7	2.1	Ig gamma-1 chain C region	P01869	1.5	1.0	2.0
Muted	NM_139063	2.0	2.1	2.1	Glycerol kinase	Q64516	1.2	1.5	2.0
Sex-lethal interacting homolog	NM_025298	1.4	1.6	2.0	Phosphatidylinositol-4-phosphate 5-kinase type1 (PI4P5K-I)	NM_008846	0.6	1.6	2.0
MO25-like protein	Q9DB16	1.5	0.9	2.0	Polyposis locus protein 1 homolog	Q60870	1.5	1.3	1.9
DNA-3-methyladanine glycolsylase	Q04841	1.1	1.6	2.0	Zinc transporter 4	O35149	1.1	1.5	1.9
Dynamin 1 like	NM_152816	1.2	1.6	2.0	DNAJ homolog subfamily B member 1	Q9QYJ3	0.9	1.3	1.8
Cyclin dependent kinase 2 interacting protein	NM_026048	1.4	1.7	2.0	Swi/snf related actin dependent regulator of chromatin D2	NM_031878	1.4	1.7	1.8
Eukaryotic translation initiating factor 3 subunit 10	P23116	1.4	1.2	2.0	Ccr4-not transcription complex, subunit 7 (Caf1)	Q60809	1.3	1.4	1.8
Flavohemoprotein B5/B5	Q99P29	1.3	2.0	2.0	Ubiquitin conjugating enzyme E2D1	NM_145420	1.4	1.4	1.8
WD-repeat protein Bing4	Q9Z0H1	1.4	1.6	2.0	Widely interspaced zinc finger motifs	NM_011717	1.2	1.5	1.8
T-complex protein 1, theta subunit	P42932	1.4	1.1	1.9	Lysosomal acid lipase/cholesteryl ester hydrolase	Q9Z0M5	1.2	1.3	1.8
Placenta specific 8	NM_139198	1.9	1.6	1.9	B Cell translocation gene 1 (Btg1)	P31607	1.5	1.4	1.8
S-adenosylmethionine decarboxylase proenzyme 1	P31154	1.7	1.1	1.9	Interleukin 21 receptor (IL-21R)	Q9JHX3	0.9	1.4	1.8
Similar to zinc finger protein 157	NM_145612	1.5	1.0	1.9	Nuclear factor of activated T cells, cytoplasmic 1 (Nfatc1)	O88942	1.1	1.4	1.8

Supplementary Table 3. GATA3 target genes differentially expressed in cluster E (continued)

Genes up-regulated ≥ 1.6 fold	Source*	G3	DO	G3:DO	Genes down-regulated ≥ 1.5 fold	Source*	G3	DO	G3:DO
PRO1853 homolog	NM_028611	1.2	1.5	1.9	High mobility group protein 1(Hmg1)	P07155	1.5	1.4	1.8
Lathosterol oxidase	O88822	1.5	1.2	1.9	T Cell activation rho GTPase activating protein	NM_145968	1.4	1.3	1.8
Kinesin like protein Kif2	P28740	1.7	1.7	1.9	Tripartite motif protein 30	P15533	1.6	1.2	1.8
Bcl2/adenovirus E1B 19-kda interacting protein 2	O54940	0.9	1.7	1.9	Tyrosine protein kinase Itk/Tsk	Q03526	1.2	1.3	1.8
Maguk P55 subfamily member 6	Q9JLB0	1.5	1.4	1.9	CUG Triplet repeat, RNA binding protein 2	NM_010160	1.5	1.4	1.7
RNA 3'-terminal phosphate cyclase-like protein	Q9JJT0	1.1	1.6	1.9	Lysosamal acic lipase/cholesteryl ester hydrolase	Q9Z0M5	0.9	1.2	1.7
Melanoma antigen, family D2	NM_030700	1.3	1.6	1.9	Lymphocye antigen Ly-6E	Q64253	1.5	1.7	1.7
Gene trap rosa 26 antisense	NM_008188	1.4	1.9	1.9	Nuclear factor of activated T cells 5 (NFAT5)	Q9WV30	1.3	1.5	1.7
Similar to zinc finger protein 118	Q91VP4	0.8	1.8	1.9	MHC class II transactivator CIITA	P79621	1.2	1.3	1.7
Elongation factor G1	Q8K0D5	1.5	1.5	1.9	Pat-12 protein	Q64154	1.5	1.4	1.7
Protein phosphatase 2, regulatory subunit b	NM_009358	1.4	1.8	1.8	Similar to rearranged L-Myc fusion sequence	Q922U7	1.4	1.2	1.7
Band 4.1-like protein 2	O70318	0.7	1.4	1.8	Signal transducer and activator of transcription 2 (Stat2)	Q9WVL2	1.5	1.2	1.7
Apoptosis antagonizing transcription factor	NM_019816	1.3	1.6	1.8	Mitogen activated protein kinase 6 (Erk3)	Q61532	1.7	1.5	1.7
RD protein (WL623)	P19426	1.6	1.1	1.8	Hypoxia inducible factor 1 alpha	Q61221	1.3	1.4	1.7
Probable mitochondrial import receptor subunit tom40	Q9QYA2	1.4	1.5	1.8	Ras-related protein rab-3D	P35276	1.2	1.4	1.7
Armadillo repeat protein	P98203	1.4	1.8	1.8	Cyfip2	NM_133769	1.4	1.3	1.7
Septin 1	P42209	1.6	1.5	1.7	Glcnac-t I	P27808	1.4	1.3	1.7
Degenerative spermatocyte homolog	NM_007853	1.1	1.8	1.7	Extacellular superoxide dismutase	O09164	1.2	1.4	1.7
DNA-binding protein Ikaros	Q03267	1.0	1.3	1.7	6-phosphofructokinase, type c	Q9WUA3	1.4	1.0	1.7
Mitogen activated protein kinase 14	P47811	1.3	1.5	1.7	Nuclear Lim interacting factor 2	NM_146012	1.4	1.3	1.6
Galectin9	O08573	1.1	1.3	1.7	P300/Cbp associated factor	NM_020005	1.3	1.3	1.6
Tyrosyl-DNA phodhodiesterase 1	NM_028354	1.4	1.4	1.7	Cell division cycle 37 homolog	NM_025950	1.3	1.3	1.6
Carnitine deficiency-associated protein	O35594	1.9	1.7	1.7	Similar to CG15168 gene product	Q8R3L0	1.3	1.5	1.6
WD-repeat protein AN11homolog	O15491	1.5	1.5	1.7	Antigen containing epitope to antibody MMS-85/12	O35243	1.0	1.4	1.6
C-ETS-1 protein	P27577	1.5	1.0	1.7	Bromodomain containing 4	NM_020508	1.4	1.7	1.6
Acethylcholine receptor protein, gamma chain	P04760	1.4	1.7	1.7	Gamma-aminobutyric acid (GABA(A))	NM_020590	1.4	1.5	1.6
Telomeric repeat factor 2 interacting protein 1	Q91VL8	1.4	1.3	1.7	TPR-containing, SH2-binding phosphpoprotein	NM_009431	1.6	1.5	1.6
Vesicle transport v-snare protein VTI1-like1	O88384	1.4	1.4	1.7	Signal transducer and activator of transcription 5a (Stat5a)	P42230	1.5	1.2	1.6
Kinesin-like 5	NM_024245	1.5	1.0	1.7	T cell surface glycoprotein CD3 epsilon chain	P22646	1.1	1.4	1.6
Small edrk-rich factor 1	O88892	1.3	1.0	1.7	Morc	NM_010816	1.2	1.3	1.6
Absent in melanoma 1	NM_172393	1.4	1.7	1.7	Myeloid leukemia sequence 1	NM_008562	1.3	1.2	1.6
Heterogeneous nuclear ribonucleoprotein H2	NM_019868	1.3	1.2	1.6	RAL Guanine nucleotide dissociation stimulator-like2	Q61193	1.4	1.2	1.6
Integral membrane protein tmie	NM_146260	1.4	1.5	1.6	Growth factor receptor-bound protein 2	Q60631	1.5	1.5	1.6
D-3-phospoglycerate dehydrogenase	Q61753	1.4	1.3	1.6	ATPsynthase mithochondrial F1 complex assembly factor 2	NM_145427	1.3	1.3	1.6
DNA-binding protein inhibitor ID-2	P41136	1.0	2.0	1.6	Palmitoyl-protein thioesterase 2	O35448	0.9	1.3	1.6
1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	Q9D1E8	1.1	1.3	1.6	Werner syndrome helicase homolog	O09053	1.4	1.5	1.6
Bcl2-like protein 11	O54918	0.7	1.1	1.6	Solute carrier family 39	NM_134135	1.3	1.2	1.6
					Segment polarity protein dishevelled homolog DVL-2	Q60838	1.3	1.4	1.6

* RefSec, Swissprot or Sptrembl.

G3 = CD2-GATA3, DO = DO11.10, G3:DO = CD2-GATA3:DO11.10

Genes sorted by G3:DO

Supplementary Table 4. Primers and probes used for RT-PCR analysis for expression of the indicated genes.

Gene	Forward primer	Reverse primer	Probe
B-Actin	TACCACTGGCATCGTGATGGACT	TTTCTGCATCCTGTCGGCAAT	
Btg1	CAA GTT CTC CGC ACC AA	GCT TTT CTG GGA ACC AGT GA	AGG AGC TG
c-Myc	GCAGCTGTTTGAAGGCTGGAT	GTCGCAGATGAAATAGGGCTGT	CACGACGATGCCCCTCAACGTGA
Ctla4	CTTGGCCTTTTGTAGCCCTG	CACATGGAAAGCTGGCGACA	AGCCATACAGGTGACCCAACCTTCAGTG
Flt3L	TGACCCTGAACTCAAAGCAA	CAGCAGGGTGGAGGAGTCT	CTCCTGCC
Gapdh	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	TGCATCCTGCACCACCAACTG
Gata3	CATTACCACCTATCCGCCCTATG	CACACACTCCCTGCCTTCTGT	CGAGGCCCAAGGCACGATCCAG
Granzyme A	TTGGACTAAACATGATTTGTGCAG	AGGTGATGCCTCGCAAAATAC	
IL-18R	ATCCAGGACACATGGCTGTATAAG	CGCAGGAGTAGTAGCCCTCATC	
Rag1	AGGCCTGTGGAGCAAGGTA	GCTCAGGGTAGACGGCAAG	ATGGCTGC
Rag2	TGCCAAAATAAGAAAGAGTATTTCAC	GGGACATTTTTGATTGTGAATAGG	GCAGGAAG
Rora	CTGGCTTCTTCCCCTACTGTTC	TTTCCAGGTGGGATTTGGATAT	
Tcf1	CAGCTCCCCCATACTGTGAG	GGCAGGGAAGTGCTGTCTAT	CTTCCTGC
TdT	GTTCAGGGTGGAAAATGAGC	CAGGACGTCTGACCCTGAGT	TGGCAGAG
Thbs2	CCAACCCATCTTTTGTGGAA	GACCAGCCCTCATCACTGTC	TGCTGTCC
Thpok	CTT TGC CTG TGA GGT CTG C	CAG TGG GGG CAC GAG TAG	CAGGAGAA
VldlR	AGCAGGCAATGCAATGGC	TGCACTTGAACTTTCCAGGGC	

6

Enforced expression of Gata3 allows differentiation of IL-17-producing cells, but constrains Th17-mediated pathology

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ABSTRACT

The zinc-finger transcription factor Gata3 serves as a master regulator of T helper 2 (Th2) differentiation by inducing expression of the Th2 cytokines IL-4, IL-5 and IL-13 and by suppressing Th1 development. Here, we investigated how Gata3 affects Th17 differentiation, using transgenic mice with enforced Gata3 expression. We activated naïve primary T cells *in vitro* in the presence of transforming growth factor-β and IL-6, and found that enforced Gata3 expression induced co-expression of Th2 cytokines in IL-17-producing T cells. Although the presence of IL-4 hampered Th17 differentiation, transforming growth factor-β/IL-6 cultures from Gata3 transgenic mice contained substantial numbers of IL-17$^+$ cells, partially because Gata3 supported Th17 differentiation by limiting IL-2 and IFN-γ production. Gata3 additionally constrained Th17 differentiation *in vitro* through IL-4-independent mechanisms, involving downregulating transcription of Stat3, Stat4, Nfatc2 and the nuclear factor RORγt, which is crucial for Th17 differentiation. Remarkably, upon myelin oligodendrocyte glycoprotein immunization *in vivo*, Gata3 transgenic mice contained similar numbers of IL-17-producing T cells in their lymph nodes as wild-type mice, but were not susceptible to autoimmune encephalomyelitis, possibly due to concomitant production of IL-4 and IL-10 induction. We therefore conclude that although Gata3 allows Th17 differentiation, it acts as an inhibitor of Th17-mediated pathology, through IL-4-dependent and IL-4-independent pathways.

INTRODUCTION

T helper cells are classically divided into two functionally distinct subsets, termed T helper 1 (Th1) and Th2[1, 2]. Th1 cells, producing IFN-γ and lymphotoxin-α, are associated with the elimination of intracellular pathogens. Two major signalling pathways facilitate Th1 development, one involving IL-12/Stat4 and the other involving IFN-γ/Stat1/T-bet. Th2 cells, producing IL-4, IL-5 and IL-13, are not only critically important for the eradication of parasitic worms, but are also implicated in allergic responses. Th2 differentiation is dependent on IL-4-induced activation of Stat6, leading to expression of the zinc-finger transcriptional regulator Gata3[3, 4]. Gata3 auto-activates its own expression and increases the accessibility of the Th2 cytokine cluster containing the genes coding for IL-4, IL-5 and IL-13[5-7]. Furthermore, Gata3 suppresses Th1 development by downregulating Stat4 and IL-12Rβ2 chain expression[8-10]. Only if T-bet is sufficiently induced in naïve cells, such Gata3 suppression is counteracted, permitting Th1 differentiation to occur. Conditional gene targeting experiments showed that Gata3 deficiency was sufficient to induce Th1 differentiation in the absence of IL-12 and IFN-γ, demonstrating that Gata3 serves as a principal Th1/Th2 switch[11].

In differentiating CD4+ effector T cells loss of Gata3 results in a substantial reduction in the generation of IL-4-, IL-5- and IL-13-producing cells[11, 12]. Gata3 binds to multiple sites within the 200 Kb Th2 cytokine locus in resting T cells; binding to the IL-5 and IL-13 promoters increases upon T-cell activation. Introduction of Gata3 into *in vitro*-cultured T cells generates Th2-specific DNAse I hypersensitive sites independent of Stat6, implicating Gata3 in the process of chromatin remodeling[6]. In activated Th2 cells Gata3, Stat6, c-Maf and the chromatin-remodeling enzyme Brg1 and RNA polymerase II are all bound across the Th2 locus, whereby densely looped, transcriptionally active chromatin is packaged by the special AT-rich sequence binding protein (Satb1)[13]. Furthermore, Gata3 activity is required during embryonic development[14] and at multiple stages of thymocyte development, including the earliest double negative stages[15, 16], β-selection[17] and CD4+ cell development[17-19].

In recent years, effector T cells distinct from the Th1 or Th2 subsets have been described, which produce IL-17 and are therefore termed Th17 cells[20, 21]. IL-17 is a pro-inflammatory cytokine involved in the control of a wide range of infections at mucosal surfaces and has been implicated in the pathogenesis of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis, autoimmune myocarditis and psoriasis (reviewed in[22, 23]). In mouse, transforming growth factor-β (TGF-β) and IL-6, an acute phase protein induced during inflammation, act together to induce Th17 differentiation[24-26]. Because TGF-β is also a critical factor for the generation of anti-inflammatory regulatory T (Treg) cells, there is a dichotomy in the generation of pathogenic Th17 T cells that induce autoimmunity and Tregs that inhibit autoimmune disease[25, 26]. The orphan nuclear receptor RORγt has been identified as a key regulator of the differentiation program of Th17 cells[27]. Th17 differentiation is regulated by several cytokines: IL-2 and IL-27 are strongly inhibitory, whereas IL-21 and IL-23 have been reported to promote IL-17 expression[27-31].

As Th1, Th2, Th17 and Treg, appear to arise in a mutually exclusive fashion, various mechanisms of counter-regulation exist, at the level of cytokines as well as transcription factors. For example, TGF-β has the capacity to block Th2 development through inhibition of Gata3 expression[32]. In Th1 cells, T-bet essentially functions as a negative regulator of Gata3 expression[10, 33]. Gata3 suppresses the induction of T-bet by downregulating Stat4 and IL-12Rβ2 chain[8-10]. In CD8 cells, the Th2 cytokine locus is silenced by expression of repressor of Gata[34]. Yet, it is currently not known whether Gata3 activity inhibits or stimulates differentiation of Th17 cells. On the one hand, Gata3 induces IL-4, which has been shown to suppress Th17 development[20, 21]. But since IFN-γ is the main inhibitor of Th17 development, Gata3 activity may, on the other hand, promote Th17-cell generation because of its capacity to negatively regulate Th1

development and thus IFN-γ production[8-10].

In this report, we investigated how Gata3 expression affects Th17 differentiation. We employed our previously characterized CD2-Gata3 transgenic mice, which have enforced Gata3 expression driven by the CD2 locus control region, resulting in enhanced Th2 differentiation of peripheral $CD4^+$ T cells[35]. We isolated naïve primary CD4 T cells from CD2-Gata3 transgenic mice, activated them *in vitro* under culture conditions supporting Th17 differentiation, and found co-expression of Th2 cytokines in IL-17-producing T-helper cells. Interestingly, we identified multiple stimulatory and inhibitory effects of Gata3 on Th17 differentiation *in vitro*. Although CD2-Gata3 transgenic mice contained normal numbers of $IL\text{-}17^+$ cells in gut-draining lymphoid tissue, they were not susceptible to Th17-mediated EAE *in vivo*.

RESULTS

Transgenic Gata3 induces IL-4 and limits IL-2 production in T helper cells

To investigate how the presence of Gata3 affects differentiation of naïve T cells toward the Th17-cell lineage, we used CD2-Gata3 transgenic mice on a C57Bl/6 background. Their phenotype was essentially similar to CD2-Gata3 transgenic FVB mice described previously[35, 36]. These mice manifested a significant reduction of thymic cellularity, affecting most stages of thymic development (see **Supplementary Fig. 1**), and contained reduced numbers of CD4 T cells and particularly CD8 T cells in spleen and lymph nodes (see **Supplementary Fig. 1**). Peripheral T cells manifested an increased surface expression of the CD3/TCR-αβ-complex and a small but consistent decrease in CD5, which is a negative regulator of TCR signalling (**Supplementary Fig. 1**). We previously found that when total peripheral CD4 T-cell fractions from CD2-Gata3 transgenic FVB mice were cultured under Th1 conditions, Th2 cytokine production was increased and IFN-γ production was hampered[35]. In these experiments, the presence of antigen-experienced Th2-committed cells might have contributed to the observed increased Th2 differentiation.

Because both IL-4 and IFN-γ are negative regulators of Th17 differentiation[20, 21, 24, 37], we first aimed to investigate the effect of Gata3 on Th17 differentiation, independent of its effects on IL-4 and IFN-γ production. To this end, we activated sorted splenic naïve $CD62L^+CD4^+$ T cells from wild-type (WT) and CD2-Gata3 transgenic C57BL/6 mice with plate-bound anti-CD3/anti-CD28 under Th17 conditions, *i.e.* in the presence of TGF-β, IL-6, anti-IFN-γ and anti-IL-4 for 7 days. We also cultured $CD62L^+CD4^+$ T cells under Th0 (no additional cytokines or mAb), Th1 (with IL-12 and anti-IL-4) and Th2 (with IL-4, anti-IFN-γ and anti-IL-12) polarizing conditions to confirm that the CD2-Gata3 transgene

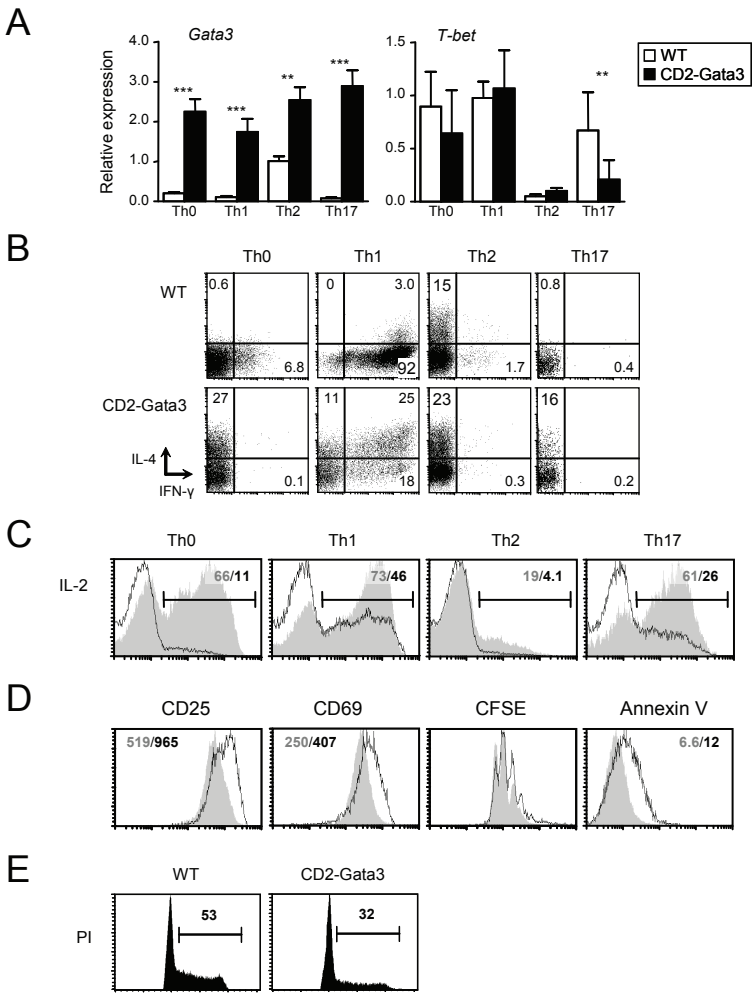

Figure 1. The CD2-Gata3 transgene induces IL-4 and limits IL-2 production in Th1, Th2 and Th17 cells. (**A**) Quantitative RT-PCR analysis of *Gata3* and *T-bet* expression in different T effector cells from wild-type (WT, white bars) and CD2-Gata3 transgenic mice (black bars), cultured for 7 days. Expression was normalized to *Gapdh* and expression levels of *Gata3* in WT Th2 cells and *T-bet* in WT Th1 cells were set to 1. Mean values and SEM are given for 5–7 mice per group (**P<0.01; ***P<0.001; *t*-test). (**B**) Flow cytometric analysis for intracellular expression of IL-4 and IFN-γ in gated CD4$^+$ T cells from day 7 cultures. Numbers indicate percentages of total cells within the quadrants. (**C**) and (**D**) Flow cytometric analysis for intracellular IL-2 expression (**C**), CD25 and CD69 expression, Annexin and carboxy-fluorescein succinimidyl ester (CFSE) (**D**) in the indicated WT (gray filled histogram) and CD2-Gata3 transgenic (black line) T-cell cultures. Cells were cultured for 3 days. Numbers indicate the percentage of total cells (**C**) or the mean fluorescence intensity (**D**) from WT (gray numbers) or CD2-Gata3 transgenic (black numbers) Th0 cell cultures. (**E**), Cell cycle distribution by PI (propidium iodide) staining of Th0 cells cultured for 3 days. Numbers indicate the proportions of cells in S/G2/M. (B–D) Data shown are representatives of four mice analyzed per group.

had the capacity to induce IL-4 and to limit IFN-γ production during differentiation of naïve T cells *in vitro*.

In all T-cell cultures from CD2-Gata3 transgenic mice, Gata3 expression levels reached values that were up to a factor of ~2–3 higher than those in WT Th2 cultures (**Fig. 1A**), irrespective of polarization conditions. Enforced Gata3 expression did not significantly affect the expression levels of T-bet in Th0, Th1 and Th2 cultures, but in CD2-Gata3 transgenic Th17 cultures the levels of T-bet were reduced (**Fig. 1A**). Whereas WT Th0 cultures contained detectable fractions of IFN-γ-producing cells, CD2-Gata3 transgenic Th0 cultures contained significant proportions of IL-4$^+$ cells, as determined by intracellular flow cytometry (**Fig. 1B**). Under Th1 polarizing conditions, a large majority of WT T cells produced IFN-γ. In CD2-Gata3 transgenic Th1 cultures, IFN-γ was produced by a significant proportion (~43%) of the cells, a large fraction of which co-expressed IL-4. Under Th2 conditions, the presence of the CD2-Gata3 transgene resulted in significantly increased proportions of IL-4$^+$ cells. In WT Th17 cultures, IL-4$^+$ or IFN-γ$^+$ cells were not detected, but in CD2-Gata3 transgenic Th17 cultures ~16% of cells produced IL-4 (**Fig. 1B**).

CD2-Gata3 transgenic Th cultures showed decreased production of IL-2, when compared with WT (**Fig. 1C**), irrespective of polarization conditions. This reduction in IL-2 appeared (at least partially) independent of Gata3-induced IL-4, as it was also seen under Th1 or Th17 conditions in the presence of anti-IL-4 antibodies. Reduced IL-2 production did not reflect impaired activation of CD2-Gata3 transgenic T cells, as at day 3 expression levels of the activation markers CD25/IL-2R and CD69 were upregulated. CD25 expression was even slightly higher in CD2-Gata3 transgenic T cells, when compared with WT (**Fig. 1D**). Nevertheless, CD2-Gata3 transgenic T-cell cultures showed increased apoptosis, as determined by Annexin V staining, and reduced proportions of cells in the S/G2/M phase of the cell cycle, but remaining cells showed a normal capacity to go through sequential cell divisions detected by carboxy-fluorescein succinimidyl ester-labeling (**Fig. 1D and E**).

Collectively, these data show that enforced Gata3 expression is sufficient to induce IL-4 expression and to reduce IL-2 production in all culture conditions. As a result, Th1 cultures contained many IL-4/IFN-γ double positive cells and Th17 cultures contained IL-4$^+$ cells, even in the presence of anti-IL-4 antibodies during cell culture.

Enforced Gata3 expression allows Th17 differentiation *in vitro*

Interestingly, Th17 cultures from WT mice and CD2-Gata3 transgenic mice contained equal proportions of IL-17-expressing cells at day 7 (**Fig. 2A**), but the CD2-Gata3 transgenic IL-17-producing cells co-expressed IL-4 and IL-13 (**Fig. 2B**). The proportions

of IL-13$^+$ cells in CD2-Gata3 transgenic Th17 cultures were in the same ranges as those in CD2-Gata3 transgenic Th0, Th1 and Th2 cultures (**Supplementary Fig. 2**). Gata3 also has the capacity to induce the immunosuppressive cytokine IL-10[35], which was

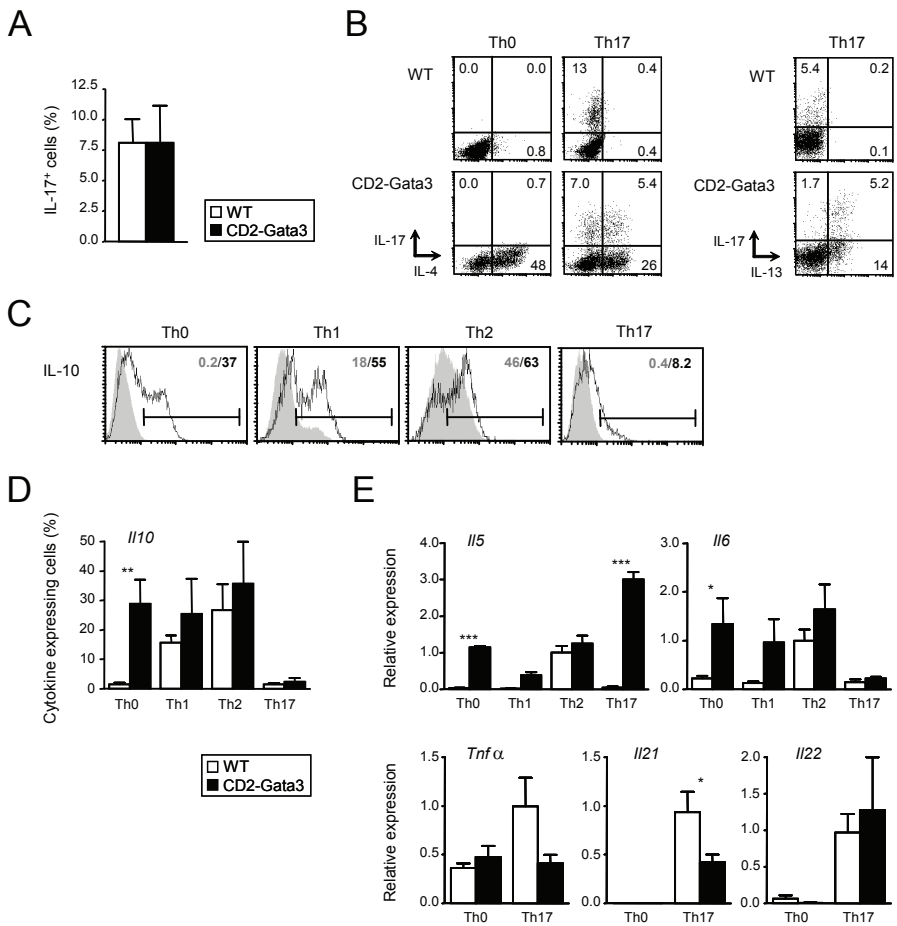

Figure 2. Enforced Gata3 expression induces Th17 cells with Th2 characteristics. (**A**) Flow cytometric analysis of IL-17 production of wild-type (WT, white bar) and CD2-Gata3 transgenic (black bar) Th17 cells. Mean values and SEM are from 17 mice per group. (**B**) Intracellular flow cytometric analysis of the indicated cytokines in WT and CD2-Gata3 transgenic T-cell cultures at day 7. Numbers indicate the percentages of total cells within the quadrants. (**C**) Flow cytometric analysis for intracellular IL-10 in WT (gray filled histogram) and CD2-Gata3 transgenic (black line) T-cell cultures. Numbers indicate the percentages of IL-10$^+$ cells. Data are representatives of 4–6 mice per group. (**D**) Quantification of flow cytometric analysis for intracellular IL-10 in gated CD4$^+$ T cells from WT (white bars) and CD2-Gata3 transgenic mice (black bars). Mean values and SEM are from 4 to 6 mice per group ($^{**}P$<0.01; *t*-test). (**E**) Quantitative RT-PCR analysis of cytokine expression in the indicated T-cell cultures. Expression was normalized to *Gapdh* and expression levels of *Il5* and *Il6* in WT Th2 and expression levels of *Tnfα*, *Il21* and *Il22* in WT Th17 cells, were set to 1. Mean values and SEM are for 5–7 mice per group ($^{*}P$<0.05;$^{***}P$<0.001; *t*-test).

originally described as a product of Th2 cells but is produced by many other cell types. We found a significant induction of IL-10$^+$ cells in CD2-Gata3 transgenic Th0 cells (**Fig. 2C and D**). In the presence of the CD2-Gata3 transgene the proportions of IL-10$^+$ cells were generally increased in Th1 and Th2 cultures. We found only a modest increase in CD2-Gata3 transgenic Th17 cultures, when compared with WT cultures (**Fig. 2C and D**). The presence of Gata3-induced expression of IL-5, irrespective of polarization conditions (**Supplementary Fig. 2** and **Fig. 2E**). IL-6, which is also associated with Th2 differentiation[1, 2, 23] was induced in Th0, Th1 and Th2 but not in Th17 cultures, as determined by quantitative RT-PCR analyses (**Fig. 2E**).

The ability of enforced Gata3 expression to induce Th2 cytokines and to suppress IL-2 production was not unique to T helper cells, as similar observations were made in cultured CD8$^+$ T cells (without additional cytokines or mAb) and Treg cells (in the presence of TGF-β, anti-IFN-γ and anti-IL-4) for 7 days (**Supplementary Fig. 3**).

To further examine the properties of the effector T cells that developed in the CD2-Gata3 transgenic Th17 cultures, we determined expression of cytokines known to be produced by Th17 cells using quantitative RT-PCR. Consistent with the observed presence of IL-17$^+$ cells in the CD2-Gata3 transgenic Th17 cultures, we found that they expressed IL-21 and TNF-α, albeit at lower levels when compared with WT Th17 cultures (**Fig. 2E**). Enforced Gata3 expression allowed normal IL-22 production in Th17 cells.

Taken together, these experiments demonstrate that – in the presence of anti-IL-4 and anti-IFN-γ antibodies – enforced Gata3 expression does not significantly affect the generation of IL-17-producing cells *in vitro*. The CD2-Gata3 transgenic cells cultured in the presence of TGF-β and IL-6 had characteristics of both Th17 cells (production of IL-17, IL-21 and IL-22, low IL-6 expression) and Th2 cells (production of the Th2 cytokines IL-4, IL-5 and IL-13, low levels of IL-2).

Transgenic Gata3 affects transcription of various regulators of Th17 cells

In our Th17 culture system, in the presence of anti-IFN-γ and anti-IL-4 antibodies, the proportions of IL-17-producing cells in WT and CD2-Gata3 transgenic cultures were similar at day 7 (see **Fig. 2B**), probably reflecting an equilibrium of stimulatory and inhibitory effects of Gata3. This enabled us to analyze the effect of Gata3 on the expression of individual factors involved in regulation of Th17 differentiation in cell populations that contained equal proportions of IL-17$^+$ cells. Day 7 WT and CD2-Gata3 transgenic Th17 cultures were activated by anti-CD3/anti-CD28 stimulation for 4h and mRNA expression levels were analyzed by quantitative RT-PCR and compared with those in Th0, Th1, Th2, Treg and CD8 cultures (**Fig. 3** and **Supplementary Fig. 4**).

Expression of the CD2-Gata3 transgene resulted in reduced transcription levels of RORγt, which is essential for Th17-cell generation. Also transcription of Nfatc2, which stimulates expression of IL-17 in human[38], was reduced. Growth factor independent-1 (Gfi-1) is a Stat6-dependent transcriptional repressor that is induced by IL-4 in activated CD4 T cells and increases Th2 cell expansion by promoting proliferation and preventing apoptosis[39]. Enforced Gata3 expression did not result in significantly increased Gfi-1 levels in Th17 cultures, but was associated with a substantial increase of Gfi-1 expression in Treg cultures (**Supplementary Fig. 4**). Gata3 expression did not have detectable effects on the expression levels of Nfatc1 or Irf4, which was recently shown to be essential for Th17 differentiation[40] (**Supplementary Fig. 4**).

Jak-Stat (signal transducer and activator of transcription) signalling is crucially involved in pathways integrating cytokine signals into T-cell differentiation programs: Stat1 and Stat4 in Th1 cells, Stat5a and Stat6 in Th2 cells and Stat3 and Stat4 in Th17 cells. In agreement with previous findings in Th1/Th2 polarization[8, 9], we found that Gata3 is capable of downregulating Stat4 in Th17 cultures (**Fig. 3**). As Stat4 was recently shown to direct Th17 cells[41, 42], this finding indicates that Gata3 might be a negative regulator of Th17 differentiation by Stat4 downregulation.

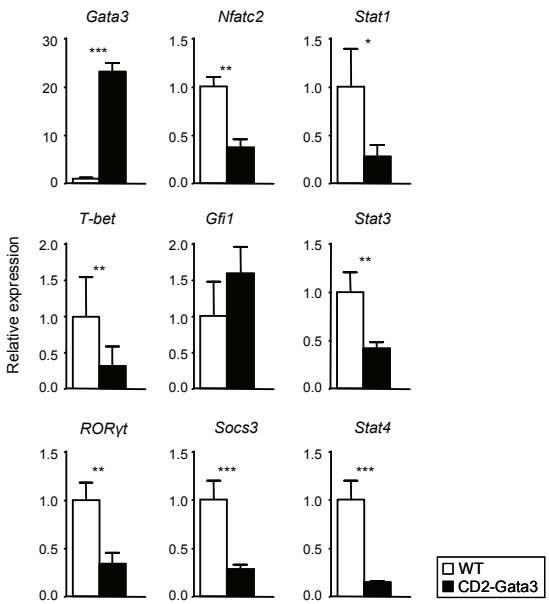

Figure 3. Enforced Gata3 expression affects factors involved in Th17 differentiation. Quantitative RT-PCR analysis of expression of the indicated genes in wild-type (WT, white bars) and CD2-Gata3 transgenic (black bars) Th17 cultures at day 7. Expression levels were normalized for *Gapdh* and values in Th17 cultures from WT mice were set to 1. Mean values and SEM are given for 6–11 mice analyzed per group ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; *t*-test).

Enforced Gata3 expression was also associated with reduced expression of Stat1, specifically in Th17 and Th0 cells, and Stat3, specifically in Th17 cells (**Fig. 3** and **Supplementary Fig. 4**). The latter finding would implicate Gata3 as a negative regulator of IL-17 differentiation, because Stat3 directly binds to the IL-17A and IL-17F promoters[43]. No significant effects of Gata3 on Stat5a or Stat6 were observed (**Supplementary Fig. 4**). IL-23-mediated phosphorylation of Stat3, and thus Th17 generation, is negatively regulated by the cytokine-inducible inhibitor Socs3 (suppressor of cytokine signalling)[43]. We found that the presence of enforced Gata3 expression is associated with significantly reduced levels of Socs3 transcripts (**Fig. 3**).

Taken together, these RT-PCR analyses show that enforced Gata3 expression is associated with significant changes in the expression levels of critical regulators of Th17 development. Gata3 has the capacity to stimulate Th17-cell generation by decreasing expression of Stat1 and Socs3 and to inhibit Th17 differentiation by limiting expression of RORγt, Stat3, Stat4 and Nfatc2.

Effects of Gata3, IL-2 and IL-4 on Th17 differentiation

As enforced Gata3 expression resulted in increased IL-4 and reduced IL-2 expression, both of which are negative regulators of Th17 differentiation[24, 28, 37], we explored the effects of Gata3, IL-2 and IL-4 on Th17-cell generation in more detail.

To this end, we stimulated primary $CD62L^+CD4^+$ T cells with plate-bound anti-CD3/anti-CD28 and cultured them with TGF-β/IL-6 and anti-IFN-γ in the presence or absence of exogenous IL-2 or IL-4 or antibodies against these cytokines, for 3 days. In these experiments, cells were re-stimulated with PMA/ionomycin for 4h before intracellular cytokine staining, which yielded higher proportions of $IL\text{-}17^+$ cells, when compared with anti-CD3 re-stimulation (compare **Fig. 2A** with **Fig. 4**). Hereby, clearance of IL-4 had only marginal effects on the generation of $IL\text{-}17^+$ cells in WT cells, probably due to the limited IL-4 expression of differentiating Th17 cells in the presence of TGF-β and IL-6. At day 3, the CD2-Gata3 transgenic Th17 cultures contained significantly reduced proportions of $IL\text{-}17^+$ T cells: ~12% in the absence and ~22% in the presence of anti-IL-4 (**Fig. 4A and B**). Although addition of external IL-4 reduced the proportions of $IL\text{-}17^+$ cells expression in WT Th17 cultures, it had only marginal effects on IL-17 expression in CD2-Gata3 transgenic cultures, probably due to substantial transgenic IL-4 production of differentiating Gata3-expressing Th17 cells (**Fig. 4A and B**).

Addition of anti-IL-2 mAb, next to anti-IL-4, significantly increased IL-17 production in differentiating WT and CD2-Gata3 transgenic Th17 cells (**Fig. 4C**). Consistent with this finding, IL-17 production was low in CD2-Gata3 transgenic Th17 cultures when exogenous IL-2 was added, irrespective of the addition of anti-IL-4 mAb. Thus, at day3

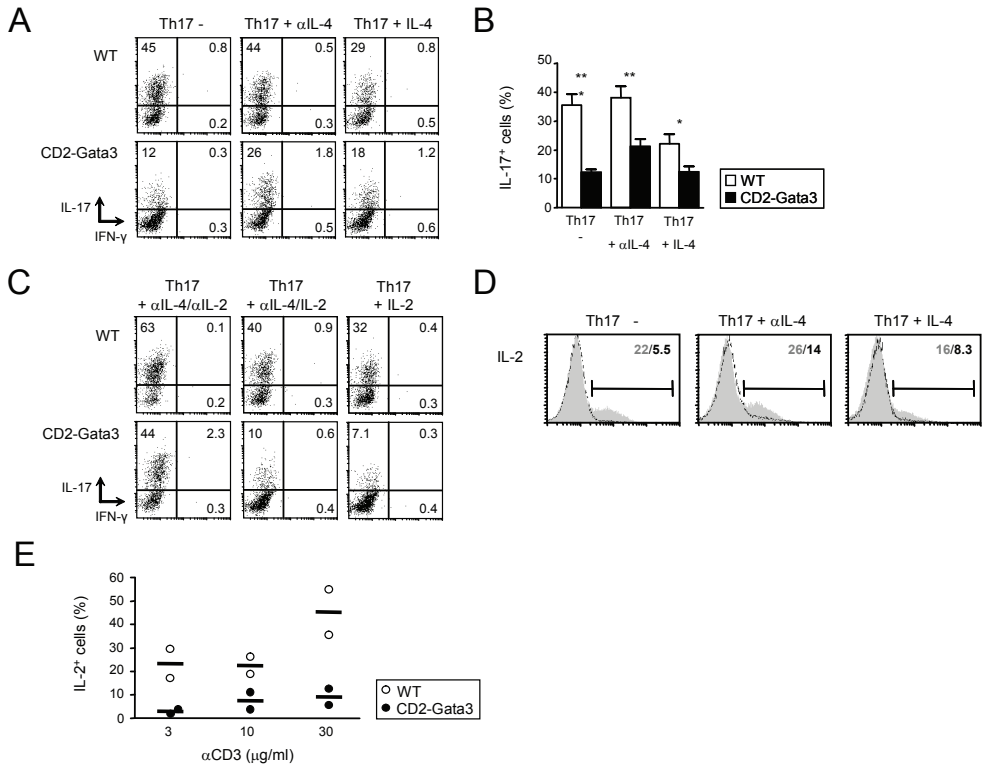

Figure 4. Enforced Gata3 expression affects Th17 differentiation *via* IL-4 and IL-2. (**A**) to (**C**) IL-17/IFN-γ expression profiles of CD4$^+$ T cells in Th17 cultures from the indicated mice. Cells were cultured for 3 days with the indicated antibodies or cytokines. Numbers indicate percentages of total cells within the quadrants (**A**, **C**) or mean values and SEM of the percentage of IL-17$^+$ cells are given ($^*P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001; *t*-test) (**B**). Data shown are representatives of 5–11 mice per group. (**D**) Flow cytometric analysis of intracellular IL-2 expression in wild-type (WT, gray filled histogram) and CD2-Gata3 transgenic (black line) Th17 cells, which were cultured for 3 days with or without αIL-4 or IL-4. Numbers indicate the percentages of IL-2$^+$ cells. Data shown are representatives of 2–10 mice per group. (**E**) Flow cytometric analysis of intracellular IL-2 in Th17 cultures from the indicated mice. Cells were cultured for 3 days and stimulated with indicated concentrations of plate-bound anti-CD3. Mean and individual data points are displayed, indicating the percentage of IL-2$^+$ cells.

CD2-Gata3 transgenic Th17 cultures contained fewer IL-17-producing cells than WT cultures, even in the presence of anti-IL-4 mAb and independent of clearance of IL-2 or addition of IL-2 (**Fig. 4C**).

To further explore whether the negative effect of Gata3 on IL-2 production in Th17 cells is regulated *via* IL-4, we investigated IL-2 production in 3-day Th17 cultures in the absence or presence of anti-IL-4 mAb or exogenous IL-4. IL-2 was expressed in ~22–26% of the WT cells, irrespective of clearance of IL-4 (**Fig. 4D**). Expression of IL-2 in CD2-Gata3 transgenic Th17 cultures was very low (~6% of cells), and the

proportion of IL-2-producing cells only moderately increased in the presence of anti-IL-4. Thus, low IL-2 expression in the presence of the CD2-Gata3 transgene is partially IL-4-dependent and partially IL-4-independent. The capacity of IL-4 to reduce IL-2 production in Th17 cells was confirmed by the finding of reduced IL-2 expression in WT Th17 cells that were cultured in the presence of IL-4 for 3 days (**Fig. 4D**). Consistent with the reported TCR signal strength-dependency of IL-2 production, and the inverse correlation between Gata3 induction and IL-2 expression in naïve T cells[44], we found that in differentiating Th17 cells IL-2 production increased when cells were stimulated with higher doses of anti-CD3 and that in CD2-Gata3 transgenic T cells increasing the anti-CD3 concentration had limited effect (in the presence of anti-CD28 mAb; **Fig. 4E**).

Taken together, we found that enforced Gata3 expression in 3-day Th17 cultures resulted in reduced proportions of IL-17-expressing cells under all conditions analyzed. Gata3 (i) stimulates IL-17 production *via* IL-2 downregulation, (ii) inhibits IL-17 production *via* induction of IL-4 and (iii) additionally constrains IL-17 production in an IL-4-independent way.

Downregulation of Stat3/4, Socs3, Nfatc2 and RORγt by Gata3 is IL-4-independent

Our finding that Gata3 has the capacity to limit expression of Stat3, Stat4, Socs3, Nfatc2 and RORγt in Th17 cultures in the presence of antibodies to IL-4 and IFN-γ (**Fig. 3**) suggested that these inhibitory effects of Gata3 were IL-4-independent. To analyze this IL-4 independence directly, we performed Th17 cultures in the presence or absence of anti-IL-4 antibodies or exogenous IL-4 and analyzed the expression of Stat3, Stat4, Socs3, Nfatc2 and RORγt at day 7 by RT-PCR. We found that the capacity of Gata3 to reduce transcription of these factors was indeed independent of IL-4 addition or clearance (**Supplementary Fig. 5A**). The presence of the Gata3 transgene did not significantly affect the *in vitro* proliferative capacity of Th17 cultures, excluding the possibility that the observed effects of Gata3 on gene transcription were unspecific (**Supplementary Fig. 5B**).

We therefore conclude that the observed inhibitory effect of Gata3 on Stat3, Stat4, Socs3, Nfatc2 and RORγt transcription is independent of IL-4.

IL-17-producing T cells are present *in vivo* in CD2-Gata3 transgenic mice

Next, we investigated whether enforced Gata3 expression would allow the differentiation of Th17 cells *in vivo*. Since Th17 cells have been associated with the mucosal lymphoid system[27], we analyzed IL-17 expression in gut-draining lymphoid tissue, including Peyer's patches, mesenteric and iliacal lymph nodes. In these tissues we found that in

the presence of the Gata3 transgene the populations of IL-17-producing T cells were maintained (**Fig. 5A**), but also IL-4 production was induced (**Fig. 5B**). Thus, enforced Gata3 expression allowed differentiation of Th17 cells *in vivo*.

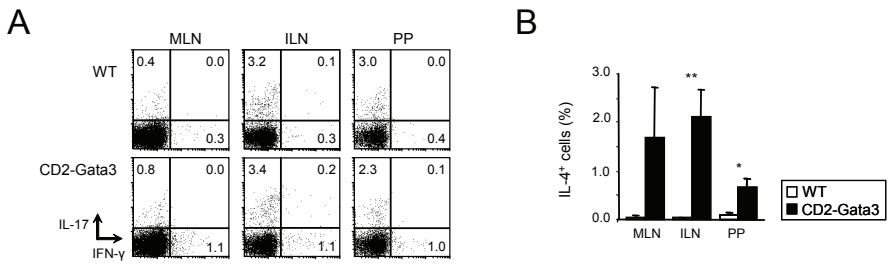

Figure 5. IL-17-producing cells in CD2-Gata3 transgenic mice *in vivo*. (**A**) Flow cytometric analysis for intracellular IL-17 and IFN-γ expression in gated CD4$^+$ T cells from mesenteric lymph nodes (MLN), iliacal lymph nodes (ILN) and Peyer's patches (PP) from wild-type (WT) and CD2-Gata3 transgenic mice. Numbers indicate the percentage of cells within the quadrants. Data are representatives of six mice analyzed per group. (**B**) Quantification of flow cytometric analysis for intracellular IL-4 in gated CD4$^+$ T cells from the indicated tissues from WT (white bars) and CD2-Gata3 transgenic mice (black bars). Mean values and SEM are from four mice per group ($^*P$<0.05; $^{**}P$<0.01; *t*-test).

Transgenic Gata3 allows *in vivo* Th17 differentiation but protects against EAE

Although our analyses in gut-draining lymphoid tissues showed that enforced Gata3 expression allowed differentiation of IL-17$^+$ T cells *in vivo*, the various stimulatory and inhibitory effects of Gata3 on Th17 differentiation identified prompted us to investigate the effect of the CD2-Gata3 transgene on development of EAE, which has been reported to be highly dependent on Th17 cells[22, 23, 29]. After MOG peptide/CFA immunization, five out of eight WT mice developed EAE. In contrast, no clinical disease symptoms were found in ten CD2-Gata3 transgenic mice (**Fig. 6A**). We determined the presence of IL-17-producing cells in a pool of axillary, brachial and inguinal lymph nodes after MOG peptide injection. Importantly, we found similar proportions of IL-17-producing CD4$^+$ T cells in WT and in CD2-Gata3 transgenic mice (**Fig. 6B**), although the values in CD2-Gata3 transgenic mice were slightly higher at day 14, and slightly lower at day 28, as compared with WT mice. Importantly, at day 14 CD2-Gata3 transgenic mice had significant numbers of IL-4 and IL-10- producing CD4$^+$ T cells in their lymph nodes (~2.0 and ~2.5%, n=5, respectively), in contrast to WT mice (both proportions were <0.2%) (**Fig. 6C**).

Next, we sorted T helper cells with the CD62L$^-$CD4$^+$ activated/memory phenotype from spleens from WT and CD2-Gata3 transgenic mice at day 14 after MOG peptide injection. Quantitative RT-PCR analyses revealed that increased expression of Gata3 was associated with reduced transcription of RORγt, Stat1, Stat3, Stat4 and

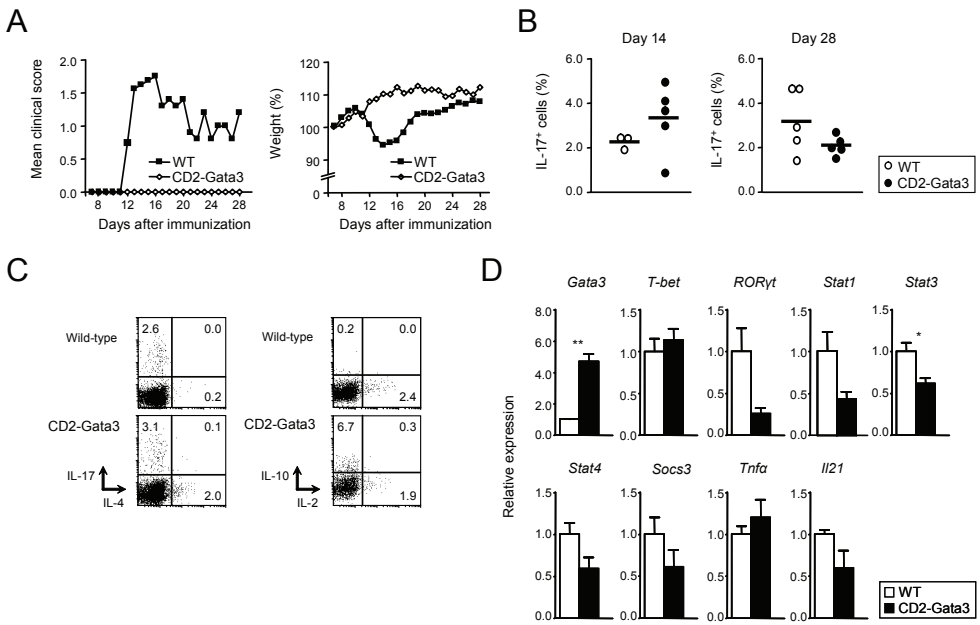

Figure 6. CD2-Gata3 transgenic mice are not susceptible to EAE induction. (**A**) Induction of EAE by immunization with $MOG_{35–55}$ peptide. Mean clinical scores and weight are given for 8–10 mice per group. (**B**) Flow cytometric analysis of intracellular IL-17 expression in gated LN $CD4^+$ T cells from wild-type (WT) and CD2-Gata3 transgenic mice 14 and 28 days after EAE induction. Mean and individual data points are displayed, indicating the percentage of IL-17^+ cells. (**C**) Flow cytometric analysis for the indicated cytokines in gated LN $CD4^+$ T cells from WT mice and CD2-Gata3 transgenic mice 14 days after EAE induction. Numbers indicate the percentage of cells within the quadrants. Data shown are representatives of 3–5 mice per group. (**D**) Quantitative RT-PCR analysis of expression of the indicated genes in sorted activated/memory $CD4^+CD62L^-$ T cells from WT (white bars) and CD2-Gata3 transgenic (black bars) mice 14 days after EAE induction. Expression levels were normalized for *Gapdh* and values of WT mice were set to 1. Mean values and SEM are given for three mice analyzed per group ($^*P<0.05$; $^{**}P<0.01$; *t*-test).

Socs3 (**Fig. 6D**), in agreement with our findings in *in vitro* Th17 cultures (**Fig. 3**). In addition, we found that T-bet and TNF-α expression were unaffected by the presence of the CD2-Gata3 transgene *in vivo*.

We therefore conclude that enforced Gata3 expression exerts a marginal effect on the generation of IL-17-producing cells in EAE. Nevertheless, CD2-Gata3 transgenic mice are not susceptible to *in vivo* EAE induction, possibly because concomitant production of IL-4 and the induction of IL-10 may restrain Th17-mediated pathology.

DISCUSSION

In this report, we studied the molecular mechanisms of counter-regulation by which the key regulator of Th2 differentiation, transcription factor Gata3, affects Th17 differentiation.

We show that enforced expression of Gata3 from a T-cell-specific transgene essentially induces Th2 cytokine production and hampers IL-2 cytokine production in various *in vitro* differentiating effector T-cell populations, including Th17 cells. The induction of the Th2 cytokine IL-4, by Gata3, had a negative effect on TGF-β/IL-6-induced Th17 differentiation, but this was partially compensated by the capacity of Gata3 to limit IL-2 production, which functions as a negative regulator of Th17 differentiation[28]. However, the Gata3-induced stimulation of IL-4 production alone could not fully explain the inhibitory effect of Gata3 on Th17 differentiation: also in the presence of anti-IL-4 antibodies CD2-Gata3 transgenic Th17 cultures contained fewer IL-17$^+$ cells, when compared with WT cultures, at day 3. Therefore, we conclude that Gata3 additionally constrained Th17 differentiation through IL-4-independent mechanisms. Our RT-PCR analyses showed that Gata3 may stimulate Th17-cell differentiation by decreasing expression of Stat1 and Socs3 and may inhibit this process by limiting expression of RORγt, Stat3, Stat4 and Nfatc2. As in CD2-Gata3 transgenic mice IL-17-producing cells were present in normal numbers *in vivo*, both in gut-draining tissue and in lymph nodes during EAE induction, we conclude that the final consequences of the various stimulatory and inhibitory effects of Gata3 were that *in vivo* the presence of enforced Gata3 expression still allows for differentiation of IL-17-producing cells. However, because CD2-Gata3 transgenic mice were not susceptible to EAE, it appears that Gata3 mainly acts as an inhibitor of Th17-mediated pathology, possibly by driving IL-4 and IL-10 production.

In single CD2-Gata3 transgenic T cells, Th2 cytokines were co-expressed together with cytokines that signify differentiation to other subsets, such as IL-17 (in Th17 cells) or IFN-γ (in Th1 cells). Therefore, these findings indicate that Gata3 expression is sufficient to induce Th2 cytokine production, irrespective of the presence of critical transcription factors such as RORγt and T-bet in Th17 or Th1 cultures, respectively. Also CD8$^+$ T-cell cultures contained substantial proportions of cells producing Th2 cytokines or IL-10 at levels, similar to those found in WT Th2 cell cultures. Thus, in CD8 T-cells transgenic Gata3 could counteract the repression of Th2 cytokine production by repressor of Gata. This finding is in apparent conflict with the report by Omori *et al*.[34], who found that ectopic expression of Gata3 in cytotoxic type 2 cells resulted only in a limited generation of IL-4-producing cells. This discrepancy might be explained by the fact that in our study Gata3 is already overexpressed in naïve CD8$^+$ T cells *in vivo*, whereas in their retrovirus gene introduction system Gata3 expression is induced during cell culture.

The finding that Gata3 had the capacity to inhibit IL-2 expression, even in Th1 or Th17 cultures in the presence of anti-IL-4 antibodies, indicates that Gata3 directly regulates IL-2, through an IL-4-independent mechanism. Gata3 expression is sufficient

to induce IL-6 in Th0 or Th1 cells, but not in Th17 cells, suggesting that this capacity of Gata3 is either specifically inhibited in Th17 cells or dependent on a co-factor that is not present in Th17 cells. It has been shown that IL-4 suppresses Th17-cell development *in vitro* and that Th2 cytokines are important for preventing or ameliorating EAE; conversely, loss of IL-25 results in accelerated EAE pathology, associated with an increased number of IL-17-producing T cells that invade the central nervous system[20, 29, 45, 46]. The prevention of EAE in CD2-Gata3 transgenic mice is consistent with our findings of (i) significant IL-4 production in lymph nodes from CD2-Gata3 transgenic mice during EAE induction *in vivo* and (ii) limited Th17 differentiation of cells from CD2-Gata3 transgenic mice, cultured for 3 days in Th17 conditions in the absence of neutralizing anti-IL-4 antibodies (**Fig. 5A**). Also, the observed induction of IL-10 by transgenic Gata3 might well contribute to the resistance of CD2-Gata3 transgenic mice to EAE. In this context, it was very recently found that TGF-β and IL-6 do not only drive Th17 commitment, but they also act together to restrain the pathogenic potential of Th17 cells, by inducing the production of IL-10, which is an important factor in the downmodulation of immune responses and EAE[47-50]. In fact, evidence was provided for the existence of Th17 subsets with effector or regulatory functions that correlate in part with their ability to produce IL-10. Although Gata3 has been shown to directly remodel the IL-10 locus in $CD4^+$ T cells independently of IL-4[51], the observed significant proportions of $IL\text{-}10^+$ CD4 T cells during *in vivo* EAE induction in CD2-Gata3 transgenic mice (**Fig. 6C**) do not necessarily point to a direct role of Gata3 in upregulating IL-10 production in Th17 cells. On the contrary, we found that transgenic expression of Gata3 had only a modest effect on IL-10 production by Th17 cells generated *in vitro* (**Fig. 2C**). Therefore, it is more likely that the induction of $IL\text{-}10^+$ T cells *in vivo* is largely indirect, *e.g.* through tolerogenic dendritic cells[47]. Further experiments are required to determine whether prevention of EAE induction by enforced Gata3 expression is indeed dependent on the induction of IL-4, IL-10 or both, or whether other molecules are involved. In this context, we found that in the presence of neutralizing antibodies to IL-10 (from day 0 to day 8), EAE can be induced in CD2-Gata3 transgenic mice, albeit with an incidence and severity that is lower than in WT mice (JP van Hamburg, unpublished data). Therefore, these preliminary experiments indicate that IL-10 induction may contribute to the effects of transgenic Gata3, but does not completely explain the reduced susceptibility of CD2-Gata3 transgenic mice.

Although in 3-day cultures transgenic Gata3 expression limited Th17 differentiation, 7-day WT and CD2-Gata3 transgenic Th17 cultures contained similar proportions of IL-17-producing cells. Apparently at day 7 inhibitory and stimulatory effects of Gata3 are in balance, which made these cultures good tools to investigate the

effect of Gata3 activity on the expression of genes involved in Th17 differentiation. We found that enforced Gata3 is associated with reduced expression of RORγt, Stat3, Stat4 and Nfatc2, but further experiments are required to establish whether these genes are direct Gata3 targets. Interestingly, Nfatc2, which is known to stimulate IL-17 in human T cells and IL-21 in Th2 cells, can be induced by IL-6 in Th2 cells[38, 52, 53] and has the ability to interact with Irf4[54], which was recently shown to be critically involved in Th17 differentiation. But, it is currently not known how Nfatc2 functions in Th17 differentiation. Furthermore, Gata3 expression resulted in downregulation of Stat1, known to suppress Th17 differentiation *via* IL-27 signalling[55] and of Socs3, which is a negative regulator of Stat3 phosphorylation[43]. The effects of Gata3 on RORγt, Socs3, Stat3, Stat4 and Nfatc2 were not influenced by the addition or clearance of IL-4, indicating that Gata3 regulates these factors in an IL-4-independent manner. This is quite remarkable for Socs3, since one of the mechanisms by which IL-4 suppresses Th17-cell development may be through induction of Socs3 expression through IL-4R signalling. This was shown to be the case in B cells by a p38 MAPK-mediated mechanism[56]. Finally, the *in vivo* development of significant numbers of IL-17$^+$ effector cells in CD2-Gata3 transgenic mice could also partially be stimulated by the ability of Gata3 to limit IL-2 production and inhibit the generation of IFN-γ-producing cells, which suppress development of Th17 cells from naïve precursor cells[20, 21]. Taken together, Gata3 has various stimulatory and inhibitory effects on Th17 differentiation (see **Fig. 7**), whereby enforced Gata3 expression still allows the generation of IL-17-producing cells *in vivo*.

It is not surprising that with the identification of two additional effector T-cell subsets, Th17 and Treg cells, the molecular mechanisms of the reciprocal interactions between T-cell subsets, involving counter-regulation at the level of transcription factors and cytokines, become more complex. For example, we found that *in vitro* generated Tregs (containing >95% CD25$^+$Foxp3$^+$ cells) expressed significant levels of Gata3 and RORγt (**Supplementary Fig. 4**), indicating that Gata3 is not Th2-specific and RORγt is not Th17-specific. As these cultured Treg cells express Foxp3, T-bet, Gata3 and RORγt, apparently without any tendency to develop into Th1, Th2 or Th17 cells, Treg differentiation seems critically dependent on the balance between these transcription factors and therefore a molecular mechanism should be present in these cells that keep transcription factors in check and prevents Th1, Th2 or Th17 development. Conversely, the presence of RORγt alone is not sufficient to induce commitment to the Th17 lineage. The simultaneous expression of Gata3, T-bet and RORγt in Treg cells are puzzling and in apparent conflict with molecular models in which key transcription factors are essential to stabilize active or silent states of cytokine loci by epigenetic modifications during polarized effector T-cell differentiation. Our findings in Gata3 transgenic mice show

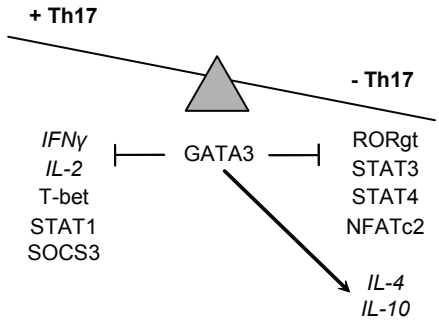

Figure 7. Regulatory effects of Gata3 on Th17 differentiation. Model showing effects of Gata3 on Th17 differentiation. Gata3 has positive effects by limiting IFN-γ and IL-2 production and inhibiting expression of T-bet, Stat1, and the Th17 repressor protein Socs3. On the other hand, Gata3 constrains Th17 differentiation by inhibiting expression of critical factors in Th17 differentiation, including RORγt, Stat3, Stat4 and Nfatc2, and limits Th17-mediated pathology by enhancing production of IL-4 and IL-10.

that Gata3 has the capacity to constrain Th17 differentiation through IL-4-dependent and IL-4-independent pathways, but neither Gata3 expression, nor the consequential production of IL-4, IL-5, IL-13 or IL-10 inhibited the generation of IL-17-producing cells *in vivo*. In this context, these findings are consistent with population-based studies in man[57-59] supporting the hypothesis that Th2-mediated diseases, such as asthma, do not necessarily protect from Th17-mediated autoimmune disorders.

METHODS

Mice. CD2-Gata3 transgenic mice[36] were backcrossed on the C57BL/6 background for at least eight generations and genotyped by PCR. Gata3 primers were: 5′-CAGCTCTGGACTCTTCCCAC-3′ and 5′-GTTCACACACTCCCTGCCTT-3′.

Flow cytometric analyses. Preparations of single-cell suspensions from thymus, spleen and lymph nodes, mAb incubations and four-color flow cytometry have been previously described[60]. Monoclonal antibodies were purchased from BD Biosciences, except PE-conjugated anti-Granzyme B (GB12) and biotinylated anti-IL-13 (BAF413), which were from Caltag Laboratories (Burlingame, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. For intracellular detection of cytokines, cells were stimulated with plate-bound anti-CD3 mAb (10 μg/mL in PBS) in the presence of GolgiStop™ (BD Biosciences) for 4h. Cells were harvested, extracellularly stained with anti-CD4 or anti-CD8 mAbs, followed by standard intracellular staining using 2% paraformaldehyde and 0.5% saponin. Samples were acquired on a FACSCalibur™ flow cytometer and

analyzed using CELLQuest™ (Becton Dickinson, Sunnyvale, CA, USA) or FlowJo™ (Tree Star, Ashland, OR, USA) research software. Live events were collected based on forward and side scatter.

Purification of naïve or activated/memory T cells and *in vitro* T-cell cultures. Naïve $CD62L^+CD4^+$ or $CD8^+$ T cells were purified from spleens obtained from 8-wk-old mice and activated/memory $CD62L^-CD4^+$ were purified from spleens from mice subjected to EAE induction. Cells were sorted, using an FACSVantage VE equipped with Diva Option and BD FACSDiva software (BD Bioscience). Purity of obtained fractions was typically >98%. $CD62L^+CD4^+$ or $CD8^+$ T-cell fractions were cultured at concentrations of 1×10^6 cells/mL in Iscove's modified Dulbecco's medium (BioWhittaker, Walkersville, MD, USA), containing 10% heat-inactivated fetal calf serum (Sigma, St. Louis, MO, USA), 5×10^{-5} M β-mercapto-ethanol (Merck, Darmstadt, Germany), supplemented with various cytokines (R&D Systems), as described below. Plates were coated with anti-CD3 and anti-CD28 (145-2C11; 37.51, BD Biosciences) at a concentration of 10 µg/mL each in PBS at 4 °C overnight. For Th1 polarizing conditions, IL-12 (10 ng/mL) and anti-IL-4 (10 µg/mL; 11B11) were added. Th2 polarizing conditions included IL-4 (10 ng/mL, R&D Systems), anti-IFN-γ (5 µg/mL; XMG1.2) and anti-IL-12/23 p40 (5 µg/mL; C17.8). Treg and Th17 polarizing conditions included TGF-β (3 ng/mL), anti-IL-4 and anti-IFN-γ. Th17 polarizing conditions additionally contained IL-6 (20 ng/mL), and in specified conditions also IL-4 (10 ng/mL), IL-2 (5 ng/mL) or anti IL-2 (10 µg/mL; JES6-1A12, Southern Biotech Associates, Birmingham, AL, USA). For Th0 conditions or CD8 T cells no cytokines or mAbs were added. On day 3 anti-CD3/CD28 activation was stopped and T-cell cultures were stained for intracellular cytokine detection or expanded in the presence of the indicated cytokines, supplemented with IL-2 (5 ng/mL) for up to 7 days.

Quantitative PCR analyses. Total RNA was extracted using the GeneElute mammalian total RNA miniprep system (Sigma) and 1 µg was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers. PCR primers spanning at least one intron–exon junction were designed manually or using ProbeFinder software (Roche Applied Science, Indianapolis, IN, USA) and probes were chosen from the universal probe library (Roche Applied Science) or designed manually and purchased from Eurogentec (Seraing, Belgium) (**Supplementary Table 1**). Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). To confirm the specificity of the amplification products, samples were analyzed

by standard agarose gel electrophoresis. Threshold levels were set and further analysis was performed using the SDS v1.9 software (Applied Biosystems). The obtained Ct values were normalized to those of glycereraldehyde-3-phosphate dehydrogenase (Gapdh).

EAE induction. EAE was induced in ~10-wk-old mice by subcutaneous injection of 200 µg MOG35–55 peptide emulsified in complete Freund's adjuvant (Difco), as described[61]. Mice were daily weighed and scored for clinical signs, as follows: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE. Paralyzed mice with EAE scores above 2 were afforded easier access to food and water.

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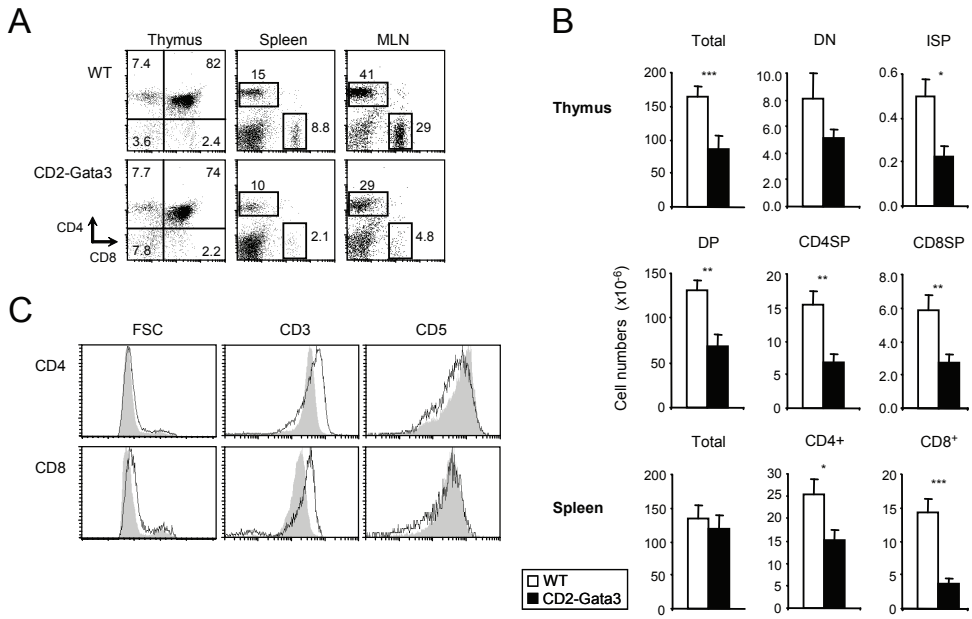
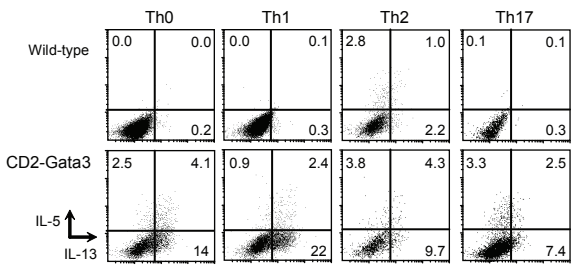
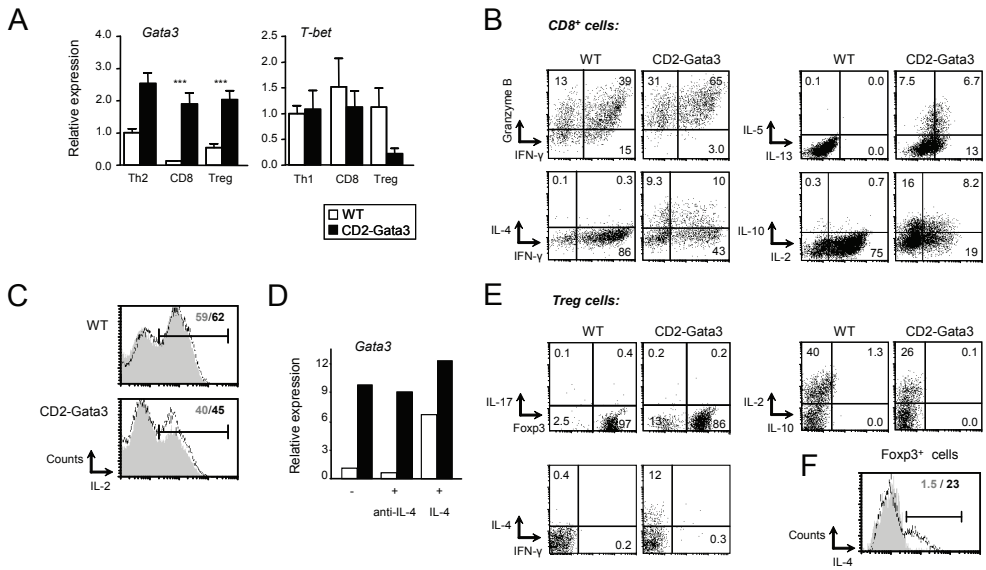
SUPPLEMENTARY FIGURES AND TABLE



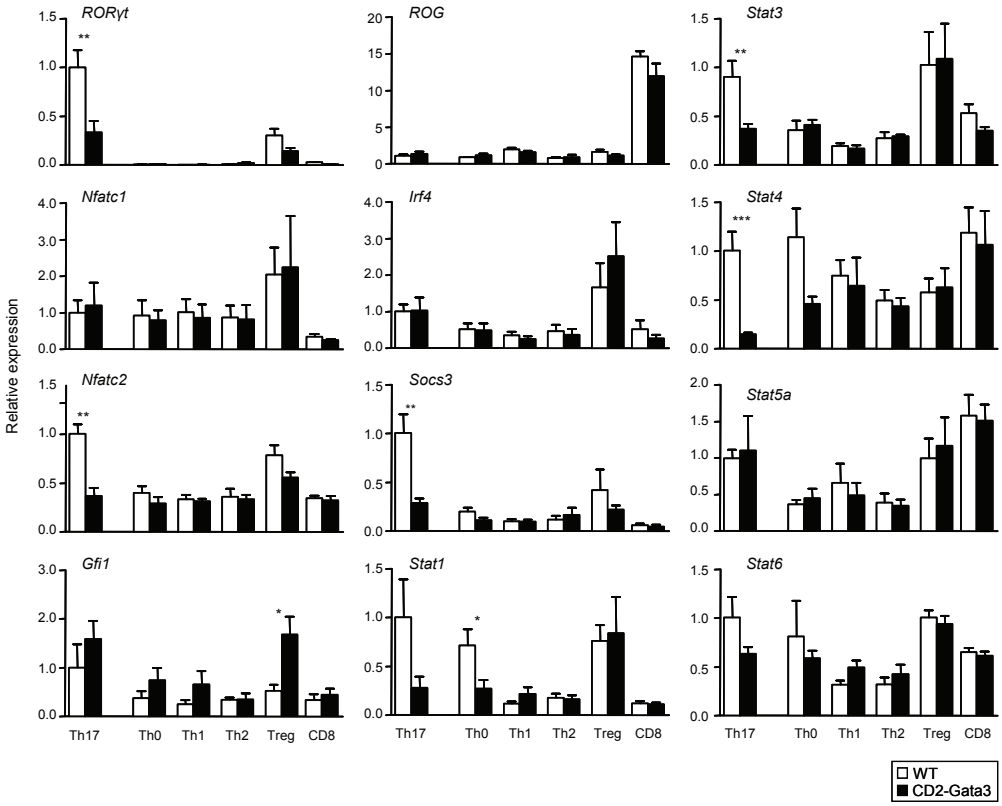
Supplementary Figure 1. Characterization of CD2-Gata3 Tg mice on the C57BL/6 background. (**A**) Distribution of T cell populations in the thymus, spleen and mesenteric lymph node (MLN) of wild-type (WT) and CD2-Gata3 transgenic mice. Results are displayed as dot plots of CD4/CD8 profiles. Percentages shown represent proportions of cells within a quadrant or gate. (**B**) Total cell numbers within the indicated cell populations in thymus and spleen. Mean values and SEM are given for eight mice analyzed per group (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; *t*-test). (**C**) Cell size (FSC), surface expression of CD3 and CD5 in splenic CD4 and CD8 T cell populations. Results are displayed as histograms indicating WT mice (gray filled histograms) and CD2-Gata3 transgenic mice (black lines). Data shown in (**A**) and (**C**) are representative for eight mice analyzed per group.



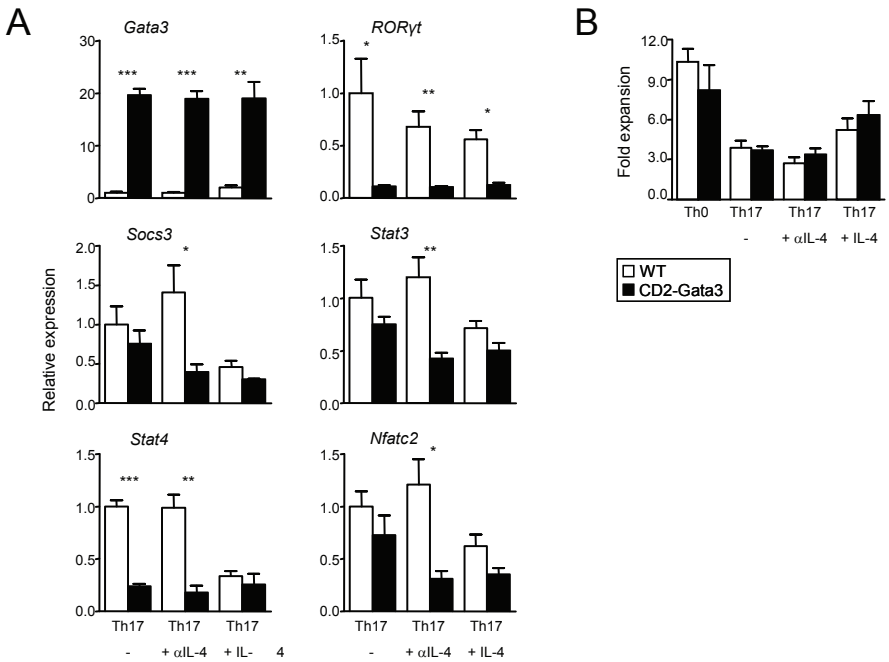
Supplementary Figure 2. Induction of IL-5 and IL13 by Gata3. Flow cytometric analysis for intracellular expression of IL-5 and IL-13 in the indicated T cell cultures from WT and CD2-Gata3 transgenic mice at day 7. $CD4^+$ T cell populations were gated and dot plots are shown, whereby numbers indicate percentages of total cells within the quadrants. Data shown are representative for four mice analyzed per group.



Supplementary Figure 3. Enforced Gata3 expression induces Th2 cytokines in CD8 T cells and Treg cells. (**A**) Quantitative RT-PCR analysis of *Gata3* and *T-bet* expression in the indicated wild-type (WT, white bars) and CD2-Gata3 transgenic (black bars) T effector cell cultures. Expression was normalized as indicated in the legend to **Figure 1A**. Mean values and SEM are given for four six mice per group (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; *t*-test). (**B**) Flow cytometric analysis of intracellular expression of granzyme B and cytokines in WT and CD2-Gata3 transgenic CD8$^+$ T cells, which were cultured for seven days. Numbers indicate the percent of total cells within the quadrants. Data shown are representative for four mice analyzed per group. (**C**) Flow cytometric analysis of IL-2 expression in WT and CD2-Gata3 transgenic CD8$^+$ T cells, stimulated by plate-bound anti-CD3/anti-CD28 and cultured for seven days (gray filled histogram) in comparison with cultures containing anti-IL-4 mAb (black line). Data shown are representative for 3-4 mice per group. (**D**) Quantitative RT-PCR of *Gata3* and *ROG* expression in WT and CD2-Gata3 transgenic CD8 T cells, cultured under the indicated conditions. Expression was normalized to *Gapdh* and levels in WT CD8$^+$ T cells were set to one. Mean values and SEM are given for 2-5 mice analyzed per group. (**E**) Flow cytometric analysis of intracellular expression of cytokines indicated and the Treg-specific transcription factor Foxp3. Numbers indicate the percentage of total cells within quadrants. (**F**) Flow cytometric analysis of intracellular IL-4 in WT (gray filled histogram) and CD2-Gata3 Tg (black line) Foxp3$^+$ cells. Numbers indicate the percentage of IL-4$^+$ cells within the Foxp3$^+$ population. Data shown are representative of four mice analyzed per group.



Supplementary Figure 4. Effect of enforced Gata3 expression on transcription levels of nuclear factors. Quantitative RT-PCR analysis of expression of the indicated nuclear factors in different T effector cell cultures. Expression levels were normalized for *Gapdh* and expression in Th17 cultures from wild-type (WT) mice were set to one. Mean values and SEM are given for 5-11 mice analyzed per group (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; *t*-test).



Supplementary Figure 5. Enforced Gata3 expression affects critical factors in Th17 differentiation in an IL-4-independent manner. (**A**) Quantitative RT-PCR analysis of expression of the indicated genes in Th17 cultures. Cells were cultured for seven days with or without αIL-4 or IL-4. Expression was normalized for *Gapdh* and values in Th17 cultures from wild-type (WT) mice were set to one. Mean values and SEM are given for eight mice analyzed per group (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; *t*-test). (**B**) Fold expansion of WT and CD2-Gata3 transgenic Th0 and Th17 cells with or without αIL-4 or IL-4. Mean values and SEM are given for eight mice analyzed per group.

Supplementary Table 1. Primers and probes used for quantitative RT-PCR analysis for expression of the indicated genes.

Gene	Forward primer	Reverse primer	Probe
Gapdh	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	TGCATCCTGCACCACCAACTG
Gata3	CATTACCACCTATCCGCCCTATG	CACACACTCCCTGCCTTCTGT	CGAGGCCCAAGGCACGATCCAG
Gfi-1	TCCGAGTTCGAGGACTTTTG	GAGCGGCACAGTGACTTCT	CTTCTCCC
Il5	ACATTGACCGCCAAAAAGAG	ATCCAGGAACTGCCTCGTC	GAGGAGAG
Il6	ATCAGGAAATTTGCCTATTGAAA	CCAGGTAGCTATGGTACTCCAGA	TTCCTCTG
Il21	CCATCAAACCCTGGAAACAA	TCACAGGAAGGGCATTTAGC	AGGAGGAG
Il22	GTGACGACCAGAACATCCAG	TCCACTCTCTCCAAGCTTTTTC	GGCTGAAG
Irf4	ACCCCATGACAGCACCTTAT	GGGTGGCATCATGTAGTTATGA	CAGCCAG
Nfatc1	TCCAAAGTCATTTTCGTGGA	CTTTGCTTCCATCTCCCAGA	GCTCCAGA
Nfatc2	CCTGCTGGTACCACCTACTTG	CCGACTGATTGGAGAGTGG	CAGCATCC
ROG	GGCTTCCTCTAGCCCCACT	CCAAGGGCTGAGGGTTTC	CCAGGGCA
RORγt	TTCACCCCACCTCCACTG	TGCAAGGGATCACTTCAATTT	TGCTGTCC
Socs3	ATTTCGCTTCGGGACTAGC	AACTTGCTGTGGGTGACCAT	CAGCCACC
Stat1	TTGTCAGATCGAACCTTCCTC	TGTCGTTCTACCACGAAGGA	CTTCCAGC
Stat3	CGTGGAGCTGTTCAGAAACTT	AACTGGACACCAGTCTTGATGA	TGGTGGAG
Stat4	CCTTAATTCAGAGCAGCTCAACA	GGTGAGGTGACCATCATTGTAG	TGGCAGAG
Stat5a	TGAAGGCGACCATCATCAG	GACGCAACAGTTGTTCAGGA	CAGCAGGC
Stat6	TCTGAGAGAGGGAGAAGATAGCAG	GGCCCCACAGAGACATGAT	GGCTGCTG
T-bet	TCAACCAGCACCAGACAGAG	AAACATCCTGTAATGGCTTGTG	GGCTGGAG
Tnfα	CCACGTCGTAGCAAACCAC	TTTGAGATCCATGCCGTTG	TGGAGGAG

7

General Discussion

Lymphocyte development and differentiation into functional effector subsets is a complex and highly regulated process. The cellular mechanisms of lineage specification and commitment, proliferation and differentiation critically depend on developmentally regulated changes in gene expression patterns. This frequently involves coordinate binding of lineage-specific transcription factors to *cis*-regulatory elements located over considerable genomic distances. For instance, three-dimensional models of gene regulation have been recently illustrated by studies at antigen receptor and T helper cytokine gene loci. Direct evidence for CTCF-mediated higher-order chromatin structure has been reported at several developmentally regulated gene loci, including the mouse *β-globin* and *H19/Igf2* loci and the human *MHCII* locus. More recently, genome-wide studies support a role for CTCF in the global organization of chromatin architecture. The main findings described in this thesis provide insight into the role of CTCF in lymphocyte proliferation, survival and differentiation, with particular emphasis on the regulation of *Ig* loci gene rearrangement and T helper cytokine gene expression.

CTCF regulation of lymphocyte proliferation, survival and differentiation

Previous studies have shown that CTCF levels markedly impact cellular functions as a consequence of the global and essential role for CTCF in regulating gene expression. Using a transgenic RNA interference (RNAi) approach, reduction of CTCF protein levels in oocytes prior to fertilization has been shown to disrupt normal progression to the blastocyst-stage embryos[1]. Further detailed analysis on the role of CTCF during early embryonic development identified misregulated transcription of hundreds of genes upon CTCF deletion[2]. Accordingly, in **Chapter 3** we show that no homozygous *Ctcf* knockout mice were born from *Ctcf*$^{+/-}$ crosses. Furthermore, the ratio of wild-type to *Ctcf*$^{+/-}$ littermates is higher than expected on a Mendelian basis in crosses among *Ctcf*$^{+/-}$ mice and between wild-type and *Ctcf*$^{+/-}$ mice, which suggested that CTCF is required in a dose-dependent manner.

In order to characterize the *in vivo* function of CTCF during B- and T-cell development we crossed *Ctcf*$^{f/f}$ mice with mice expressing Cre recombinase specifically in the early stages of B- and T-cell lineage - *mb1-cre Ctcf*$^{f/f}$ and *Lck-Cre Ctcf*$^{f/f}$ mice, respectively. We demonstrated in **Chapter 2** that early B cell development is impaired in *mb1-cre Ctcf*$^{f/f}$ mice due to markedly defective proliferation and differentiation of CD2^{-} cytoplasmic Igμ^{+} pre-B cells. Likewise in **Chapter 3** we found that deletion of CTCF in thymocytes from *Lck-Cre Ctcf*$^{f/f}$ mice specifically hampers αβ T-cell development, namely the proliferation of β-selected cells (DN4 and ISP cells) and their differentiation from ISP to DP cells. Interestingly, both analyses show that CTCF is essential at early stages of lymphocyte development for the cellular proliferation and differentiation events occurring

after the first step of antigen receptor loci rearrangements – pre-BCR checkpoint after *Igh* rearrangement in B-cells and pre-TCR checkpoint after *Tcrβ* rearrangement in T-cells. Although a striking parallel between CTCF role in early B- and T-cell development can be drawn when comparing *mb1-cre Ctcf$^{f/f}$* and *Lck-Cre Ctcf$^{f/f}$* mice, it is important to note that the developmental arrest observed during T-cell development is apparently less complete and appears to occur later. Pre B-cells from *mb1-cre Ctcf$^{f/f}$* mice show reduced cell size, impaired proliferation and a complete developmental arrest (**Chapter 2**). In contrast, in *Lck-Cre Ctcf$^{f/f}$* mice, TCRβ^{+} DN3 cells have the capacity to increase their cell size and proliferate at the developmental progression of TCRβ^{-} DN3E to TCRβ^{+} DN3L cells (**Chapter 3**). However, from the DN3L-to-ISP stage, CTCF-deficient cells become smaller and less proliferative than wild-type cells, and although CTCF-deficient DP cells are observed in *Lck-Cre Ctcf$^{f/f}$* mice, differentiation from the ISP-to-DP stage is strongly impaired. This does not necessary reflect that CTCF plays and intrinsically different role during B- and T-cell development and can be simply due to different kinetics of *Ctcf* deletion or CTCF protein depletion in *mb1-cre Ctcf$^{f/f}$* and *Lck-Cre Ctcf$^{f/f}$* mice. In this context, *Ctcf* deletion was monitored in *mb1-cre Ctcf$^{f/f}$* and *Lck-Cre Ctcf$^{f/f}$* mice by analysis of *LacZ* expression, a reporter gene present in the *Ctcf$^{f/f}$* allele. In the B-cell lineage, deletion of *Ctcf$^{f/f}$* alleles by *mb1-cre* occurs earlier and is more complete (**Chapter 2**), when compared to *Lck-Cre*-mediated *Ctcf$^{f/f}$* deletion in the T-cell lineage (**Chapter 3**). Further elucidation on the role of CTCF in T-cell proliferation came from *in vitro* studies with peripheral CD4 and CD8 T cells from *CD4-Cre Ctcf$^{f/f}$* mice described in **Chapter 4**. TCR-stimulation of CTCF-deleted peripheral T-cells with plate-bound anti-CD3 and anti-CD28 antibodies results in severely impaired cell size increase, proliferation and upregulation of the activation markers CD25 and CD69. Interestingly, these defects can be rescued to a great extent when TCR-proximal signalling is circumvented with PMA and ionomycin, although cell recovery is still reduced when compared with wild-type cultures. These results suggest that CTCF controls the expression of genes encoding for proteins involved in TCR-proximal signalling events required for full T-cell activation. Importantly, they show that CTCF is not absolutely required for T-cell proliferation. In line with this, *in vitro* proliferation of γδ T-cells is completely CTCF-independent (**Chapter 3**).

Changes in the expression levels of several genes may contribute to the defects observed in CTCF-deleted cells. In **Chapter 2** we performed genome-wide gene expression profiling of B-cell progenitors from *mb1-cre Ctcf$^{f/f}$* and wild-type mice, to more comprehensively analyze changes in gene activity regulated by CTCF. We identified around 200 genes with misregulated transcription in CTCF-deficient B-cell progenitors. Some of these genes code for proteins with biological functions such as

signal transduction, proliferation, survival or metabolism. Dysregulated expression of these genes may explain proliferation, survival and differentiation defects in CTCF-deficient B- and T-lymphocytes. A remarkable example is the *Gimap4* gene, which we also showed to be upregulated in *Lck-Cre Ctcf$^{f/f}$* DP cells by RQ-PCR (**Chapter 3**). Gimap4 is a member of a novel protein family of putative small GTPases called Gimap/Ian (GTPase of the immunity associated protein family, also termed immune-associated nucleotide-binding proteins), previously implicated in thymocyte development, survival of T lymphocytes and T helper cell differentiation[3]. *Gimap4* expression is detected upon pre-TCR signalling, TCR-mediated positive selection and in peripheral T- and B-cells[4-6]. Gimap4 is a lymphocyte signalling molecule, which GTPase activity is regulated by Calmodulin (Calcium modulated protein) binding and phosphorylation mediated by PKC[5, 7]. Gimap4 seems to be dispensable for T cell development but is associated with increased apoptosis in peripheral T-cells[5, 8]. Thus, upregulated *Gimap4* expression could contribute to survival defects in CTCF-deficient B- and T-lymphocytes. Eight functional mouse *Gimap* genes cluster on chromosome 6 spanning a region of ~140 Kbp[9]. Interestingly, we also detected upregulated expression of *Gimap3* and *Gimap6* in CTCF-deficient B-cell progenitors micro-array analysis described in **Chapter 2**. *Gimap3* expression is increased during the transition from DP into CD4$^+$ or CD8$^+$ SP thymocytes and was shown to support positive selection of SP CD4$^+$ and CD8$^+$ T cells[8]. In rats, *Gimap6* expression is the highest in DP thymocytes but its role in T-cells remains to be investigated[10]. It would be of interest to study if CTCF is a direct regulator of *Gimap* gene cluster expression and the significance of upregulated *Gimap4*, *Gimap3* and *Gimap6* expression in the phenotype observed in CTCF-deleted B- and T-cells.

Cell-type-specific changes in gene expression may also explain the proliferation and differentiation defects observed in B- and T-cells deleted for CTCF. For example, β-selected cells from *Lck-Cre Ctcf$^{f/f}$* mice have highly increased expression of the cyclin-CDK inhibitor encoding genes *p21* and *p27*, while in CTCF-deficient mouse embryonic fibroblasts (MEFs) expression of *p21*, but not of *p27*, is increased (**Chapter 3**). In *mb1-cre Ctcf$^{f/f}$* B-cell progenitors we do not find misregulated expression of *p21* or *p27* (**Chapter 2**). Previously it has also been shown that in WEHI 231 immature B-cells, reduction of CTCF levels decreased rather than increased the expression of *p21* and *p27* [11]. This suggests that CTCF function is context dependent. In fact, ISP cells are highly proliferating as a result of pre-TCR stimulation, whereas crosslinking of the BCR on WEHI 231 immature B-cells results in cell-cycle arrest and apoptosis, which could explain a different effect of CTCF in *p21* and *p27* gene expression. CTCF binds to two adjacent sites in the first intron of *p21*, but no correlation between CTCF binding and *p21* expression is observed in T-cells (**Chapter 3**). Therefore, whether CTCF is a direct

transcriptional regulator of *p21* and/or *p27* remains to be seen. It is possible that CTCF cooperates with distinct transcription factors for positive or negative regulation of *p21* and/or *p27* expression in different cell-types. Similarly, expression of genes required for cell differentiation might depend on the cooperation between CTCF and lineage-specific transcription factors. In line with this, ISP and DP cells from *Lck-Cre Ctcf$^{f/f}$* mice express low levels of TCRβ (**Chapter 3**) and CTCF binding at the *Tcrβ* and *Tcrα/δ* loci has been previously reported[12-14]. The T-cell transcription factor Gata3 binds to *Tcr* enhancer elements and plays an important role in the regulation of T-cell-specific expression of the *Tcrβ* and *Tcrα/δ* loci[15, 16]. In **Chapter 5** we found that overexpression of Gata3 resulted in increased occupancy of Gata-3-binding sites in the *Tcrα* enhancer region and enhanced TCR expression. It would be interesting to investigate whether Gata3 regulates *Tcrβ* and *Tcrα/δ* loci expression in a CTCF-dependent fashion.

In conclusion, global role of CTCF in regulating gene expression causes difficulties to the identification of specific CTCF target genes underlying B- and T-lymphocyte proliferation and differentiation defects. Further complexity arises from the fact that some of these genes may be regulated in a cell-type-specific manner. A comparative analysis of genome-wide gene expression profiling studies in different CTCF-deleted cell populations might be elucidative. In this context, γδ T-cells are unique in the sense that CTCF is not required for their development or *in vitro* proliferation (**Chapter 3**), which makes this cell population a prime candidate for genome-wide gene expression analysis.

CTCF regulation of *Ig* loci gene rearrangements

It is well-established that antigen receptor loci undergo large-scale contraction by chromatin looping as part of the mechanisms regulating V(D)J recombination[17-22]. This ensures that the multiple antigen receptor gene segments scattered over a large genomic region encounter each other with the appropriate frequencies. Thus far, only a few molecular components have been identified that modulate antigen receptor loci topology. These include Pax5, YY1 and Ikaros, which have been shown to control *Igh* locus contraction and distal V_H-to-DJ_H recombination[23-25]. However, the precise mechanism by which these factors act to regulate chromatin structure remains elusive and other proteins certainly collaborate with them to modulate chromatin structure.

The Igh locus

In **Chapter 2** we found that $CD2^-$ cytoplasmic $Ig\mu^+$ pre-B cells generated in *mb1-cre Ctcf$^{f/f}$* mice have an apparently normal V_H family repertoire and no obvious recombination bias to proximal V_H gene segments. The question arises of whether CTCF protein is

completely absent when *Igh* recombination, in particular V_H-to-DJ_H joining, is initiated at the pro-B cell stage[20]. Analysis of *LacZ* expression in B-cell lineage cells of *mb1-cre Ctcf$^{f/f}$* mice shows that *Ctcf* deletion is efficiently induced and almost complete at the pro B-cell stage (**Chapter 2**). As for CTCF protein levels, western blot analysis clearly demonstrates that CTCF is essentially undetectable in $B220^+CD19^+CD2^-$ cells (pro-/pre-B cell fractions) from *mb1-cre Ctcf$^{f/f}$* mice (**Chapter 2**). Importantly, the analysis of CTCF protein levels in total pro-/pre-B cell fractions does not lead to an underestimation of the actual amounts of CTCF protein in pro-B cells since cytoplasmic $Ig\mu^+$ pre-B cells only represent a minor fraction of $B220^+CD19^+CD2^-$ cells in *mb1-cre Ctcf$^{f/f}$* mice (~25%), probably due to strong proliferation and/or survival defects (**Chapter 2**). Moreover, *mb1-cre*-mediated *Yy1* gene deletion has previously provided evidence that YY1 is critical for *Igh* locus contraction and distal V_H-to-DJ_H rearrangement at the pro-B cell stage[24]. Based on these findings, we propose that CTCF is not essential for distal V_H-to-DJ_H rearrangement at the pro-B cell stage, which is further supported by the binding pattern of CTCF in the *Igh* locus. A total of 53 CTCF binding sites span the entire V_H region, although CTCF occupancy is mostly similar in pro-B and pre-B cells[26] (**Fig.1A**). Thus, it is unlikely that CTCF alone accounts for changes in *Igh* locus topology that localize distal V_H regions within close proximity of DJ_H elements occurring in pro-B cells[20]. However, we cannot formally exclude a role for CTCF in *Igh* locus conformation. It is possible that CTCF determines the establishment of a higher-order chromatin structure at the *Igh* locus before CTCF deletion in *mb1-cre Ctcf$^{f/f}$* mice is complete. For example, CTCF could epigenetically mark the *Igh* locus at the pre-pro-B cell stage and thereby determine the recruitment of other proteins directly involved in changing *Igh* locus topology in pro-B cells. In line with this, the recruitment of cohesin to CTCF binding sites at the *Igh* locus is largely pro-B cell specific[26] and CTCF capacity to direct histone modification was previously observed at the mouse *β-globin* locus[27]. Further experiments are required to investigate if earlier deletion of CTCF affects *Igh* locus rearrangement.

Other CTCF binding sites identified in the *Igh* locus locate in the V_H-D_H intergenic region and at the 3' regulatory region (3'RR)[26, 28]. A recent study has demonstrated that CTCF binding sites in the V_H-D_H intergenic region have enhancer-blocking activity and mark a sharp decrease in Eμ-dependent antisense transcription over the D_H-J_H region[29]. Therefore, it was proposed that CTCF binding to the V_H-D_H intergenic region prevents chromatin opening extending into the independently activated V_H chromatin domain. Interestingly, fluorescence in situ hybridization (FISH) experiments suggest that CTCF binding sites in the V_H-D_H intergenic region and at the 3'RR are in close spatial proximity during the pre-pro-B cell stage, when D_H-to-J_H rearrangement occurs, thereby forming a DJ_H chromatin loop[20]. Based on these findings it is tempting to speculate that

CTCF is implicated in the control of ordered V(D)J recombination at the *Igh* locus. It is generally accepted that V_H-to-DJ_H rearrangement is the step that is regulated in the context of lineage-specificity and allelic exclusion. Accordingly, D_H-to-J_H but not V_H-to-DJ_H rearrangements can be detected in T cells[30] and D_H-to-J_H joining occurs on both *Igh* alleles in developing pro-B cells[31]. It would be interesting to investigate whether CTCF is involved in the regulation of lineage-specificity and allelic exclusion of the *Igh* locus V(D)J recombination process. Finally, CTCF binding sites at the 3'RR also show enhancer-blocking activity[28] and within the 5' flank of the *Igh* locus a binding motif for CTCF maps to a constitutive hypersensitive site, but CTCF occupancy or enhancer-blocking activity has not been tested[32]. Interestingly, genome-wide gene expression

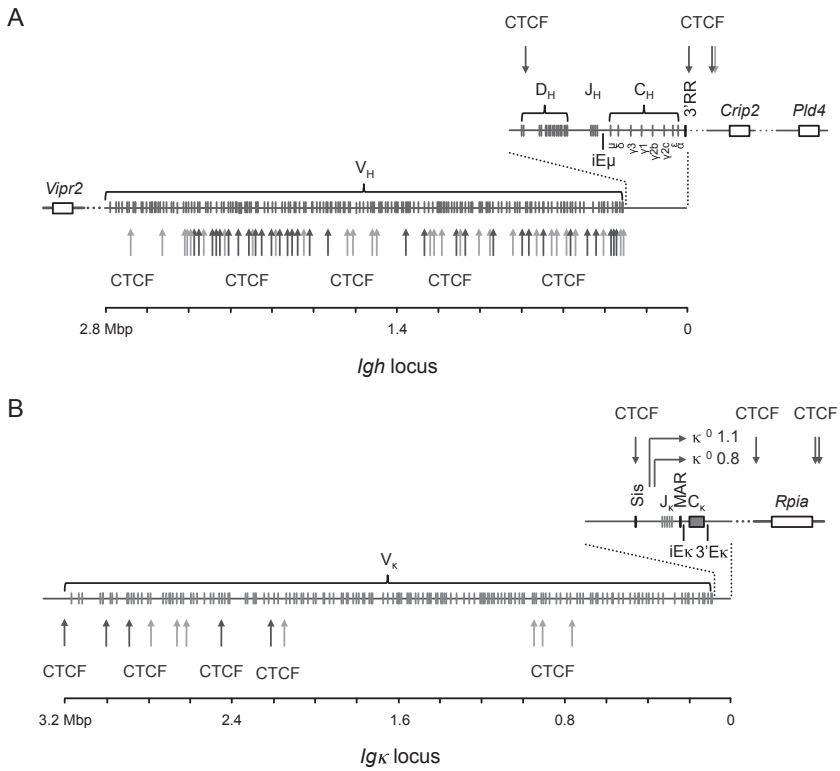


Figure 1. Schematic representation of CTCF binding sites in the mouse *Igh* and *Igκ* loci. CTCF binding in the *Igh* and *Igκ* loci have previously been described in different B-cell lineage subsets (pro-B and pre-B cells)[26]. Black arrows represent CTCF binding sites present in both pro-B and pre-B cells. Grey arrows represent CTCF binding sites present in: (**A**) only in pro-B cells or pre-B cells, (**B**) only in pre-B cells. CTCF binds to the V_H-D_H or V_κ-J_κ intergenic region and the 3' and 5' flanks of both the *Igh* and *Igκ* loci. The CTCF binding site indicated between the *Igκ* locus and the *Rpia* gene was not reported by Degner *et al* [26] but is detected by CTCF ChIP-Seq in mouse I11 erythroid cells (R. Stadhouders and E.Soler, personal communication).

profiling of B-cell progenitors from *mb1-cre Ctcf$^{f/f}$* mice show upregulated expression of genes located in the vicinity of the *Igh* locus – *Vipr2*, *Pld4* and *Crip2* (**Chapter 2**). Thus, CTCF binding sites flanking the *Igh* locus probably function to protect neighboring sequences from recombination events or accessibility changes associated with *Igh* locus recombination.

The Igκ locus

Results described in **Chapter 2** support a role for CTCF-mediated enhancer-blocking activity between the iEκ and 3'Eκ enhancers and the $V_κ$ region or genomic regions outside the *Igκ* locus. In small pre-B cells from *mb1-cre Ctcf$^{f/f}$* V_H81X transgenic mice we observe (i) increased proximal $V_κ$ and reduced $J_κ$ germline transcription ($κ^0$0.8 and $κ^0$1.1), (ii) increased recombination to proximal $V_κ$ gene segments, although recombination to distal $V_κ$ gene segments still occurred, and (iii) increased interactions of the iEκ and 3'Eκ enhancers with the proximal $V_κ$ region and with genomic elements outside the *Igκ* locus, although iEκ and 3'Eκ enhancers interactions with distal $V_κ$ gene segments also occurred. Nevertheless, one criticism we could consider in our findings is the fact that CTCF-deletion likely has an impact in pre-B cells viability, which can be critical if we take into account that the *Igκ* locus can undergo multiple rounds of $V_κ$-to-$J_κ$ recombination. In this view, CTCF-deficient pre-B cells would get only one opportunity of $V_κ$-to-$J_κ$ recombination and these primary rearrangements would favor proximal $V_κ$ gene segments. In contrast, longer-lived wild-type pre-B cells may undergo several rounds of $V_κ$-to-$J_κ$ recombination using progressively more distal $V_κ$ to rearrange with more downstream $J_κ$ gene segments. However, crosses of *mb1-cre Ctcf$^{f/f}$* V_H81X transgenic mice with mice carrying the anti-apoptotic Eμ-Bcl2 transgene demonstrate that increased survival cannot restore pre-B cell numbers and thus, the developmental arrest observed at this stage cannot be explained by defective survival only (C.R.A., unpublished). Moreover, when analyzing $V_κ$-to-$J_κ$ recombination data (**Chapter 2**) for joints using $J_κ$1, by definition the $J_κ$ gene segment used in primary recombination events, we still observe increased usage of proximal $V_κ$ gene segments in small pre-B cells from *mb1-cre Ctcf$^{f/f}$* V_H81X transgenic mice. Another major concern in our analysis is the use of RAG-sufficient *mb1-cre Ctcf$^{f/f}$* V_H81X transgenic mice. Particularly, one could argue that increased interactions of proximal $V_κ$ gene segments with iEκ and 3'Eκ enhancers observed in 3C assays (**Chapter 2**) simply results from their increased recombination to $J_κ$ gene segments and subsequent genomic proximity to iEκ and 3'Eκ enhancers. This would however not be supported by the finding that interactions between the 3'Eκ enhancer and the $J_κ$ gene segments is reduced to background levels in *mb1-cre Ctcf$^{f/f}$* V_H81X transgenic small pre-B cells (**Chapter 2**). Further 3C experiments using *mb1-*

cre Ctcf$^{f/f}$ V_H81X transgenic small pre-B cells on a RAG-deficient background should definitively elucidate on this.

In a striking parallel to the *Igh* locus, strong CTCF binding sites locate to the V_κ-J_κ intergenic region and flank the *Igκ* locus[26] (**Fig. 1B**). Based on our findings and the work by Featherstone *et al*[29] we propose that CTCF binding sites in the V_κ-J_κ intergenic region contain enhancer-blocking activity that limit interactions between the iEκ and 3'Eκ enhancers and the promoters of proximal V_κ gene segments (**Fig. 2**). Similarly, CTCF binding sites flanking the *Igκ* locus likely prevent inappropriate communication between the iEκ and 3'Eκ enhancers and genomic regions outside the *Igκ* locus. A remarkable example is the neighboring *Rpia* gene, which transcriptional unit is contacted by both the iEκ and 3'Eκ enhancers in *mb1-cre Ctcf*$^{f/f}$ V_H81X transgenic small pre-B cells, consistent with upregulated *Rpia* expression found in genome-wide gene expression profiling of B-cell progenitors from *mb1-cre Ctcf*$^{f/f}$ mice (**Chapter 2**).. Interestingly, CTCF binding sites in the V_κ-J_κ intergenic region locate to the recombination silencer Sis[26], which deletion results in reduced levels of $\kappa^0$0.8 and $\kappa^0$1.1 germline transcripts (**Fig. 2**), although transcription from germline proximal V_κ gene segments was not assessed[33]. Sis has been demonstrated to recruit one *Igκ* allele to pericentromeric heterochromatin by association with Ikaros, a repressor protein that binds to heterochromatin[33]. It would be interesting to test if Sis-bound CTCF cooperates with Ikaros in the pericentromeric heterochromatin recruitment of *Igκ* alleles, for example by FISH experiments. Further experiments are required to investigate whether mutation of CTCF binding sites in the V_κ-J_κ intergenic region and/or flanking the *Igκ* locus mimic the phenotype observed for CTCF-deficient small pre-B cells.

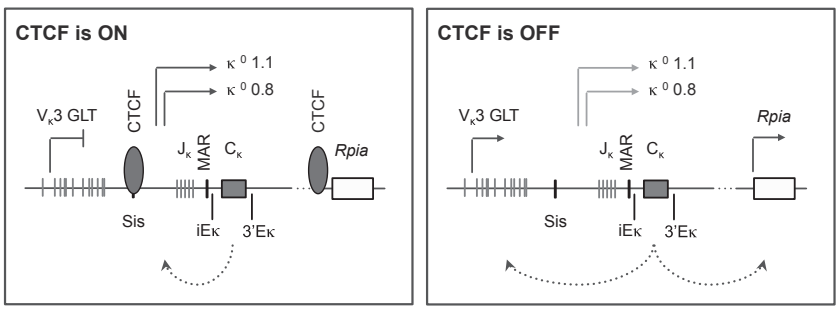


Figure 2. Model for CTCF function at the *Igκ* locus. CTCF binding at the V_κ-J_κ intergenic region and the 3' flank of the *Igκ* locus prevent inappropriate communication between the *Igκ* enhancers and the proximal V_κ gene segments (V_κ3) or the juxtaposed house-keeping gene *Rpia* (*left*). In the absence of CTCF, increased interactions (dashed arrows) between *Igκ* enhancers and the proximal V_κ gene segments or the juxtaposed house-keeping gene *Rpia* are observed (*right*). This results in increased V_κ3 germline transcription (GLT) and *Rpia* expression, and reduced levels of $\kappa^0$1.1 and $\kappa^0$0.8 germline transcripts (grey arrows).

Enhancer-blocking CTCF-driven insulation usually occurs through the formation of long-range chromatin interactions between CTCF-bound insulators[34-36]. In **Chapter 2** we hypothesized that (i) CTCF binding sites flanking the *Igκ* locus and in the $V_κ$-$J_κ$ intergenic region partition off the locus into two main chromatin loop domains, separating the $V_κ$ region from the $J_κ$-$C_κ$ cluster containing the iEκ and 3'Eκ enhancers, and (ii) dynamic scanning of the $V_κ$ region for recombination depends on further regulatory sub-loops bringing $V_κ$ gene segments into close spatial proximity with the $J_κ$-$C_κ$ cluster. This model is strongly supported by a recent study where the *in vivo* binding pattern of RAG1 and RAG2 proteins to antigen receptor loci was assessed[37]. This study demonstrates that binding of RAG proteins occurs at small regions of highly active chromatin encompassing J (and where present J-proximal D) gene segments of *Igκ*, *Igh*, *Tcrα* and *Tcrβ* loci, referred to as recombination centers. Recombination centers are formed in a lineage- and developmental stage-specific manner and are thought to coordinate V(D)J recombination by providing a discrete site within which V gene segments compete for stable capture by RAG proteins. In **Chapter 2** we found that recombination and iEκ and 3'Eκ enhancers interactions to distal $V_κ$ gene segments occur in the absence of CTCF, which suggests that CTCF is largely dispensable for distal $V_κ$ gene segments to contact with the $J_κ$-$C_κ$ cluster containing the iEκ and 3'Eκ enhancers (or recombination center). Only 11 CTCF binding sites were identified in the $V_κ$ region and the majority appeared to be pre-B cell specific[26]. It is possible that CTCF cooperates in a redundant fashion with lineage-specific transcription factors bound to $V_κ$ promoters (such as, Ebf1 or OcaB[38, 39]) or to iEκ and/or 3'Eκ enhancers (for example, E2A, Pax5 or Irf4[40-52]) for cell-type-specific regulation of chromatin looping in the $V_κ$ region. In line with this, a recent study has demonstrated coordinate binding of E2A and CTCF to a small subset of *cis*-regulatory elements genome-wide, which raises the possibility that E2A and CTCF act in concert to modulate long-range chromatin structure[53]. Further experiments using 3C assays are required to identify CTCF-based chromatin contacts in the *Igκ* locus in rearranging small pre-B cells and non-Igκ-expressing cells. It would also be interesting to study in CTCF-deficient small pre-B cells the possible additional effects of lineage-specific transcription factors deletion or genetic disruption of their target sequences on the structural organization of the *Igκ* locus. Alternatively, CTCF can function to direct local histone modifications at the *Igκ* locus[27], and thereby regulated RAG protein targeting in the $J_κ$ region. ChIP experiments to determine patterns of histone modifications in the *Igκ* locus in CTCF-deficient small pre-B cells would be required to elucidate this.

Taken together, our findings suggest that CTCF is not absolutely required for distal V-to-(D)J rearrangements at *Ig* loci in rearranging B-cells. Therefore, large-scale reorganization

of the chromatin fiber that brings distal V gene segments into close proximity of (D)J elements might not be dependent on CTCF. CTCF may predominantly function as an enhancer-blocking protein that orchestrates proper functional communication between enhancers and promoters within the *Ig* loci, while limiting the actions of enhancers elsewhere in the genome. Enhancer-blocking activity mediated by CTCF possibly evolved to have distinct functions in the *Igh* and *Igκ* loci – control of ordered V(D)J rearrangement at the *Igh* locus and limit of proximal $V_κ$ gene segment usage at the *Igκ* locus. Interestingly, proximal $V_κ3$ family members usage has previously been associated with the development of auto-reactive B1-cells[54], while in the *Igh* locus proximal V_H7183 family members are frequently used[55].

CTCF regulation of T helper cytokine gene expression

During T helper cell differentiation, coordinate regulation of cytokine gene expression depends on long-range chromatin loops bringing distal *cis*-regulatory elements into intimate contact with cytokine genes[56-59]. Both T helper cell lineage-specific transcription factors and Stat proteins have been implicated in the control of these intrachromosomal interactions[56-59]. However, our current understanding of the mechanisms underlying cell type-specific differences in higher-order chromatin structure at cytokine gene loci and their contribution to gene expression is far from complete.

The Ifng locus and Th1 lineage commitment

In **Chapter 3** we found ~50% reduction in IFN-γ-producing cells when naïve $CD4^+$ T cells from *CD4-Cre Ctcf$^{f/f}$* mice are cultured in Th1-polarized conditions. The mechanism for reduced IFN-γ production in the absence of CTCF has been illuminated by a recently reported study. This study demonstrates that T-bet promotes *Ifng* expression in part by facilitating CTCF binding and chromatin looping at the *Ifng* locus[59]. Particularly, CTCF acts to establish a Th1-cell specific *Ifng* locus architecture, which helps to drive the juxtaposition of T-bet-binding enhancers and the flanking CTCF-binding elements to *Ifng* and to promote *Ifng* expression (**Fig. 3**). Another study in human Th1 cells demonstrated that cohesins form the topological and mechanistic basis for CTCF-mediated chromatin looping at the *IFNG* locus[60]. Consistently, we also observed reduced IFN-γ production in CTCF-deficient $CD8^+$ T-cell cultures (C.R.A., unpublished). Given that in $CD8^+$ T-cells T-bet acts in concert with other, highly homologous, T-box transcription factor called Eomes to promote IFN-γ production[61-63], it would be interesting to investigate if CTCF cooperates with T-bet and/or Eomes for *Ifng* locus architecture in $CD8^+$ T-cells.

In all vertebrates except rodents, the *IFNG* gene is not clustered with other co-expressed cytokines and the nearest upstream genes are *IL26* and *IL22*, which are

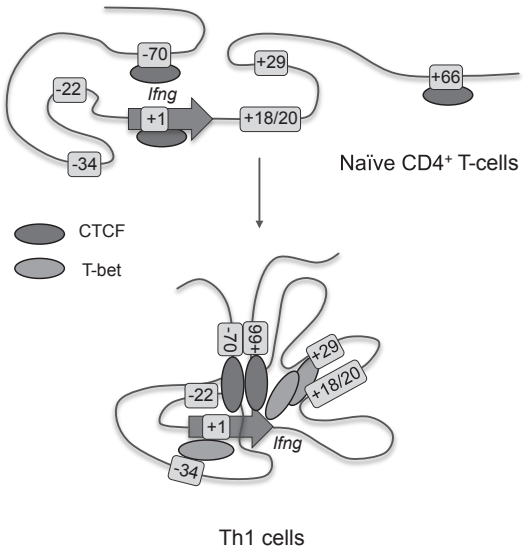


Figure 3. Model for CTCF-mediated higher-order chromatin conformation of the *Ifng* locus. In naive CD4$^+$ T-cells, CTCF is bound primarily at hypersensitive site (HS) -70 and T-bet is not bound. Upon Th1 cell differentiation, T-bet binds to the *Ifng* promoter and to the conserved non-coding sequence (CNS) -34, CNS+18/20, and CNS+29 enhancers, and CTCF binds strongly at HS +1 (in intron 1 of *Ifng*) and at HS +66 in addition to HS -70. This binding contributes to and is required for the juxtaposition of each of these distal regulatory elements to *Ifng* and its promoter. In this active locus conformation, the CNS -34 enhancer is in close proximity to *Ifng* gene. Figure adapter from[59]. **See Appendix for full-color figure.**

mainly expressed by Th17 cells. In rodents, complex structural rearrangements resulted in near complete deletion of *IL26*, except for a distal regulator element located ~70 Kbp upstream of *Ifng*[64]. The mouse -70 Kbp region exhibits both barrier and enhancer-blocking insulator activity and CTCF occupancy is detected in naïve, Th1 and Th2 CD4$^+$ T-cells[59]. Interestingly, in **Chapter 3** we observed a small population of IFN-γ$^+$ cells in CTCF-deficient Th17 cultures suggesting that binding of CTCF to the -70 Kbp region insulate the *Ifng* locus from the neighboring *Il22* gene. Expression of IFN-γ can result in impaired differentiation of Th17 cells[65, 66], although *in vitro* this might be masked by the presence of IFN-γ neutralizing antibodies in Th17-polarizing culture conditions. It would be interesting to investigate whether differentiation of Th17 cells in *CD4-Cre Ctcf$^{f/f}$* mice is impaired in vivo, for example upon induction of Th17-mediated inflammatory or autoimmune disorders, such as inflammatory bowel disease (IBD), experimental autoimmune encephalomyelitis (EAE) or collagen induced arthritis (CIA). Similarly, whether *Il22* expression is upregulated in CTCF-deficient Th1 cultures and impairs Th1 polarization remains to be tested.

The Th2 cytokine locus and Th2 lineage commitment

Results described in **Chapter 3** support a critical role for CTCF in the control of Th2 cytokine gene expression. We found that transcription of *Il4*, *Il5* and *Il13* is strongly reduced when naïve CD4$^+$ T cells from *CD4-Cre Ctcf$^{f/f}$* mice are cultured in Th2-polarizing conditions. Consistent with a Th2 cytokine defect, *CD4-Cre Ctcf$^{f/f}$* mice have reduced serum levels of the IL-4 dependent isotypes IgG1 and IgE. Importantly, in CTCF-deficient Th2 cultures, cells were polarized towards the Th2 lineage as substantiated by induction of the key transcriptional regulators Gata3 and Satb1 and low expression of T-bet and Stat4. Based on these findings we propose that CTCF is essential for Gata3- and Satb1-dependent regulation of Th2 cytokine gene expression. However, the mechanisms by which CTCF might facilitate Th2 cytokine production remain largely elusive.

We identified four constitutive CTCF binding sites (CBS) in the mouse Th2 cytokine locus (**Chapter 3**): two sites upstream of *Il5* (CBS-6 and CBS-7) and one downstream of *Il4* within *Kif3a* (CBS-1) flanking the Th2 cytokine locus and one site in the intergenic region between *Il13* and *Il4* (CBS-3) located to the DNaseI hypersensitive site Hss3, which is of unknown function[67]. Previous studies have shown that both Gata3 and Stat6 are required for the establishment and/or maintenance of a “poised” chromatin configuration in the Th2 cytokine locus, where cytokine gene promoters and the LCR come into close spatial proximity[56, 57]. Further changes to this conformation are induced upon expression of Satb1, which mediates the formation of a more densely looped, transcriptionally active chromatin structure at the Th2 cytokine locus[58]. One possible explanation for the Th2 cytokine expression defect observed in CTCF-deficient Th2 cells is that in the absence of CTCF the recruitment of key transcription factors for Th2 development, such as Gata3, Stat6, Satb1, c-Maf or Irf4, to the Th2 cytokine locus is impaired (**Fig. 4**). Indeed, one of the CTCF binding sites we identified (CBS-3) is located near to CNS-1, which has been shown to bind the C-terminal Zn-finger of Gata3 and to be critical for Th2 cytokine expression[67, 68]. Preliminary experiments using ChIP assay suggested that CTCF deficiency in Th2 cells does not result in decreased occupancy of Gata3 at the Th2 cytokine locus in several Gata3 binding sites (C.R.A., unpublished). Another possibility is that CTCF organizes higher-order chromatin conformation at the Th2 cytokine locus required for transcription of *Il4*, *Il5* and *Il13* genes. This hypothesis can be tested using 3C-type experiments to interrogate Th2 cytokine locus interactions in CTCF-deficient and wild-type naïve, Th1 and Th2 CD4$^+$ T-cells. For instance, upstream and downstream CTCF-binding elements could cooperate with the CTCF-binding element in the *Il13-Il4* intergenic region to help approximate Th2 cytokine gene promoters and *cis*-regulatory elements (such as the LCR or interposed enhancers) during Th2 differentiation. In this context, it would be interesting to investigate if similarly

to the *Ifng* locus[59], Th2 cell-specific CTCF binding and chromatin looping also occurs at the Th2 cytokine locus. It also remains to be tested whether CTCF has a role in the maintenance of a transcriptionally active chromatin structure at Th2 cytokine locus. Further experiments where CTCF is targeted *in vitro* once polarization to the Th2 lineage is complete (for example using RNAi or recombinant soluble Cre approaches) would elucidate this.

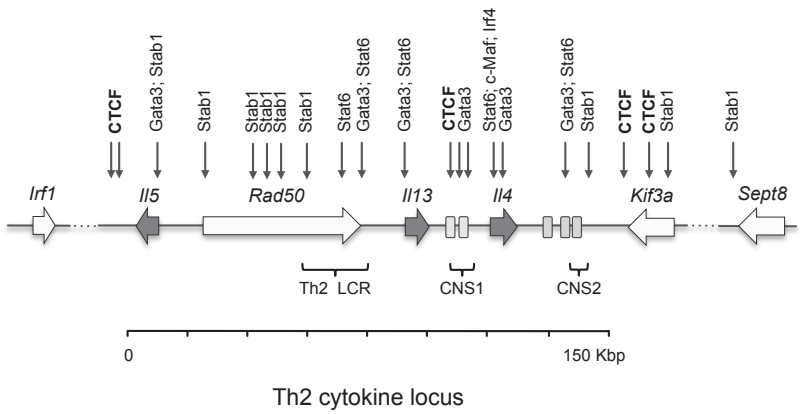


Figure 4. Schematic representation of the binding sites for CTCF and key Th2 transcription factors in the mouse Th2 cytokine locus. CTCF binding sites in the Th2 cytokine locus are interspersed with the binding sites for key Th2 transcription factors such as Gata3, Stat6, Satb1, c-Maf and Irf4. CTCF binding sites (CBS) upstream of *Il5* (CBS-6 and CBS-7) and downstream of *Il4* within *Kif3a* (CBS-1), flank the Th2 cytokine locus. CTCF binding in the intergenic region between *Il13* and *Il4* (CBS-3) locates to the DNaseI hypersensitive site Hss3 near to CNS-1. **See Appendix for full-color figure.**

During Th2 differentiation Gata3-mediated counter-regulatory mechanisms are required for the suppression of alternative T helper lineage fates. For example, Gata3 suppresses Th1 differentiation by downregulation of *Stat4* [69, 70]. Gata3 has also been shown to inhibit *Foxp3* expression during iTreg polarization[71]. In **Chapter 6** we found that enforced Gata3 expression counteracts Th17 differentiation via (i) induction of IL-4 expression and (ii) downregulation of *Stat3*, *Stat4*, *Nfatc2* and *RORγt* transcription in an IL-4-independent way. It is therefore conceivable that these transcription factors are direct Gata3 targets. One intriguing possibility to be tested is whether CTCF also cooperates with master transcription factors for the negative regulation of genes essential for alternative T helper lineage fates.

Taken together, our findings demonstrate that CTCF is required for coordinate regulation

of *Ifng* and Th2 cytokine gene expression during Th1 and Th2 cell differentiation, respectively. In particular, studies at the *Ifng* locus show that T-bet-dependent CTCF occupancy is on the basis of a Th1-cell specific *Ifng* locus architecture and *Ifng* expression[59]. Importantly, these findings demonstrate a presumably widespread mechanism of cooperation between a ubiquitous architectural factor (CTCF) and a lineage-specific transcription factor (T-bet) in cell type-specific gene regulation.

Concluding remarks

The study of lymphocyte development is important for understanding how lymphocytes acquire their antigen specificity and function, and at the same time constitute an excellent model system to study developmentally regulated gene expression. In this thesis we used a conditional gene targeting approach to study the *in vivo* function of CTCF in B- and T-lymphocytes. We provide evidence that CTCF is important for gene regulation in the lymphocyte lineage, particularly during *Ig* loci gene rearrangement and T helper cytokine gene expression. Similarly to the imprinted *H19/Igf2* locus, CTCF may predominantly function at the *Ig* loci as an enhancer-blocking protein that orchestrates proper functional communication between enhancers and promoters (**Fig. 5**). As for the regulation of T helper cytokine gene expression, the role of CTCF at the *Ifng* locus is parallel to its role in the *MHCII* locus. In both loci CTCF cooperates with cell type-specific factors (T-bet or CIITA/RFX) for enhancer-promoter long-range interactions and gene expression. It remains to be established if at the Th2 cytokine locus CTCF operates in a similar manner for coordinate regulation of gene expression, involving e.g. Gata3 and Satb1. An alternative view is that CTCF-based chromatin loops are required for the establishment and/or maintenance of a transcriptionally active chromatin structure at the Th2 cytokine locus. A similar mechanism has been predicted at the *β-globin* locus, although destabilization of CTCF contacts has no effect on gene expression. Taken together, these studies demonstrate that CTCF-mediated chromatin loops can facilitate communication between *cis*-regulatory elements but can also lead to the exclusion of interactions between them. The spatial positioning of CTCF binding sites with respect to genes and other regulatory elements and the affinity of long-range interactions dictates the types of CTCF-based chromatin loops formed and final outcome in terms of gene regulation.

Dissecting the molecular mechanisms underlying the generation of a wide repertoire of antigen receptors and the expression of effector cytokines may (i) facilitate the development of clinical strategies for the generation of a functional lymphoid compartment, able to fight a vast array of potential pathogens while maintaining

tolerance to self (for example, in the context of HSC transplantation) and, (ii) contribute to understand the etiology of Th2-mediated diseases (such as, asthma and allergy) and improve current therapies. Despite CTCF being a ubiquitously expressed transcription factor, the possibility of topical treatment with CTCF-inhibitors for pulmonary Th2 diseases such as asthma is a realistic one, paralleling e.g. inhaled corticosteroids.

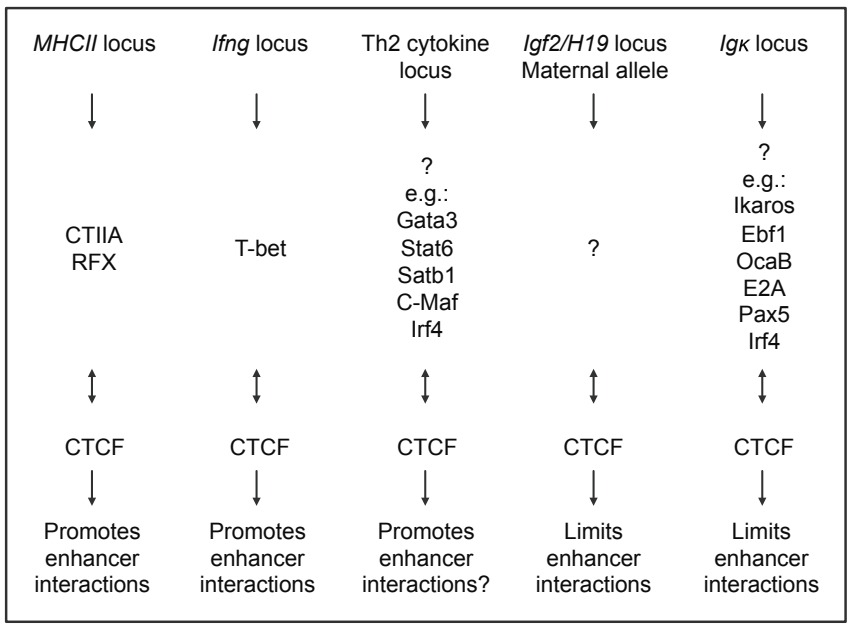


Figure 5. Comparison between CTCF function at several developmentally regulated loci. CTCF-mediated chromatin loops can facilitate the communication between *cis*-regulatory elements such as enhancers and promoters. At the human *MHCII* and mouse *Ifng* loci CTCF has been shown to cooperate with cell-type specific transcription factors (CIITA/RFX and T-bet) for long-range interactions between distal *cis*-regulatory elements and gene expression. We speculate that a similar mechanism accounts for CTCF function at the Th2 cytokine locus. At the *Igf2/H19* and *Igκ* loci, CTCF prevents inappropriate communication between enhancers and promoters, which might also depend on the collaboration between CTCF and cell type-specific transcription factors.

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Summary
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Samenvatting

SUMMARY

The adaptive arm of the immune system is responsible for highly effective immune responses against pathogens, due to the unique and virtually limitless capacity of B- and T-lymphocytes to specifically recognize antigens. This relies on the generation of a wide repertoire of antigen receptors – B-cell receptor (BCR) in B-lymphocytes and T-cell receptor (TCR) in T-lymphocytes – during lymphocyte development. Additionally, effective immune responses against pathogens depend on differentiation of naïve B- and T-lymphocytes into mature effector B- and T-cells, once they encounter their cognate antigen.

Proper lymphocyte development and differentiation into functional effector subsets depends on the expression of lineage-associated genes, which is controlled by the combinatorial activity of multiple signalling pathways and transcription factors in a complex gene regulatory network. In addition, the formation of a diverse repertoire of antigen receptors during lymphocyte development is a tightly regulated process. During this process, which is refered to as V(D)J recombination, recombination takes place between large genomic loci, containing over hundred V gene segments. Similarly, coordinate regulation of cytokine gene loci underlies the function of effector T-cells, namely T helper cells. When inappropriate it can result in immune pathologies e.g. asthma or autoimmune diseases, including rheumatoid arthritis or multiple sclerosis. At these complex loci, regulation of gene expression involves the coordinate binding of lineage-specific transcription factors to *cis*-regulatory elements, such as promoters, enhancers, silencers or LCRs, located over considerable genomic distances. Emerging evidence suggests that long-range interactions between *cis*-regulatory elements, by looping of the intervening DNA fragment, are part of the mechanism of gene regulation at these developmentally regulated loci.

The CCCTC-binding factor (CTCF) is a highly conserved and ubiquitously expressed DNA-binding protein among vertebrates, which is implicated in genome-wide organization of chromatin architecture and non-canonical gene regulatory functions. Direct evidence for CTCF-mediated higher-order chromatin structure has been reported at several developmentally regulated gene loci, including the mouse *β-globin* and *H19/Igf2* loci and the human *MHCII* locus. Cell-type-specific regulation of chromatin looping possibly arises through cooperation between the ubiquitous architectural factor CTCF and lineage-specific transcription factors. This thesis describes the results from studies aimed to unravel the function of CTCF throughout B- and T-lymphocyte development and effector T helper cell differentiation, while further exploring the role of the transcription factor Gata3 in the T cell lineage.

In **Chapters 2** and **3** we studied the *in vivo* function of CTCF during B- and T-cell

development through conditional deletion of the *Ctcf* gene, using the Cre-loxP system. In **Chapter 2** we found that CTCF is critical at the pre B-cell developmental checkpoint, probably as a regulator of genes involved in cellular proliferation and differentiation. Pre-B cells generated in *mb1-cre Ctcf$^{f/f}$* mice have apparently normal *Ig heavy chain* (*Igh*) gene recombination. In contrast, at the *Igκ* light chain locus we observed enhanced proximal $V_κ$ germline transcription, while recombination to proximal $V_κ$ gene segments is significantly increased. We demonstrated that CTCF limits interactions of the intronic (iEκ) and 3'Eκ enhancers with the proximal $V_κ$ region and prevents inappropriate interactions between these enhancers and genomic elements outside the *Igκ* locus. Based on these findings we propose a predominant role for CTCF as an enhancer-blocking protein at the *Igκ* locus. In **Chapter 3** we found that CTCF is essential for αβ T-cell development, in particular for proper proliferation of β-selected cells and their differentiation from immature single positive (ISP) to double-positive (DP) cells. In *Lck-Cre Ctcf$^{f/f}$* mice, β-selected cells are arrested in the cell cycle due to highly increased expression of the cyclin-CDK inhibitors p21 and p27. Remarkably, in *Lck-Cre Ctcf$^{f/f}$* mice γδ T-cell development appeared to occur independently of CTCF.

In **Chapter 4** we investigated the role of CTCF in T cell activation and cytokine gene expression upon polarization of *CD4-cre Ctcf$^{f/f}$* naïve CD4$^+$ T-cells into different T helper cell subsets *in vitro*. We found that CTCF-deleted peripheral T-cells have impaired activation and proliferation upon anti-CD3/anti-CD28 stimulation *in vitro*. Interestingly, these defects could be rescued to a great extent when TCR-proximal signalling was circumvented with phorbol ester (PMA) and ionomycin, suggesting that CTCF controls the expression of genes encoding for proteins involved in TCR-proximal signalling events required for full T-cell activation. Rescue of signaling cascade downstream of the TCR by PMA and ionomycin stimulation allowed *in vitro differentiation of CTCF-deleted* naïve CD4$^+$ T-cells into different T helper cell subsets. We found strongly reduced transcription of *Il4*, *Il5*, and *Il13* in Th2 polarization cultures, despite normal induction of key Th2 cytokine transcriptional regulators, Gata3 and special AT-rich binding protein 1 (Satb1). In contrast, IFN-γ production in Th1 cultures was only moderately reduced and IL-17 production in Th17 cultures was unaffected. Consistent with a Th2 cytokine defect, *CD4-cre Ctcf$^{f/f}$* mice had very low levels of IgG1 and IgE in their serum. Chromatin immunoprecipitation analysis revealed multiple CTCF binding sites in the Th2 cytokine locus. Taken together, these findings demonstrate that CTCF is essential for Gata3- and Satb1-dependent regulation of Th2 cytokine gene expression.

Gata3 expression is induced at the DP stage and has been implicated in positive selection towards the CD4 lineage. In **Chapter 5**, a comparison between gene expression profiles of DP cells from CD2-Gata3 transgenic and wild-type mice

revealed new putative Gata3 target genes that may clarify Gata3 function at the DP stage. Interestingly, overexpression of Gata3 resulted in increased occupancy of Gata-3-binding sites in the TCRα enhancer region and enhanced TCR expression. Differentiation towards Th2 lineage is critically dependent on Gata3, which has previously been shown to negatively regulate the differentiation of alternative lineage fates, such as Th1 or iTreg. In **Chapter 6**, naïve $CD4^+$ T-cells with enforced Gata3 expression were differentiated into Th17 cells *in vitro*. Gata3 was shown to support Th17 differentiation by limiting IL-2 and IFN-γ production and constrain Th17 differentiation, both through IL-4 production and by IL-4-independent mechanisms. These detailed analyzes on the molecular mechanisms underlying the role of Gata3 in the T-cell lineage represent an important step for future studies on the possible cooperation between Gata3 and CTCF in the regulation of Gata3 target genes. In particular, because parallel studies in Th1 cells have shown a presumably widespread mechanism of cooperation between CTCF and a lineage-specific transcription factor in cell type-specific gene regulation.

Finally, in the **Discussion** we consider the implications of our findings for the knowledge on the role of CTCF in B- and T-lymphocytes proliferation, survival and differentiation, particularly on *Ig* loci gene rearrangement and T helper cytokine gene expression. We identified CTCF as a critical factor that influences the usage of V_κ gene segments for recombination, contributing to the mechanism that gives nearby and remote V_κ regions equal opportunities to rearrange with J_κ elements in the *Igκ* locus. Parallel to findings in Th1 cells, we demonstrate that CTCF is essential for Th2 cytokine gene expression. We speculate that CTCF might cooperate with key Th2 transcription factors such as Gata3, in the regulation of Th2 cytokine gene expression.

Importantly, these findings contribute to our understanding of the molecular mechanisms underlying the generation of a wide repertoire of antigen receptors and the expression of effector cytokines. In the future, our findings might have implications for the development of clinical strategies for the generation of a functional lymphoid compartment, for example in the context of hematopoietic stem cell (HSC) transplantation. Moreover, they can contribute to understanding the etiology of Th2-mediated diseases, such as asthma and allergy, and improve current therapies.

SAMENVATTING

Het immuunsysteem bestaat uit twee delen, namelijk een deel dat is aangeboren en een deel dat zich aanpast aan de ziekteverwekker. Dit laatste deel van het immuunsysteem, het aanpassingsimmuunsysteem, bestaat uit B- en T-lymfocyten. Deze B- en T-lymfocyten hebben de unieke eigenschap dat ze virtueel elke ziekteverwekker, ook wel een antigen genoemd, kunnen herkennen. Hierdoor is dit deel van het immuunsysteem in staat om zeer effectieve afweerreacties tegen ziekteverwekkers uit te voeren. De B- en T-lymfocyten kunnen veel verschillende antigenen herkennen doordat elk B- en T-lymfocyt een receptor tot expressie brengt die specifiek gericht is tegen een uniek antigen. Op B-cellen (B-lymfocyt) heet deze receptor de B-cel receptor (BCR), op T-cellen heet deze receptor de T-cel receptor (TCR). Zodra B- of T-cellen hun antigen herkennen worden ze geactiveerd en differentiëren de nog naïeve B-of T-cellen respectievelijk in effector B- of T-cellen.

De vorming en differentiatie van B- en T-lymfocyten wordt aangestuurd door middel van celtype specifieke genen die op hun beurt weer worden gereguleerd door een netwerk van signaleringscascades en transcriptie factoren. Ook de vorming van het brede repertoire van BCRs en TCRs tijdens respectievelijk de B- en T-cel ontwikkeling is nauwkeurig gereguleerd. Tijdens dit proces, dat V(D)J recombinatie wordt genoemd, vindt er recombinatie plaats tussen verschillende genomische gebieden waarin meer dan honderd V-gen segmenten liggen. Ook de regulatie en activatie van de cytokine genen en loci in de effector T-cellen wordt gereguleerd door een complex netwerk van factoren. Wanneer de activiteit van deze cytokine loci niet goed wordt gereguleerd kan dat leiden tot immunologische ziektes, zoals auto-immuunziekten (waaronder reumatische artritis en multiple sclerosis) en astma.

De genen in de hierboven genoemde loci worden gereguleerd door middel van celtype specifieke transcriptie factoren die binden aan cis-regulatore elementen die over het genoom verspreid kunnen liggen. Voorbeelden van cis-regulatore elementen zijn promotoren, enhancers, silencers of locus control regions (LCR). Steeds meer waarnemingen laten zien dat de interacties tussen ver uit elkaar liggende cis-regulatore elementen essentieel zijn voor de juiste regulatie van deze genen en loci. Wanneer ver uit elkaar liggende cis-regulatore elementen een interactie/binding aangaan vormt het tussenliggende DNA een lus.

De CCCTC-binding factor (CTCF) is een eiwit dat aan DNA bind. Het eiwit is zeer geconserveerd binnen de groep van gewervelde dieren. Het CTCF eiwit komt in eigenlijk elk celtype tot expressie. CTCF is genoom-breed betrokken bij het tot stand brengen of juist tegen gaan van interacties tussen cis-regulatore elementen. CTCF reguleert de expressie van vele genen door de vorming van DNA structuren en DNA

lussen te dirigeren. Zo hebben onderzoekers laten zien dat CTCF betrokken is bij de regulatie van meerdere loci, waaronder de *β-globin* en *H19/Igf2* loci en het humane *MHCII* locus. Celtype specifieke regulatie van de vorming van DNA structuren en lussen is mogelijk door de samenwerking tussen de alomvertegenwoordigd CTCF factor en celtype specifieke transcriptie factoren. Dit proefschrift beschrijft de resultaten van studies die zijn uitgevoerd om uit te zoeken welke functie(s) CTCF uitvoert tijdens de B- en T-cel ontwikkel en gedurende de differentiatie van geactiveerde T-cellen. Tevens beschrijft dit proefschrift studies die zijn uitgevoerd om de rol van de transcriptie factor Gata3 in T-cellen verder op te helderen.

In de **Hoofstukken 2** en **3** hebben we bestudeerd welke *in vivo* functie CTCF tijdens de B- en T-cel ontwikkeling uitvoert. Dit hebben we gedaan door middel van een conditionele deletie van het *Ctcf* gen, door gebruik te maken van het Cre-loxP systeem. In **Hoofdstuk 2** vonden we dat CTCF een essentiële rol vervult op het controle punt in de ontwikkeling van de precursor-B cel (pre-B cel). CTCF reguleert dit controle punt waarschijnlijk door genen te reguleren die de celdeling en differentie aansturen. Pre-B cellen in *mb1-cre Ctcf$^{f/f}$* muizen hebben een normale recombinatie van de *immunoglobuline zware keten* (*Igh*) genen. Tevens vonden we versterkte expressie van de proximale V_{κ} steriele transcripten en een significant verhoogde gebruik van de proximale V_{κ} genen. Wij hebben laten zien dat CTCF de interacties met de intronic (iEκ) en 3'Eκ enhancers met de proximale V_{κ} gebieden remt. Tevens laat onze data zien dat CTCT onjuiste interacties voorkomt tussen deze enhancers en genomische gebieden buiten het *Igκ* locus. Op basis van deze bevindingen stellen wij de theorie voor dat het CTCT eiwit een dominante rol speelt als enhancer-blokkerend eiwit op de *Igκ* locus. De studie die is beschreven in **Hoofdstuk 3** heeft ons laten zien dat CTCF noodzakelijk is voor de αβ T-cel ontwikkeling. CTCF vervult namelijk een belangrijke rol in de celdeling van β-geselecteerde cellen en in hun differentiatie van immature enkel positieve in dubbel-positieve (DP) T-cellen. We vonden namelijk in *Lck-Cre Ctcf$^{f/f}$* muizen dat β-geselecteerde cellen geblokkeerd zijn in hun celdeling als gevolg van sterk verhoogde expressie van de cyclin-CDK remmers p21 en p27. Daarin tegen is de ontwikkeling van γδ T-cellen normaal in *Lck-Cre Ctcf$^{f/f}$* muizen wat suggereert dat de ontwikkeling van deze cellen onafhankelijk is van CTCF.

In **Hoofdstuk 4** hebben we onderzocht welke rol CTCT vervult tijdens de activatie van T-cellen. Daarnaast hebben we in T-helper cellen gekeken welke functie CTCF vervult in de expressie van de cytokine genen. Hiervoor hebben we T-helper cellen bestudeerd die *in vitro* werden gedifferentieerd vanuit *CD4-Cre Ctcf$^{f/f}$* naïve $CD4^+$ T-cellen. We vonden dat perifere T-cellen die deficiënt waren voor CTCF een verminderde activatie en celdeling hadden wanneer deze cellen *in vitro* gestimuleerd

werden met anti-CD3/anti-CD28. Opmerkelijk is dat deze defecten grotendeels konden worden opgeheven wanneer de TCR-signaleringscascade werd omzeild door middel van stimulatie met phorbol ester (PMA) en ionomycin. Dit suggereert dat CTCF de expressie reguleert van eiwitten die noodzakelijk zijn voor het tot stand brengen van de signaleringscascade achter de TCR. Het herstel van de T-cel activatie doormiddel van PMA en ionomycin zorgde ervoor dat de CTCF-deficiënte $CD4^+$ T-cellen *in vitro* wel konden differentiëren in verschillende T-helper (Th) subsets. Analyse van de transcipties van *Il4*, *Il5* en *Il13* cytokine genen in Th2 gepolariseerde kweken liet zien dat de expressie van deze cytokines sterk was verlaagd in CTCF-deficiënte Th2-cellen. Dit ondanks dat de inductie van eiwitten die essentieel zijn voor de regulatie en expressie van Th2-cytokine genen, zoals Gata3 en Satb1, normaal was in CTCF-deficiënte Th2-helper cellen. Daarin tegen was the IFN-γ productie in Th1-kweken alleen ligt verlaagd en was de productie van IL-17 in gekweekte Th17-cellen normaal. In overeenstemming met het gevonden defect in de productie van Th2-cytokines hadden de *CD4-Cre Ctcf*$^{f/f}$ muizen een verlaagde hoeveelheid IgG1 en IgE in hun serum. Immuno precipitatie van het chromatin van CTCF-deficiente $CD4^+$ T-cellen liet zien dat er meerde CTCF bindingsposities aanwezig zijn in het Th2-cytokine locus. Al deze bevindingen bij elkaar laten zien CTCF essentieel is voor de Gata3- en Satb1-afhankelijke regulatie van de expressie van de Th2-cytokine genen.

De expressie van transcriptie factor Gata3 wordt geïnduceerd op het DP stadium van de T-cel ontwikkeling. Gata3 is op het DP stadium betrokken bij de positieve selectie in de richting van de $CD4^+$ T-cellen. **Hoofdstuk 5** beschrijft de bevindingen van de vergelijking tussen de genexpressie patronen in DP cellen van CD2-Gata3 transgene en wild-type muizen. Deze genexpressie analyse heeft genen onthuld die mogelijk door Gata3 worden gereguleerd. Met deze data hopen we te begrijpen welke rol Gata3 vervult op het DP-stadium. Zo vonden we dat overexpressie van Gata3 resulteerde in verhoogde bezetting van de Gata3-bindingsites in het TCRα enhancer gebied en in een verhoogde expressie van de TCR. Eerder onderzoek heeft laten zien dat Gata3 een belangrijke factor is voor het ontstaan van T-helper subsets: het ontstaan van Th2-cellen is afhankelijk van Gata3 terwijl de vorming van Th1-en regulatore T-cellen wordt geremd door Gata3. In **Hoofdstuk 6** hebben we gekeken hoe Gata3 expressie de differentiatie van Th17-cellen reguleert door *in vitro* $CD4^+$ T-cellen met versterkte Gata3 expressie te differentiëren in Th17-cellen. De resultaten lieten zien dat Gata3 de Th17-differentiatie ondersteund door de productie van IL-2 en IFN-γ te verminderen en door Th17-differentiatie te remmen door middel van zowel de productie van IL-4 als ook door IL-4 onafhankelijke mechanismen. Deze data over de moleculaire rol van Gata3 in de T-cel ontwikkeling vormt de basis voor toekomstige studies om te onderzoeken

hoe Gata3 en CTCF mogelijk samen de expressie van genen reguleren die voor hun expressie afhankelijk zijn van Gata3. Voor Th1-cellen zijn al mogelijke interacties tussen CTCF-en Th1-specifieke transcriptie factoren beschreven die samen the Th1-differentiatie vormgeven.

Tot slot, in de **Discussie** overwegen en wegen wij de implicaties van onze bevindingen voor de kennis en de kijk op de rol van CTCF in de ontwikkeling, proliferatie, overleving en differentiatie van B-en T-cellen. Hierbij ligt het accent op de rol van CTCF in de recombinatie van de *Ig* loci genen en de expressie van de T-helper cytokine genen. We hebben ontdekt dat CTCF een kritische factor is die de recombinatie van de Vκ-gen segmenten stuurt waardoor zowel dichtbij als verliggende V_{κ}-gen segmenten een gelijke kans hebben om te recombineren met een Jκ-element in het Igκ locus. Daarnaast vonden we dat CTCF, net zoals in Th1-cellen, ook essentieel is voor de expressie van the Th2-cytokine genen. We veronderstellen dat CTCF samen met essentiële Th2- transcriptie factoren, waaronder Gata3, de expressie van de Th2-cytokine genen reguleert.

De bevindingen die staan beschreven in dit proefschrift dragen bij aan het begrijpen van de moleculaire mechanismen die de vorming van het brede repertoire van antigen receptor en de expressie van de cytokine genen reguleren. We spreken de hoop uit dat onze bevindingen in de nabije toekomst kunnen bijdragen aan de ontwikkeling van therapeutische methoden voor het maken van functionele lymfoïde populaties, bijvoorbeeld voor de transplantatie van hematopoëtische stamcellen. Daarnaast kunnen onze bevindingen bijdragen aan het verbeteren van de huidige therapieën en aan het begrijpen van de etiologie van ziektes die worden veroorzaakt Th2-cellen, zoals astma en allergieën.

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LIST OF PUBLICATIONS

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To my master student João, thanks for allowing me to talk Portuguese in the lab and being my test tube in supervision.
Marthe and Ingrid, if I would be allowed to choose roomies, I wouldn't be able to pick better

ones… that makes me realize how lucky I was to have you the last years of my PhD. You showed me the true meaning of *gezelligheid* and our DE coffees made me almost feel at home. Thank you for this and much more... I will miss you, A LOT ☺!
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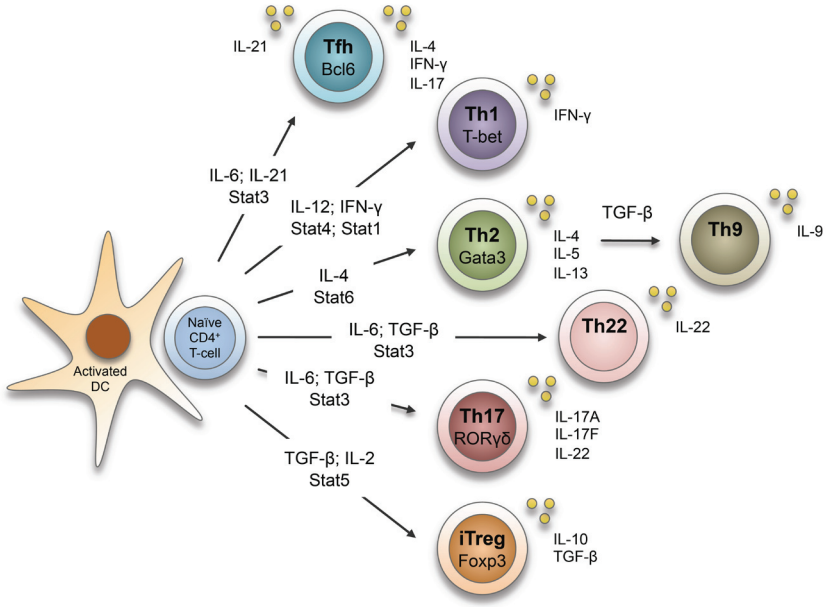
Andrea, obrigada pela amizade e pelo carinho nos difíceis momentos antes de cortar a meta!

Ao meu pai, à minha mae e a minha mana Fátima, obrigada por tudo mas principalmente por acreditarem em mim e me apoiarem incondicionalmente, apesar das imensuráveis saudades.

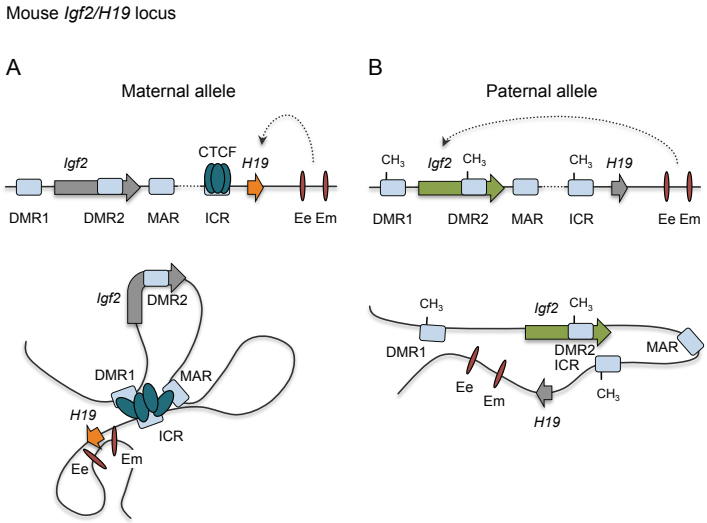
Tiago, we can do it!

Thank you,
Claudia

Appendix

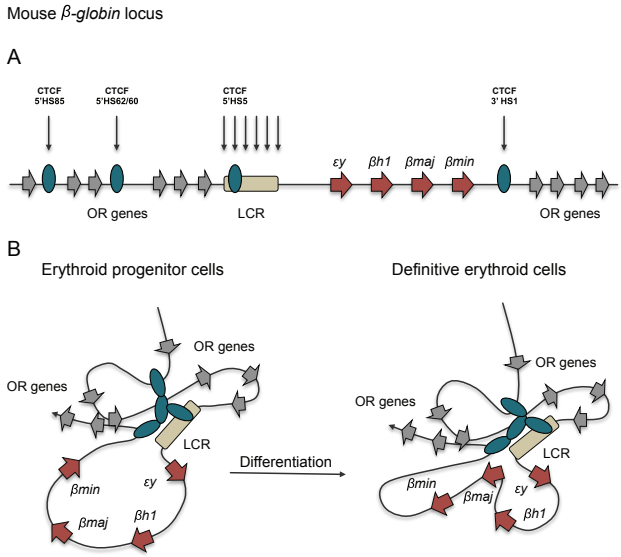


Chapter 1 - Figure 7. Differentiation of effector CD4⁺ T-cells subsets in the mouse. During activation in a particular cytokine milieu, naïve CD4⁺ T-cells may differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17, and inducible regulatory T-cells (iTregs). Different differentiation programs triggered by TCR and cytokine signalling are mainly based on the activation of Stat proteins and induction of master transcription factors that collaborate in the regulation of unique patterns of cytokine production. However, cytokine production by Th cells might be more flexible than previously though and recently T follicular helper (Tfh), Th9 and Th22 cells have been described. Whether these subsets represent distinct lineages remains to be elucidated.

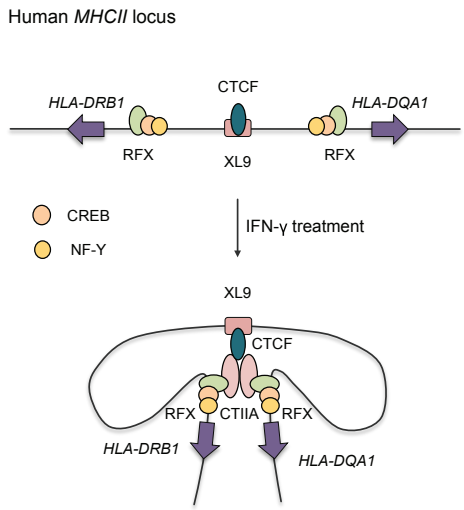


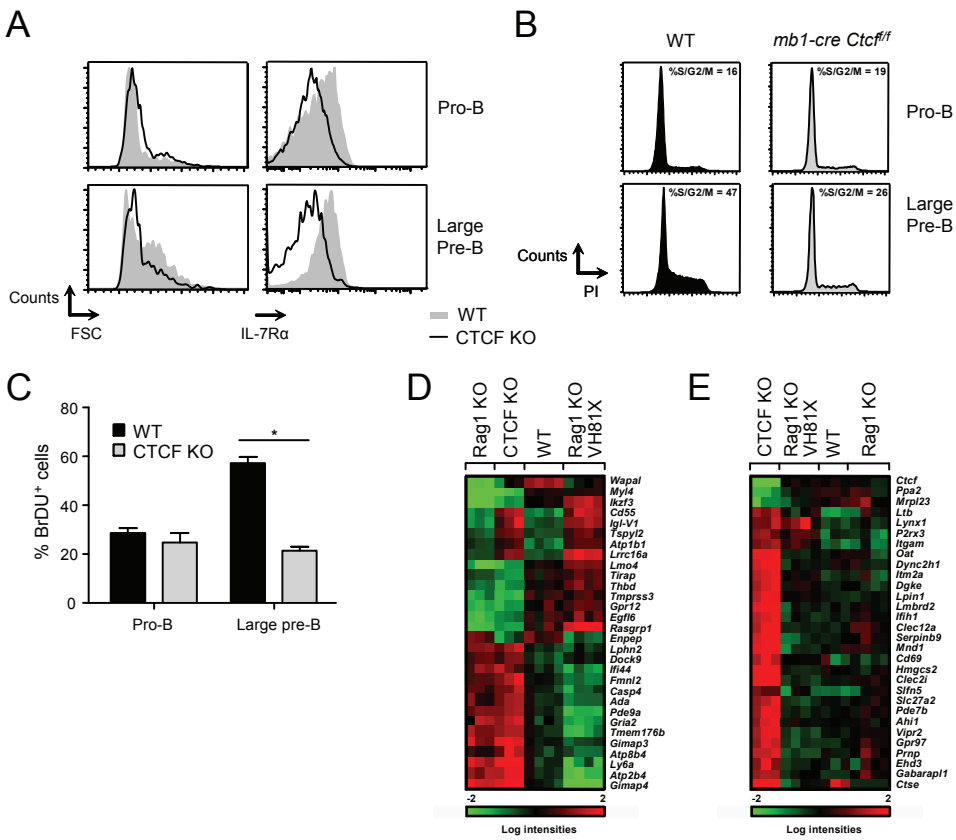
Chapter 1 - Figure 10. Schematic representation of the mouse *Igf2/H19* locus. The maternally expressed noncoding *H19* gene (**A**) is located approximately 90 Kbp downstream from the gene encoding Insulin-like growth factor 2 (*Igf2*) that is expressed exclusively from the paternal allele (**B**). The imprinting control region (ICR) ~2 Kbp upstream of *H19* contains four CTCF binding sites and is essential for the regulation of the entire locus. Differentially methylated regions (DMRs) such as DMR1 upstream of *Igf2* promoters and DMR2 within *Igf2* exon 6, act in concert to regulate reciprocal, allele-specific expression patterns from a shared set of downstream enhancers at 8 Kbp (Ee, endodermal tissue enhancer) and 25 Kbp (Em, mesodermal tissue enhancer) downstream of the H19 gene. Allele-specific patterns of CTCF binding, DNA methylation ($-CH_3$) and chromatin looping account for parent-of-origin *H19* and *Igf2* gene expression patterns during development. Figure adapter from[316].

Chapter 1 - Figure 12. Schematic representation of the human *MHCII* locus.
In the human *major histocompatibility complex class II* (*MHCII*) locus, CTCF binds to the XL9 enhancer element between two coregulated genes, *HLA-DRB1* and *HLA-DQA1*, driven by divergent promoters. Transcription factors RFX, CREB and NF-Y bind to regulatory sequences within the proximal promoter of *MHCII* genes when they are transcriptionally inactive. Interferon-γ (IFN-γ) treatment induces transcription in non-expressing cell types by upregulating the non-DNA binding coactivator CIITA, which subsequently forms an heterodimer with RFX-CREB-NF-Y-bound promoters and the CTCF-bound enhancer in parallel with *HLA-DRB1* and *HLA-DQA1* gene activation. Figure adapter from[316].

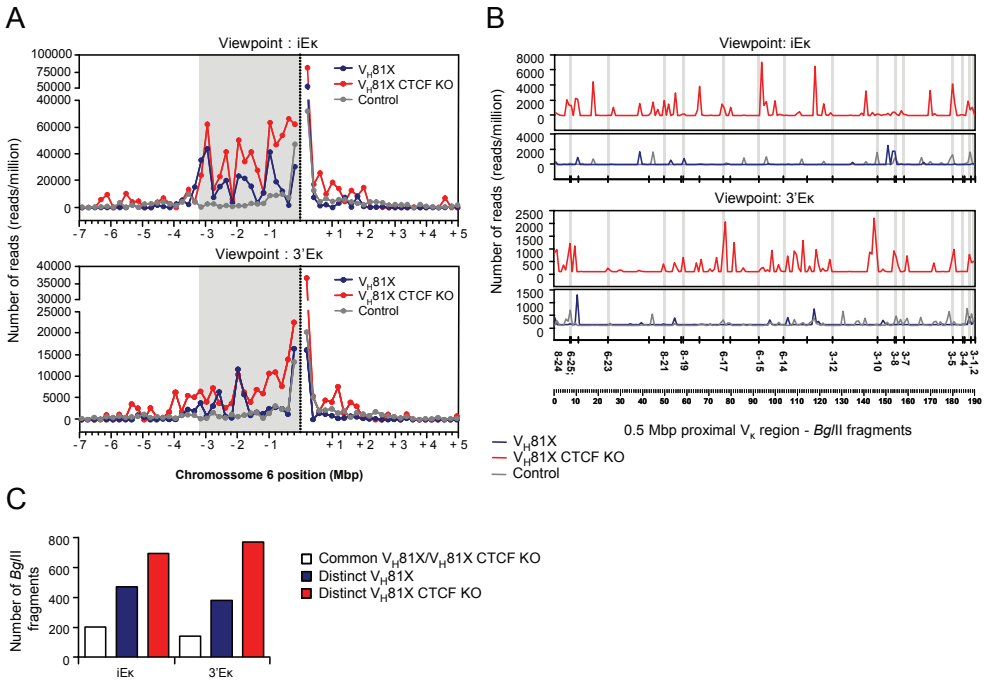


Chapter 1 - Figure 11. Schematic representation of the mouse *β-globin* locus. (**A**) Four globin genes are embedded within a larger olfactory receptor (OR) gene cluster. Developmentally regulated globin expression (*εy* and *βh1* in primitive erythroid cells; *β-major* (*βmaj*) and *β-minor* (*βmin*) in definitive erythroid cells) is regulated in part by a series of *cis*-regulatory elements surrounding the locus (indicated by arrows). An upstream locus control region (LCR) containing six DNaseI hypersensitive sites (HS) is required for high-level transcription. Three CTCF binding sites have been identified upstream (5'HS85, 5'HS62/60 and 5'HS5) and one 20 Kbp downstream (3'HS1) of *globin* genes. (**B**) Cell type-specific CTCF-mediated intrachromosomal interactions in the mouse *β-globin* locus. Although loops are illustrated by CTCF multimerization, it is not clear whether CTCF binds directly to each site or through interaction with other proteins that are not represented for simplicity. In definitive erythroid cells full expression of, for example, the *βmaj* gene depends on stable LCR-gene contacts. Figure adapter from[316].

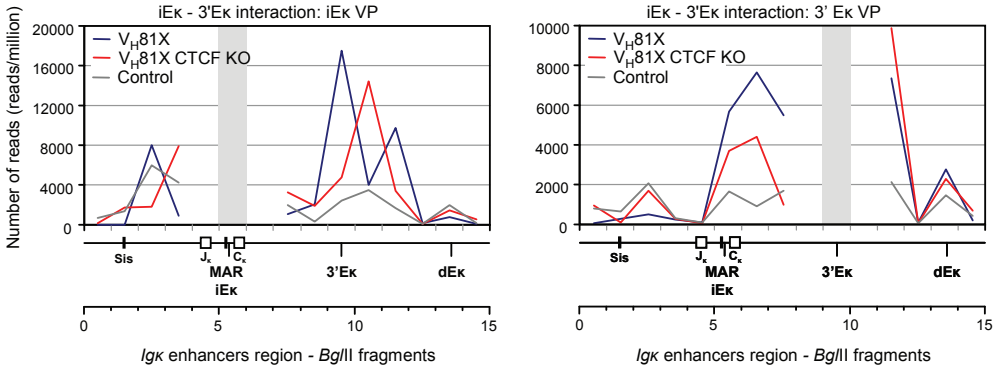




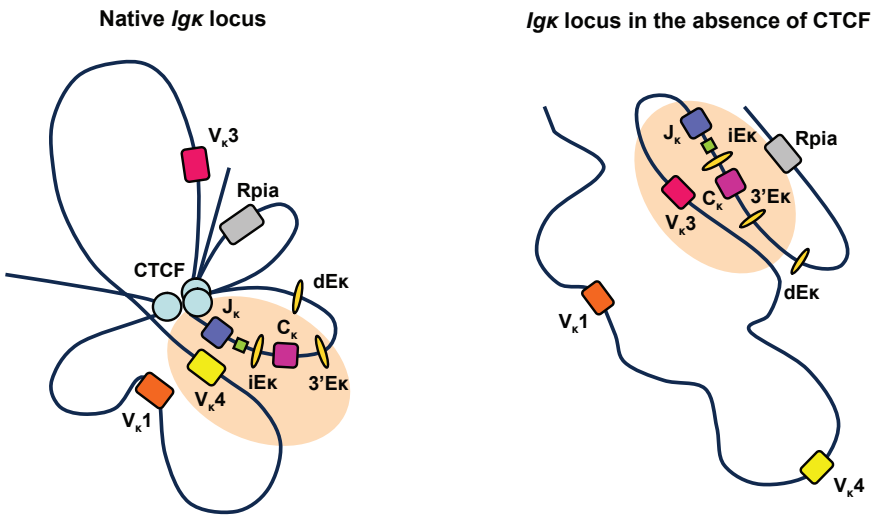
Chapter 2 - Figure 2. Defective pre-B cell proliferation and differentiation in *mb1-cre Ctcf*$^{f/f}$ mice. (**A**) Flow cytometric analysis of wild-type (WT) and *mb1-cre Ctcf*$^{f/f}$ (CTCF KO) B220^{+}CD19^{+} pro-B cells (CD2^{-} intracellular Igμ^{-}) and large pre-B cells (CD2^{-} intracellular Igμ^{+}) for cell size (FSC, forward side scatter) and IL-7Rα. Results are displayed as histogram overlays (4-6 mice per genotype). (**B**) Propidium iodide (PI) cell cycle analysis of B220^{+}CD19^{+} pro-B cells (CD2^{-} intracellular Igμ^{-}) and large pre-B cells (CD2^{-} intracellular Igμ^{+}) purified from WT and *mb1-cre Ctcf*$^{f/f}$ BM. Percentages of cells in cycle (S/G2/M; >2N DNA content) are shown (representative of 2 mice per genotype). (**C**) *In vivo* proliferation analysis of WT and *mb1-cre Ctcf* $^{f/f}$ B220^{+}CD19^{+} pro-B cells (CD2^{-} intracellular Igμ^{-}) and large pre-B cells (CD2^{-} intracellular Igμ^{+}). Mice were i.p. injected with a single dose of BrdU and after 4 hr the percentages of BrdU^{+} cells were determined by flow cytometry (mean values and SD for 3-4 mice per genotype; *, P<0.001). (**D**) and (**E**) DNA microarray analysis of total mRNA from purified B220^{+}CD19^{+}CD2^{-} pro-B/large pre-B cell fractions in WT and *mb1-cre Ctcf*$^{f/f}$ mice. Genes differentially expressed between the two genotypes were subdivided into two groups: genes which expression did (**D**) or did not differ (**E**) between control *Rag1*$^{-/-}$ pro-B cell and V_H81X *Rag1*$^{-/-}$ pre-B cell fractions. Heatmaps for the 30 genes with highest fold change in expression between WT and *mb1-cre Ctcf*$^{f/f}$ B cell progenitors are shown (for complete gene lists: see **Supplementary Tables 1 and 2**). Bottom, logarithmic quantitative scale for gene expression (3-4 pools of 3-7 mice per genotype).



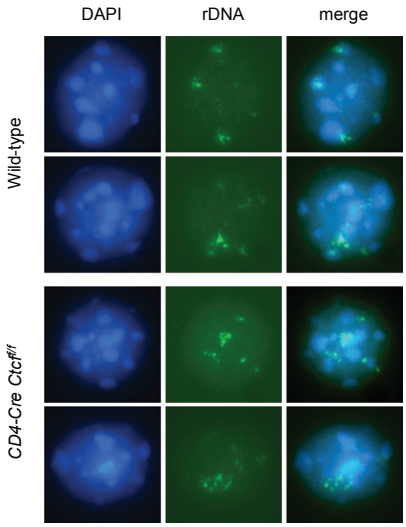
Chapter 2 - Figure 7. Increased interactions between the iEκ/3'Eκ enhancers and the $V_κ$ proximal region in the absence of CTCF. (**A**) to (**C**) 3C-Seq analysis of iEκ and 3'Eκ interactions in B220$^+$CD19$^+$CD2$^+$IgM$^-$ small pre-B cell populations purified from V_H81X and V_H81X *mb1-cre Ctcf$^{f/f}$* (CTCF KO) BM and total fetal liver cells. Cross-linked and *Nla*III-digested DNA fragments were ligated and subsequently digested by *Bgl*II followed by re-ligation. Primers on the fragment of interest (containing iEκ or 3'Eκ, viewpoint) and facing outwards were used on inverse PCR. PCR products representing the genomic environment of the fragment of interest were further characterized by high-throughput sequencing. (**A**) Number of reads per million in 0.2 Mbp intervals in the *Igκ* locus (shaded area) plus 3.8 Mbp upstream and 5 Mbp downstream genomic regions. The dashed line represents the viewpoint. 3C-Seq counts obtained for fragments adjacent to the viewpoints were not considered. (**B**) Number of reads per million obtained for each of the 190 *Bgl*II fragments that cover the proximal 0.5 Mbp $V_κ$ region. In each panel the X-axis shows the location of various $V_κ$ gene segments present in the proximal 0.5 Mbp $V_κ$ region. Shaded lines highlight the *Bgl*II fragments containing the $V_κ$ gene segments shown to be used in unproductive and productive alleles in **Figure 6**. (**C**) Total number of *Bgl*II fragments on chromosome 6 interacting with iEκ or 3'Eκ that were found both in V_H81X and V_H81X *mb1-cre Ctcf$^{f/f}$* small pre-B cells, unique for V_H81X small pre B cells or unique for CTCF-deficient V_H81X small pre-B cells.



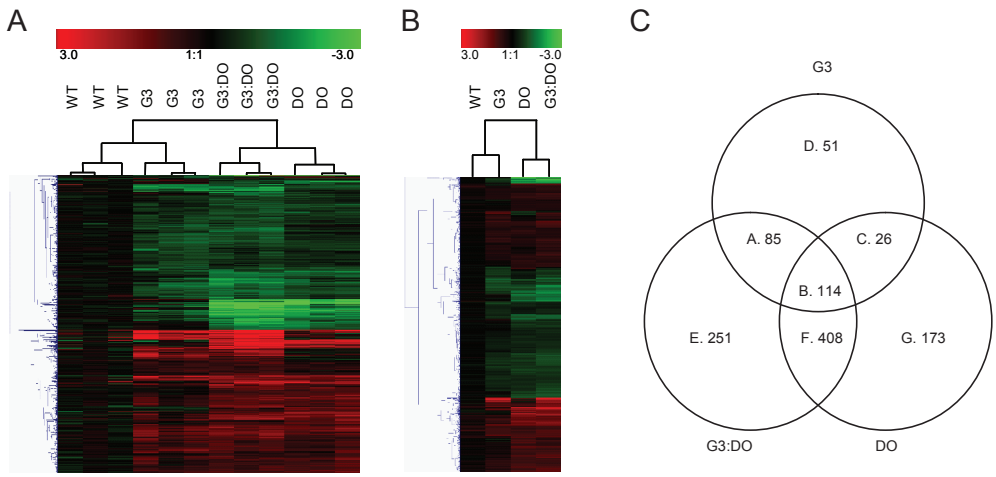
Chapter 2 - Supplementary Figure 10. Interactions between *Igκ* enhancers in B220^{+}CD19^{+}CD2^{+} IgM^{-} small pre-B cell populations purified from V_H81X and V_H81X *mb1-cre Ctcf$^{f/f}$* (CTCF KO) BM and total fetal liver cells, obtained by 3C-Seq analysis. Data represent number of reads per million obtained for the *Bgl*II fragments that cover the *Igκ* enhancers region. Shaded areas indicate the fragments containing the iEκ (left) and 3'Eκ (right) (viewpoint, VP). 3C-Seq counts obtained for fragments adjacent to the viewpoint are not shown. *Bottom*, schematic representation of the *Igκ* enhancers region showing the relative location of Sis, $J_κ$, MAR, iEκ, $C_κ$, 3'Eκ and dEκ. Data is from 5-6 mice per genotype.



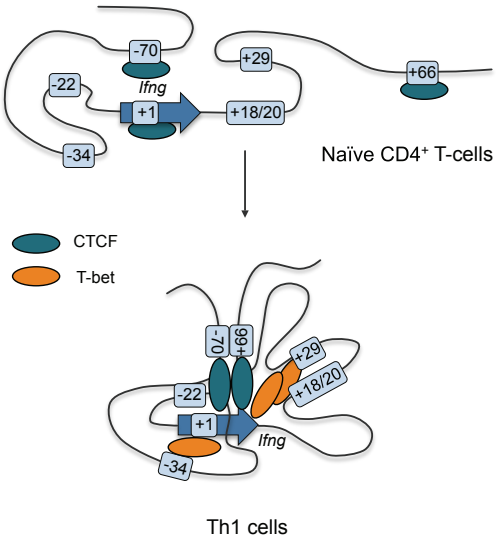
Chapter 2 - Supplementary Figure 11. Model for CTCF function at the *Igκ* locus. CTCF binding sites flanking the *Igκ* locus and in the $V_κ$-$J_κ$ intergenic region (Degner *et al*, 2009) prevent inappropriate communication between the *Igκ* enhancers and the proximal $V_κ$ gene segments or the juxtaposed house-keeping gene *Rpia* by positioning the $J_κ$-$C_κ$ cluster containing the iEκ and 3'Eκ enhancers in a separate chromatin loop domain. CTCF possibly cooperates in a redundant fashion with other proteins for further regulatory sub-loops within $V_κ$ region, bringing into close spatial proximity $V_κ$ and $J_κ$ gene segments in recombination centers (Ji *et al*, 2010) (shaded area). The absence of CTCF (right) favors interactions between *Igκ* enhancers and proximal $V_κ$ gene segments or the *Rpia* gene.



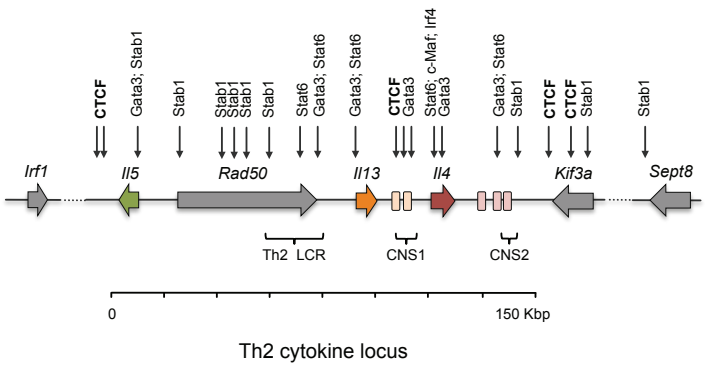
Chapter 3 - Supplementary Figure 6. CTCF deletion does not affect nucleolar organization in resting T cells. FISH analysis of nucleolar organization in FACS-sorted naive CD62L+ peripheral T cells from the indicated mice. Slides were hybridized with a DIG-labeled rDNA probe *(green)* containing non transcribed rDNA (Akhmanova *et al*, 2000). Cells were counterstained with DAPI (blue).



Chapter 5 - Figure 3. Identification of specific gene expression signatures in DP cells as a result of enforced Gata3 expression. Gene expression profiles were generated from FACS-sorted DP cells from wild-type (WT), CD2-Gata3 transgenic (G3), DO11.10 TCR transgenic (DO) and CD2-Gata3:DO11.10 double transgenic (G3:DO) mice. (**A**) Non-supervised hierarchical clustering of both genes (rows) and individual mice (columns). Gene expression profiles were analyzed for 3 individual mice per group. (**B**) Non-supervised hierarchical clustering of both genes (rows) and grouped (*n*=3) mice of the indicated genotype. Dendograms above and on the left side of the matrixes indicate the average linkage clustering of respectively individual mice and genes. Green and red colors indicate the level of downregulation or upregulation of genes, when compared with a pool of DP cells obtained from WT mice (*n*=6). The color scale above the matrix correlates with gene expression and the given value numbers represent ^{2}log values. (**C**) Differentially expressed genes in DP thymocytes from CD2-Gata3 and DO11.10 single transgenic and CD2-Gata3:DO11.10 double transgenic mice, displayed in a Venn diagram. Genes are either up- or downregulated 1.4-fold (P<0.0001) and are not differentially expressed in WT mice. Numbers of differentially expressed genes per cluster are given.



Chapter 7 - Figure 3. Model for CTCF-mediated higher-order chromatin conformation of the *Ifng* locus. In naive CD4⁺ T-cells, CTCF is bound primarily at hypersensitive site (HS) -70 and T-bet is not bound. Upon Th1 cell differentiation, T-bet binds to the *Ifng* promoter and to the conserved non-coding sequence (CNS) -34, CNS+18/20, and CNS+29 enhancers, and CTCF binds strongly at HS +1 (in intron 1 of *Ifng*) and at HS +66 in addition to HS -70. This binding contributes to and is required for the juxtaposition of each of these distal regulatory elements to *Ifng* and its promoter. In this active locus conformation, the CNS -34 enhancer is in close proximity to *Ifng* gene. Figure adapter from[59].



Chapter 7 - Figure 4. Schematic representation of the binding sites for CTCF and key Th2 transcription factors in the mouse Th2 cytokine locus. CTCF binding sites in the Th2 cytokine locus are interspersed with the binding sites for key Th2 transcription factors such as Gata3, Stat6, Satb1, c-Maf and Irf4. CTCF binding sites (CBS) upstream of *Il5* (CBS-6 and CBS-7) and downstream of *Il4* within *Kif3a* (CBS-1), flank the Th2 cytokine locus. CTCF binding in the intergenic region between *Il13* and *Il4* (CBS-3) locates to the DNaseI hypersensitive site Hss3 near to CNS-1.