Central immune tolerance depends on crosstalk between the classical and alternative NF-κB pathways in medullary thymic epithelial cells

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Medullary thymic epithelial cells (mTECs) contribute to self-tolerance by expressing and presenting peripheral tissue antigens for negative selection of autoreactive T cells and differentiation of natural regulatory T cells. The molecular control of mTEC development remains incompletely understood. We here demonstrate by TEC-specific gene manipulation in mice that the NF-κB transcription factor subunit RelB, which is activated by the alternative NF-κB pathway, regulates development of mature mTECs in a dose-dependent manner. Mice with conditional deletion of RelB lacked mature mTECs and developed spontaneous autoimmunity. In addition, the NF-κB subunits RelA and c-Rel, which are both activated by classical NF-κB signaling, were jointly required for mTEC differentiation by directly regulating the transcription of RelB. Our data reveal a crosstalk mechanism between classical and alternative NF-κB pathways that tightly controls the development of mature mTECs to ensure self-tolerance.

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1. Introduction

Most T lymphocytes develop in the thymus, consisting of the outer cortex and the inner medulla. In the cortex T cell progenitors with random α/β T cell receptor specificities are generated, including cells recognizing self-antigens. In the thymic medulla most autoreactive T cells are either deleted by apoptosis (negative selection) or they differentiate into natural regulatory T cells (nTregs). Both mechanisms contribute to the establishment of self-tolerance and require the recognition of antigen/MHC complexes displayed on antigen-presenting cells (APCs) [1]. Medullary thymic epithelial cells (mTECs), which are the major stromal component of the medulla, can act as APCs, and are the main source for non-ubiquitous peripheral tissue antigens (PTAs) in the thymus [2]. Expression of numerous PTAs, such as insulin-2 and salivary protein-1 by mature mTECs is dependent on the transcription factor Aire, and mutations in the Aire gene can lead to autoimmunity in mice and men [3]. In addition, dendritic cells (DCs) transport self-antigens from the periphery into the thymus and are involved in self-tolerization [4,5]. Both mTECs and DCs are able to delete self-reactive T cells and induce Treg cells, creating two distinct T cell pools with divergent TCR repertoires via both tolerization modes [6]. Furthermore, presentation of mTEC-derived self-antigens by DCs plays an important role in tolerance induction.

mTECs and cortical TECs (cTECs) have been postulated to derive from a bipotent epithelial progenitor [7]. However, recently it has been suggested that mTECs do not derive from a lineage-negative progenitor but rather from precursors expressing hallmarks of cTECs, such as CD205 and the thymoproteasome subunit β5t [8,9]. Furthermore, downstream of a common TEC progenitor a lineage-restricted mTEC stem cell seems to exist, which gives lifelong rise to CD80+MHCIi immature and CD80+MHCIi mature mTECs [10]. Dependent on LTβR signaling mature mTECs can further progress into post-Aire stages, which are CD80+MHCIi again. Terminally differentiated Aire− mTECs have high expression of involucrin and...
form Hassels’ corpuscle-like structures in mouse thymus [11]. Lymphoid tissue inducer cells and Vγ5+ γδ T cells in the embryo as well as iNKT cells and positively selected CD4 single-positive thymocytes in adult mice have been shown to induce commitment and maturation of the mTEC lineage [12–15]. This occurs via activation of the TNF receptor superfamily members RANK, CD40, and LTβR, which can trigger both the classical and the alternative NF-κB pathways [12,16–18].

In vertebrates the NF-κB/Rel transcription factor family comprises five members, RelA (p65), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2), which form different homo- and heterodimers. In resting cells the dimers are kept in the cytoplasm by interaction with inhibitory IκB proteins [17]. The classical NF-κB pathway can be induced by many different stimuli, such as TNF, IL-1β or LPS. Via activation of IκKβ it leads to phosphorylation and degradation of IκBζ, and the nuclear translocation of RelA/p50 and c-Rel/p50 heterodimers [17]. In contrast, the alternative NF-κB pathway is only activated together with the classical pathway by a few members of the TNFR superfamily, such as RANK, CD40 and LTβR. The signaling occurs via NIK and IKKα, leads to the processing of the inhibitory NF-κB2 precursor p100 and culminates in the activation of RelB/p52 complexes [17]. Alternative NF-κB signaling has been shown to play a major role in mTEC differentiation. Mice deficient for components of this pathway have a reduction in mTEC numbers and develop autoimmune syndromes [19–24]. However, systemic inactivation of alternative NF-κB signaling also leads to defects in the development of secondary lymphoid organs and DCs, which might contribute to the autoimmune phenotypes in these mice [25,26]. Indeed, mice with TEC-specific ablation of Tra6, which exclusively lack mTECs, develop only a rather mild autoimmune hepatitis [27].

Tra6, which binds to the intracellular domains of RANK and CD40, has been shown to play a role in multiple signaling pathways [28]. However, it is not involved in alternative NF-κB signaling, indicating that an unknown pathway downstream of Tra6 is essential for mTEC differentiation. Although classical NF-κB signaling can be transduced by Tra6, its implication in mTEC differentiation has not yet been genetically investigated.

We show here that mTEC development is critically dependent on the activation of RelA and c-Rel via the classical NF-κB pathway. Both factors transcriptionally regulate the expression level of RelB, which determines the total number of mTECs per thymus in a dose-dependent manner. Complete absence of mature mTECs by TEC-specific inactivation of Relb results in symptoms of systemic autoimmunity. All together, these results show that the crosstalk between both NF-κB pathways culminating in the activation of a high amount of RelB protein is important for the development of sufficient mTECs to guarantee the establishment of self-tolerance.

2. Materials and methods

2.1. Mice

Foxn1-Cre [29], Relaflax [30], c-Relflax [31], Tra6flax [32] and xB-EGFP [33] mice have been described previously. Generation of Relbfllox mice: To construct the targeting vector a floxed neomycin (neo) resistance cassette was cloned into the Ascl site of the 4th intron of the Relb gene (Supplemental Fig. 1). A single loxp site was placed into the BglII site 184 bp upstream of exon 4. The length of the homologous sequence upstream of the 5’ loxP site was 2.75 kb. The HSV thymidine kinase gene was inserted 0.8 kb downstream of the alternative NF-κB cassette for selection against random integration events. E14 embryonic stem (ES) cells that were cultured in ES medium (DMEM, 15% FCS, 1× sodium pyruvate, 1× Pen/Strep, 1× l-glutamine, 1× non-essential amino acids, 1 µM 2-mercaptoethanol, 1000 units/ml LIF (ESGRO®; Chemicon) were electroporated with the linearized targeting vector and selected with G418 (200 µg/ml; Invitrogen) and gancyclovir (Cyméven; 2 µM; Roche). Resistant clones were screened for homologous recombination by Southern blot analysis of BamHI-digested genomic DNA using an external 3’-flanking probe. Positive clones were further analyzed by Southern blotting using a neo probe to exclude clones with additional random integrations of the targeting construct in the genome. The neo cassette was deleted by transient transfection of a plasmid (pMC-cre) expressing Cre recombinase. Clones containing the floxed allele were identified by Southern blots of BamHI/HindIII-digested genomic DNA and PCR. The presence of loxp sites was verified by sequencing. Properly targeted ES cell clones were injected into C57BL/6 blastocysts and chimeric mice were tested for germline transmission of the Relbfllox allele. Generation of Rosa26-Relb transgenic mice: For targeting the Relb transgene into the Rosa26 locus we used the TV-Rosa26 vector (kindly provided by U. Zimber-Strobl, Helmholtz Zentrum München) containing a loxP-flanked stop cassette with a transcription and translation termination site (Supplemental Fig. 2). A murine Relb cDNA was inserted downstream of the stop cassette. The linearized targeting vector was electroporated into E14.1 ES cells. C418 resistant clones were screened for homologous recombination by PCR and Southern blot analysis of EcoRI-digested genomic DNA using an external 5’-flanking probe. Targeted ES cells were injected into C57BL/6 blastocysts to obtain chimeric mice, which were backcrossed with C57BL/6 mice to obtain germline transmission of the Relb transgene. Generation of Relb-Cre-P2A-Katashka mice: Generation of the BAC transgenic mice was performed by PolyGene (Switzerland). A BAC of 93 kb (bMQ-258kO8) containing the complete Relb locus with 45 kb of upstream region was modified by introducing the gene coding for a Cre-P2A-Katashka fusion protein into the stop exon of the Relb gene (Supplemental Fig. 3). The construct was injected into pronuclei from fertilized C57BL/6 oocytes and four founder mice were obtained, which transmitted the transgene to the progeny. In one of these founder lines (#53) transgene expression nicely reflected that of endogenous Relb (Riemann et al., manuscript in preparation).

All mouse lines were on a C57BL/6J background. The mice were analyzed at the age of 4–6 weeks unless otherwise indicated. Floxed littermates without Foxn1-Cre transgene were used as controls since analysis of the pure Foxn1-Cre mice did not show any significant alterations in the thymus. All experiments were performed in accordance with the ethic committee guidelines.

2.2. Histology and immunohistochemistry

Tissues were fixed in 10% buffered formalin, embedded in paraffin blocks, sectioned at 6 µm, and stained with hematoxylin and eosin (H&E). For immunohistochemistry, thymi were embedded in Neg-50 frozen section medium (Richard Allen Scientific) and frozen on dry ice. Ten µm thick sections were cut, air-dried, and fixed in cold acetone. Staining was performed either with biotinylated Ulex europaeus agglutinin-1 (UEA-1) (Vector Laboratories) or with antibodies against Aire (M–300; Santa Cruz), claudin-3 and –4 (Zymed); keratin-5 (Covance), or RelB (C-19; Santa Cruz) followed by biotinylated anti-rabbit IgG (Vector Laboratories). Staining with MDC8 antibody (Antikoerper-online) was followed by biotinylated anti-rabbit IgG (Vector Laboratories). All sections were then incubated with Vectastain ABC peroxidase (Vector Laboratories). Diaminobenzidine was used to visualize the immunostaining and counterstaining was performed with Mayer’s hematoxylin. All sections were examined by light microscopy using an Olympus BX41 microscope.
2.3. Flow cytometry and sorting of thymic epithelial cells

Single-cell suspensions of thymic stromal cells were prepared in 5 digestion steps with collagenase D, dispase, and DNase I (Roche) as described [34]. For TEC analysis, cells were stained with anti-CD45-PE-Cy7 (clone 30-F11), anti-EP-CAM-PE (G8.8), anti-Ly51(B1)-FITC/Biotin (6C3), and anti-CD80-APC/PerCP-Cy5.5 (BD) (16-10A1). Streptavidin-PerCP-Cy5.5 was used as secondary reagent. For intracellular staining with anti-Aire-AF647 (SH12), anti-c-Rel-PE (1RELAH5), anti-Ki67-FITC (B56), or anti-cleaved caspase-3 (Asp175; Cell Signaling) followed by anti-rabbit-IGG-Pacific Blue (Invitrogen), cells were fixed and stained using FoxP3 fixation and permeabilization kit (eBioscience). Analyses were performed with a FACSCan II (BD). For TEC sorting, stromal cells were enriched by depletion of CD45+ cells using a magnetic cell sorter (Miltenyi Biotec) prior to sorting with a FACSAria II (BD). Antibodies were obtained from ebioscience unless otherwise stated.

2.4. Flow cytometry of infiltrating cells in lung and liver

For isolation of infiltrating cells, lung tissues were digested for 1 h with collagenase IV-S (Sigma). Liver tissues were minced by pressing through a metal sieve and subsequently suspended by several passages through a 21-gauge needle. Suspensions of both tissues were filtered through 40 μm cell strainers and purified using Percoll density gradients. Red blood cells were depleted with ACK lysing buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.3). Percoll density gradients. Red blood cells were depleted with ACK buffer (see above) cells were filtered through a 40 μm cell strainer. DCs were stained with anti-CD11c-APC (N418), anti-SIRP α,6.7) antibodies and 5 μl of 5 digestion steps with collagenase D, dispase, and DNase I (Roche) as described [34]. For TEC analysis, cells were stained with anti-Aire-AF647 (SH12), anti-c-Rel-PE (1RELAH5), anti-Ki67-FITC (B56), or anti-cleaved caspase-3 (Asp175; Cell Signaling) followed by anti-rabbit-IGG-Pacific Blue (Invitrogen), cells were fixed and stained using FoxP3 fixation and permeabilization kit (eBioscience). Analyses were performed with a FACSCan II (BD). For TEC sorting, stromal cells were enriched by depletion of CD45+ cells using a magnetic cell sorter (Miltenyi Biotec) prior to sorting with a FACSAria II (BD). Antibodies were obtained from ebioscience unless otherwise stated.

2.5. Flow cytometry of thymic DCs and T cells

Thymi were minced with scissors into small pieces and digested with collagenase D and DNase I (Roche). After erythrocyte lysis in ACK buffer (see above) cells were filtered through a 40 μm cell strainer. DCs were stained with anti-CD11c-APC (N418), anti-sIgR (CD172a)-Biotin (P84) followed by Streptavidin-PE and anti-PDCA1-FITC (eBio927). For T cell analysis staining was performed with anti-TCRα-PE (H57-597), anti-CD4-PE (GK1.5), anti-CD8a-eFluor450 (53-6.7), anti-CD62L-FITC (MEL-14), and anti-CD44-APC (IM7) (all antibodies from ebioscience). Analyses were performed with a FACSCan II (BD).

2.6. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from thymic or sorted mTECs using the RNeasy Minikit (Qiagen) or the RNAqueous-Micro kit (Ambion), respectively. First-strand cDNA synthesis was conducted with 1 μg of RNA using oligo(dt)12-18 primer and M-MLV reverse transcriptase (H-) (Promega). For relative quantification of RNA using oligo(dT)12-18 primer and M-MLV reverse transcription (H-) and washed 3 times with TBST. Immuno-reactive proteins were detected using an ECL visualization system (GE Healthcare).

2.7. Western blots of 3T3 mouse embryonic fibroblasts (MEFs)

For activation of classical NF-κB MEFs was analyzed by chromatin immunoprecipitation using the “ChIP-IT Express Enzymatic” Magnetic Chromatin Immunoprecipitation Kit (Active Motif). The ChIP was carried out according to the manufacturer’s instructions using 5 μg chromatin enzymatically sheared for 6 min and 3 μg antibody (α-RelA (C-20), α-c-Rel (C), and α-Reib (C-19); all from Santa Cruz). One-twentieth of the DNA was used in each PCR reaction (40 cycles) applying the following primers for the Relb promoter (−440/−293), sense, 5'-CTTGGTCTGTGAGGTAAGCCTG-3'; antisense, 5'-CGCGGCTCGTCGAAGCCTG-3'; and 5'-GGAGG-TGAGGAGCCTC-3'.

2.8. Western blots

Liver tissue from Rag1−/− mice was homogenized in lysis buffer (20 mM Tris-HCl pH 8.8, 10 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% SDS, protease inhibitor cocktail from Roche) and lysates were centrifuged for 5 min at 16,100 g. Supernatants were mixed 1/1 with sample buffer (250 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 20 mM DTT, 0.01% bromophenol blue), 10 μg of protein per lane were separated by SDS-PAGE (10%), and electroblotted onto Immobilon P membranes (Millipore). Membranes were blocked with 5% non-fat milk-TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% Tween 20) for 2 h and incubated with sera from individual mice diluted 1/200 in 5% non-fat milk-TBST overnight. After three washes with TBST, membranes were incubated with HRP-conjugated goat-anti-mouse IgG (1:10,000; Dako) in TBST for 2 h and washed 3 times with TBST. Immuno-reactive proteins were detected using an ECL visualization system (GE Healthcare).

2.9. Chromatin immunoprecipitation (ChIP)

In vivo binding of NF-κB subunits to the Relb promoter in 3T3 MEFs was analyzed by chromatin immunoprecipitation using the “ChIP-IT Express Enzymatic” Magnetic Chromatin Immunoprecipitation Kit (Active Motif). The ChIP was carried out according to the manufacturer’s instructions using 5 μg chromatin enzymatically sheared for 6 min and 3 μg antibody (α-RelA (C-20), α-c-Rel (C), and α-Reib (C-19); all from Santa Cruz). One-twentieth of the DNA was used in each PCR reaction (40 cycles) applying the following primers for the Relb promoter (−440/−293), sense, 5'-AGG-3'; antisense, 5'-CTTGGTCTGTGAGGTAAGCCTG-3'; and 5'-GGAGG-TGAGGAGCCTC-3'.

2.10. Statistical analysis

Results are depicted as mean values ± SD. Statistical analysis was performed using Student’s two-tailed unpaired t-test for
comparisons between two groups with unequal variances. Differences were considered significant at *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

3. Results

3.1. Both RelA and RelB are required for proper development of mTECs

Systemic Relb−/− mice lack mature mTECs and develop a severe autoimmune syndrome with inflammatory infiltrates in multiple organs [19,20]. To investigate whether TEC-specific deletion of RelB is sufficient to ablate mTECs resulting in severe autoimmunity, we generated Relbfl/− mice and crossed them with Foxn1-Cre mice to obtain conditional RelbTEC-KO mice. In addition, we generated RelaTEC-KO mice to investigate the role of the classical NF-κB pathway in mTEC development and the establishment of self-tolerance.

In contrast to Relb−/− mice, which exhibit severe thymic atrophy predominantly affecting the medulla [20], both RelaTEC-KO and RelbTEC-KO mice displayed only a minor reduction of medullary areas in H&E stained sections (Fig. 1A). However, RelbTEC-KO mice completely lacked mature UEA-1+ and Aire− mTECs while they were partially reduced and unevenly distributed in RelaTEC-KO thymi. Keratin 5 staining confirmed the absence of TECs in medullary areas of RelbTEC-KO mice, whereas they were only partially reduced in RelaTEC-KO mice. Claudin-3 and -4 (Cld3,4) are expressed already by early precursors of mature Aire+ mTECs [36]. Thus, the absence of Cld3,4+ cells in RelbTEC-KO thymi indicated that mTEC differentiation was blocked at an early maturation stage. Cld3,4+ cells were also reduced in RelaTEC-KO thymi, although less pronounced. We next quantified immature and mature subpopulations of total mTECs by flow cytometry. For detection of these cells we used antibodies against the following markers: (1) CD45, which is highly expressed on hematopoietic, but not on stromal cells, (2) EpCAM, which is specifically expressed on all thymic epithelial cells, (3) Ly51, which is expressed on cTECs but not on mTECs and (4) CD80, which shows high expression on mature mTECs (13).

Fig. 1. Both RelA and RelB are required for proper development of mTECs. (A) Thymus sections from control, RelaTEC-KO, and RelbTEC-KO mice were stained with H&E, UEA-1, or antibodies against Aire, claudin 3 and 4, keratin 5, or RelB. Scale bars: 50 μm (Aire), 100 μm (RelB), or 500 μm. (B) Thymus single-cell suspensions were stained with antibodies against CD45, EpCAM, Ly51, and CD80 and analyzed by flow cytometry. CD45 EpCAM+ TECs (upper row) were segregated into Ly51 CD80hi mature mTECs, Ly51 CD80lo immature mTECs, and Ly51− CD80hi cTECs (middle row). Aire expression was analyzed by intracellular staining (lower row). Numbers in the respective gates indicate frequencies of cells within each TEC subpopulation. (C) Quantitative summary of total cell numbers of indicated TEC subpopulations per thymus from control (n = 10), RelaTEC-KO (n = 8), and RelbTEC-KO (n = 4) mice. Data represent mean values ± SD.
mTECs have been implicated in the establishment of immunological self-tolerance due to their capability to express and present a wide range of PTAs, which drives both negative selection as well as differentiation of nTregs [1]. In agreement with the complete lack of Aire+ mTECs in RelbTEC-KO thymi, mRNA expression of the Aire-dependent PTAs casein-\(_a\), salivary protein-1, and insulin-2 was strongly decreased compared to control thymi (Fig. 2A). While expression of the Aire-independent PTA Fabp-9 was also almost absent, the mRNA encoding C-reactive protein was slightly higher decreased in the absence of mature mTECs. The proportion of SIRP\(_a\)-expressing conventional DCs (cDCs) as well as PDCA1\(^+\) plasmacytoid DCs (pDCs) were not affected. These results indicated that RelA was involved in the regulation of PTA expression itself. Besides mTECs, the absence of Aire expression in mTECs has been implicated in the establishment of immunological self-tolerance due to their capability to express and present a wide range of PTAs, which drives both negative selection as well as differentiation of nTregs [1]. In agreement with the complete lack of Aire+ mTECs in RelbTEC-KO thymi, mRNA expression of the Aire-dependent PTAs casein-\(_a\), salivary protein-1, and insulin-2 was strongly decreased compared to control thymi (Fig. 2A). While expression of the Aire-independent PTA Fabp-9 was also almost absent, the mRNA encoding C-reactive protein was slightly higher expressed, which is in agreement with low level expression of this PTA in cTECs [2]. In contrast, RelaTEC-KO thymi expressed PTAs at reduced but higher levels than RelbTEC-KO thymi. However, the reduction in PTA expression was more pronounced than the decrease of mTEC numbers, suggesting that RelA may also be involved in the regulation of PTA expression itself. Besides mTECs, DCs are involved in the induction of central tolerance in the thymus [1]. SIRP\(_a\)^+ cDCs, which develop in the thymus, are involved in negative selection by cross-presentation of autoreactive T cells and differentiation of nTregs [5]. Furthermore, SIRP\(_a\)^+ cDCs, which develop in the thymus, are involved in negative selection by cross-presentation of mTEC-derived antigens to CD8^+ T cells [37]. Immunohistochemical analysis of thymus sections using MDC-8 antibody revealed markedly reduced DC staining in RelbTEC-KO mice (Fig. 2B). RelaTEC-KO mice had only slightly reduced numbers of DCs, which were not evenly distributed in the medulla, reminiscent of the distribution of UEAI^+ mTECs in RelbTEC-KO thymus. Flow cytometric analysis of the three DC subpopulations revealed a specific loss of thymic SIRP\(_a\)^+ cDCs in RelbTEC-KO mice (Fig. 2C). In contrast, the percentage of SIRP\(_a\)^+ cDCs was not affected and pDCs were only slightly decreased in the absence of mature mTECs. The proportion of SIRP\(_a\)^+ cDCs was also diminished in RelbTEC-KO thymus, while SIRP\(_a\)^+ cDCs and pDCs were not affected. These results indicated that development and/or localization of SIRP\(_a\)^+ cDCs in the thymus is dependent on the presence of mTECs.

Analysis of different T cell subpopulations showed normal frequencies of CD4 and CD8 single-positive \(\gamma\delta\) T cells and of \(\gamma\delta\) T cells in both RelaTEC-KO and RelbTEC-KO mice (Supplemental Fig. 6). However, both mouse lines had a significant reduction of FoxP3^+ nTregs,
which was more pronounced in Relb\textsuperscript{TEC-KO} animals (Fig. 2D).

Notably, thymic iNKT cells were almost completely absent in both Rela\textsuperscript{TEC-KO} and Relb\textsuperscript{TEC-KO} mice. Thus, while mTECs were dispensable for the development of normal numbers of ab and gd T cells, they were required for the generation of FoxP3\textsuperscript{+} nTreg and iNKT cell populations.

### 3.3. RelbTEC\textsuperscript{-/} but not RelaTEC\textsuperscript{-/} mice develop autoimmunity

Histopathological examination of multiple tissues from Relb\textsuperscript{TEC-KO} mice showed inflammatory cells preferentially infiltrating liver and lung (Fig. 3A). Flow cytometric analysis furthermore revealed increased frequencies of activated/memory CD4\textsuperscript{+}CD44\textsuperscript{high}CD62\textsuperscript{low} T cells in both tissues of Relb\textsuperscript{TEC-KO} mice, indicating an on-going autoimmune response (Fig. 3B). Moreover, sera from Relb\textsuperscript{TEC-KO} mice reacted against liver proteins of RAG1\textsuperscript{-/-} mice indicating the presence of autoantibodies. Western blots of liver protein extracts from RAG1\textsuperscript{-/-} mice were incubated with serum from three mice per genotype. Asterisks mark protein bands recognized only by sera from Relb\textsuperscript{TEC-KO} mice indicating the presence of autoantibodies.

Compared to age-matched Relb\textsuperscript{TEC-KO} mice (Fig. 3B). These results showed that Relb is essential in mTECs, but is also important in other cell types to establish full self-tolerance. In contrast to Relb\textsuperscript{TEC-KO} mice, Rela\textsuperscript{TEC-KO} mice neither showed autoimmune infiltrates nor autoantibodies (Fig. 3A, B and C). Thus, low levels of thymic PTA expression in combination with only moderately reduced Sirp\textsuperscript{x} DCs were sufficient to establish self-tolerance in Rela\textsuperscript{TEC-KO} mice.

### 3.4. RelA controls the number of differentiating mTECs by regulating the expression of RelB

The ubiquitin-ligase Traf6, which can activate the classical NF-kB pathway, has been shown to be essential for the differentiation of mTECs, possibly via induction of RelB expression [38]. To investigate the molecular function of RelA in mTEC differentiation, we compared Relb and Nrk2 mRNA levels in mTECs, which were sorted by FACS from Rela\textsuperscript{TEC-KO} and control mice (Supplemental Fig. 7). Whereas Nrk2 expression was unaltered, expression of the RelB gene was reduced in mTECs from Rela\textsuperscript{TEC-KO} mice (Fig. 4A). Interestingly, we observed that thymi from heterozygous Relb\textsuperscript{TEC-KO} mice, harboring only one functional Relb allele, contained half the...
number of mTECs compared with control mice, suggesting a direct relationship between RelB dose and the number of differentiating mTECs (Fig. 4B). This notion we tested by expressing either one or two copies of a Relb transgene specifically in the thymic epithelium of RelbTEC-KO mice. Therefore, we generated Rosa26-Relb mice, which expressed a Relb transgene from the Rosa26 locus in a Cre-dependent manner. These mice were crossed with RelbTEC-KO mice to obtain RelbTEC-KO;Rosa26-Relb mice with either one or two Rosa26-Relb alleles. Importantly, TEC-specific expression of only one allele of the Relb transgene was sufficient to release the block in differentiation of mature Aire⁺ mTECs in RelbTEC-KO mice (Fig. 4C and D). The finding that expression of an additional Relb transgene allele almost doubled the number of immature and mature mTECs confirmed the assumption that RelB regulates the number of mTECs in a dose-dependent manner.

To genetically investigate whether reduced mTEC differentiation in RelaTEC-KO mice was due to reduced RelB levels, we also expressed two alleles of the Relb transgene specifically in RelA-deficient TECs. Transgenic expression of RelB led to a marked restoration of both immature and mature mTEC numbers in RelaTEC-KO;Rosa26-Relb animals (Fig. 4E). Thus, normal differentiation of mTECs was dependent on the dose of RelB and transcriptional upregulation of RelB via activation of RelA represented a function of the classical NF-κB pathway in mTECs. However, RelbTEC-KO mice completely lacked mTECs while RelaTEC-KO mice had only reduced numbers still expressing detectable levels of RelB. This indicated that besides RelA another transcription factor must be involved in the regulation of RelB in mTECs.

3.5. c-Rel partially compensates for the loss of RelA in transcriptional regulation of RelB expression and mTEC development

Since we found that expression of the NF-κB subunit c-Rel is upregulated in a subpopulation of mature mTECs (Supplemental Fig. 8), it was tempting to speculate that RelA and c-Rel cooperate in the regulation of RelB. To reveal a potential role of c-Rel in mTEC development mice with TEC-specific deletion of either the c-Rel gene alone (c-RelTEC-KO) or in combination with deletion of the Rela gene (Rela;c-RelTEC-DKO) were generated. The thymic epithelia from both mouse lines were analyzed by FACS in parallel with RelaTEC-KO and control thymi. While mTEC numbers in c-RelTEC-DKO mice were not significantly altered compared to controls, the Rela;c-RelTEC-DKO animals showed a striking reduction of mature and immature mTEC numbers, which was much stronger than in the RelaTEC-KO mice (Fig. 5A). Rela;c-RelTEC-DKO animals had only few Aire⁺ mature mTECs but did not show any signs of spontaneous autoimmunity.
Fig. 5. c-Rel partially compensates the loss of RelA in transcriptional regulation of RelB expression and mTEC development. (A) CD45 EpCAM+ epithelial cells from control (n = 7), RelaTEC-KO (n = 3), c-RelTEC-KO (n = 4), and Rela;c-RelTEC-DKO (n = 4) mice were classified into immature, mature, and Aire+ mTECs as well as cTECs as described in Fig. 1B and C. Bar diagrams display total cell numbers of respective TEC subpopulations per thymus and represent mean values ± SD. (B) Induction of RelB protein in MEFs of the indicated genotypes after stimulation with TNF (10 ng/ml). Whole-cell protein extracts were prepared at the times indicated and analyzed by Western blot analysis with antibodies specific for RelA, c-Rel, RelB and β-actin. (C) Interaction of RelA, c-Rel and RelB with IκBα in unstimulated MEFs of the indicated genotypes. (Upper panel) Western blot analysis was performed with whole-cell protein extracts using antibodies specific for IκBα and β-actin. (Lower panel) Whole cell protein extracts were incubated with α-IκBα antibody-coated agarose beads and co-immunoprecipitated proteins RelA, c-Rel and RelB were detected by Western blot analysis. (D) Activation of NF-κB subunits RelA, c-Rel, and RelB in MEFs of the indicated genotypes. Nuclear protein extracts were prepared at different timepoints after stimulation with TNF (10 ng/ml) and analyzed by Western blot analysis with the indicated antibodies. (E) ChIP experiments were performed with 5 μg chromatin of wild-type MEFs at the indicated times after stimulation with TNF (10 ng/ml) using antibodies specific for RelA, c-Rel and RelB. Precipitated DNA was analyzed by PCR using primers encompassing the indicated position in the Relb promoter. PCR was also performed with 10 ng of input chromatin to ensure equal loading. In (B), (C), (D) and (E) representative figures from three experimental replicates are shown.
such as inflammatory infiltrations in the liver, indicating that even these few mTECs were sufficient to establish central tolerance. Nevertheless, Rela−/− c- Rel TECKO mice showed a very similar defect in mTEC development as RelbTECKO mice. This finding suggested that RelA and c-Rel regulate differentiation of mTECs by cooperatively controlling the expression of RelB. RelA and c-Rel are both activated via the classical NF-κB signaling pathway. To investigate the respective contribution of both factors to the induction of RelB we stimulated 3T3 mouse embryonic fibroblasts (MEF) from wild-type, Rela−/−, c-Rel−/− and Rela−/−;c-Rel−/− mice with TNF, which induces classical but not alternative NF-κB signaling, and measured RelB induction by Western Blot analysis. In wild-type cells, TNF strongly increased RelB protein levels starting at 1 h with further increase at 4 and 8 h after stimulation (Fig. 5B). Compared to wild-type cells, RelB induction was slower but not much weaker in Rela−/− cells and unchanged in c-Rel−/− cells. Interestingly, in cells lacking both RelA and c-Rel, RelB levels were only slightly upregulated by TNF, indicating that RelA and c-Rel regulate RelB expression in a redundant but pivotal manner.

In unstimulated cells RelA is complexed with the NF-κB inhibitor IκBα, which is degraded upon induction of the classical NF-κB pathway releasing RelA for nuclear translocation and target gene regulation [17]. To analyze alterations in the NF-κB/IκBα complex in the absence of RelA and/or c-Rel an IκBα-Western-blot as well as IκBα-immunoprecipitations were performed using whole cell extracts from the unstimulated wild-type and mutant MEFs. The co-immunoprecipitated NF-κB subunits RelA, c-Rel and RelB were analyzed by Western blot. While in wild-type and c-Rel−/− cells IκBα was mainly complexed with RelA, the depletion of RelA caused a reduction in IκBα protein levels but an increased recruitment of c-Rel into the IκBα-complex (Fig. 5C). In the absence of both RelA and c-Rel the IκBα-protein amount decreased strongly.

Fig. 6. Traf6 regulates expression of RelB in mTECs. (A) Thymus single-cell suspensions from control and Traf6TECKO mice expressing two alleles of a Rosa26-Relb transgene were stained and analyzed as described in Fig. 1B. (B) Bar diagrams display total cell numbers of respective TEC subpopulations per thymus from control (n = 7) and Traf6TECKO (n = 6), as well as from Traf6TECKO mice expressing two alleles of a Rosa26-Relb transgene (n = 4) and represent mean values ± SD.
proteins are able to interact with the Rel. While RelA is dissociated from the only 15 min after stimulation with TNF it is replaced by RelA and c-3.6. Traf6 regulates expression of RelB in mTECs
control the transcription of
of RelB via the classical NF-
function of both receptors on mTECs is to upregulate the expression signals from RANK and CD40, we conclude that one important independent role in mTEC development. Since Traf6 mediates the addition at least one other unknown factor, which plays a RelB-
This indicates that Traf6 not only activates expression of RelB, but in
in a clear increase in the numbers of immature and mature mTECs
of RelB protein were constitutively present in the nucleus of control
nuclear translocation after stimulation with TNF. While low levels
Interestingly, due to the interaction of RelB with I
expression of the red
mice, a BAC transgenic mouse line, in which
expression of the red
expression of the red
mice could also be the reason for the finding that, in contrast to RelbTEC-KO mice, thymi from Rela;c-RelTEC-DKO mice still had few mature mTECs (Fig. 5A). Since the Relb promoter contains three potential NF-κB recognition motifs, we investigated whether Rela, c-Rel and RelB regulate the transcription of RelB by direct binding to its promoter. Chromatin-immunoprecipitations (CHIP) were performed using wild-type cells. Interestingly, we found that in unstimulated cells the RelB promoter is occupied with Rela protein itself (Fig. 5E). However, only 15 min after stimulation with TNF it is replaced by Rela and c-Rel. While Rela is dissociated from the Relb promoter already after 60 min, the interaction of c-Rel is longer lasting and still present after 240 min. Together, these results clearly show that all three Rel proteins are able to interact with the Relb promoter and directly control the transcription of Relb.

3.6. Traf6 regulates expression of RelB in mTECs
Similar to Rela;c-RelTEC-DKO and RelbTEC-KO mice, Traf6TEC-KO mice have recently been shown to lack mature mTECs [27]. Since Traf6 transduces the stimulation of RANK and CD40 to classical NF-κB signaling but also to several other pathways [28], we investigated whether loss of mTECs in Traf6TEC-KO mice was also due to inefficient induction of RelB expression. For this, we bred Traf6TEC-KO,Rosa26-Relb mice and compared their mTEC numbers with those from Traf6TEC-KO mice. Expression of two Relb transgene alleles resulted in a clear increase in the numbers of immature and mature mTECs compared to the Traf6TEC-KO mice, verifying that Traf6 controls mTEC development via induction of RelB (Fig. 6A and B). However, this rescue in mTEC numbers by the Relb transgene was less efficient in Traf6TEC-KO mice than in RelbTEC-KO mice (Figs. 6B and 4D). This indicates that Traf6 not only activates expression of RelB, but in addition at least one other unknown factor, which plays a RelB-independent role in mTEC development. Since Traf6 mediates the signals from RANK and CD40, we conclude that one important function of both receptors on mTECs is to upregulate the expression of RelB via the classical NF-κB pathway.

3.7. High NF-κB activity and RelB expression in the majority of mature mTECs
Our data showed that the transcriptional regulation of RelB by RelA and c-Rel is essential for the differentiation of both immature and mature mTECs. To find out at which stage of mTEC differentiation classical NF-κB signaling induces the expression of Relb, we analyzed two reporter mouse lines, (1) xB-EGFP mice, which express EGFP under control of three copies of the NF-κB binding site from the immunoglobulin kappa light chain enhancer, (2) Relb-Cre-P2A-Katushka mice, a BAC transgenic mouse line, in which expression of the red fluorescence protein Katushka is regulated by the endogenous regulatory sequences of the Relb gene. Flow cytometric analysis of mTECs from xB-EGFP mice showed the highest NF-κB activity in the majority of CD80hi mature mTECs, whereas a couple of immature mTECs displayed low basal NF-κB activity (Fig. 7A). Similarly, RelB expression was also mainly confined to CD80hi mature mTECs with low expression in some immature cells (Fig. 7B). This overlap of NF-κB activity and RelB expression not only corroborates the link between both events in mature mTECs, but also indicates that RANK-, CD40-, and LTβR-induced signaling occurs predominantly in these cells.

4. Discussion
While the importance of alternative NF-κB signaling in differentiation of mature mTECs and in induction of self-tolerance has been well characterized by the analysis of mice with systemic inactivation of its components [19–24], the mTEC-intrinsic role of this pathway as well as the function of the classical NF-κB signaling pathway in both processes is less clear. Here, we used TEC-specific inactivation of NF-κB transcription factors RelA, c-Rel and RelB to delineate and compare the respective contributions of both pathways to these issues. We found that RelbTEC-KO mice lacked all mature mTECs, verifying the cell-intrinsic role of alternative NF-κB signaling in mTEC development. Surprisingly, these mice developed only mild autoimmunity compared to systemic Relb+/− mice, indicating that Relb is important in other cell-types, which act together with mTECs to prevent autoimmunity. Indeed Relb+/− mice have additional defects in stromal cells of secondary lymphoid organs [25]. These tissues also maintain self-tolerance either through ectopic PTA expression and presentation by stromal cells or through presentation of tissue-derived antigens by immature DCs [39]. In addition, Relb is intrinsically required for proper development and function of DCs [26,40,41]. It was reported that injection of RelB-proficient DCs can ameliorate the severe inflammation of Relb−/− mice [26]. The similar inflammatory phenotypes of Traf6TEC-KO and RelbTEC-KO mice further corroborate that specific
depletion of mTECs alone elicits mild autoimmunity affecting predominantly only liver and lung [27].

The selective depletion of all mature and most immature mTECs by TEC-specific ablution of Relb makes Relb<sup>TEC-KO</sup> mice an ideal tool to investigate the distinct roles these cells play in the thymus. Differentiation of nTregs in thymus can be induced by the presentation of self-antigen on mTECs or DCs [4,6]. However, DCs are not essential for the differentiation of nTregs in thymus since depletion of DCs does not lead to decreased nTreg numbers [42]. In contrast to DCs, mTECs were required for the generation of a normal FoxP3<sup>+</sup> nTreg population. However, significant numbers of nTregs still developed in the absence of mTECs in Relb<sup>TEC-KO</sup> thymi. One possible explanation for this might be that in the absence of mTECs DCs have taken over their function to drive nTreg differentiation. Notably, SIRP<sup>−/−</sup> cDCs, which have been shown to be the most potent DC subpopulation for nTreg instruction [4], were not affected in Relb<sup>TEC-KO</sup> mice, while CD8<sup>+</sup> SIRP<sup>−/−</sup> cDCs, which were reduced in Relb<sup>TEC-KO</sup> mice, have very recently been shown to be dispensable for nTreg induction [43].

The strong reduction of mTECs in Rela<sup>C-Rel</sup>TEC-DKO mice demonstrates that classical NF-κB signaling plays a cell-intrinsic role in mTEC development, which seems to be as important as the alternative NF-κB pathway. The finding that Relab<sup>−/−</sup> Relb<sup>−/−</sup> TEC-DKO mice still contained few mature mTECs and didn’t develop spontaneous autoimmunity might be due to the compensation of the RelA and c-Rel function in classical NF-κB signaling by Relb. This is supported by the finding that Relb is recruited into a complex with IkBz in Rela<sup>/−</sup> c-Rel<sup>/−</sup> MEFs, rendering it inducible by classical NF-κB signaling after TNF stimulation. A similar mechanism was previously described for DCs, in which strong upregulation of Relb during maturation elicited its incorporation into an IkBz–complex [41].

Since ablation of RelA resulted in decreased Relb mRNA levels in a diminished number of mTECs, which could be partly rescued by TEC-specific expression of a Relb transgene, our data show that one main function of classical NF-κB signaling in mTECs is to upregulate the expression of Relb. The experiments with TNF-stimulated MEFs further show that this crosstalk between classical and alternative NF-κB signaling is based on a direct transcriptional regulation of Relb by RelA and c-Rel and probably also underlies the overlapping roles of RelA and c-Rel in mTEC development.

Classical NF-κB signaling is one pathway among others that can be activated by several receptors via the ubiquitin ligase Traf6 [28]. Traf6 has been shown to be essential for mTEC development [27,38], however the involved downstream signaling pathways were not clear. Our finding that the block in mTEC development due to Traf6-deficiency can be overcome by expression of a Relb transgene indicates that Relb upregulation by RelA and c-Rel acts downstream of Traf6 in mTECs. However, this does not exclude that other Traf6-dependent pathways as well might play a role. This is suggested by the less efficient rescue of mature mTEC numbers by Relb transgene expression in Traf6<sup>TEC-KO</sup> compared to Relb<sup>TEC-KO</sup> mice. For instance, IRF7, which can be activated by Traf6 [28], has recently been reported to be important for mTEC development [44].

Interestingly, a recent publication showed that activation of alternative NF-κB signaling in mTECs by inactivation of Trafβ could completely revert the strong decrease of mTEC numbers in Cd40<sup>−/−</sup> LTr<sup>−/−</sup> mice, but not in Rankl<sup>−/−</sup> mice, demonstrating that a pathway distinct from alternative NF-κB signaling is essential for mTEC development downstream of Rankl [45]. This suggests that the induction of Relb by RelA and c-Rel downstream of Traf6 is initiated primarily by Rankl stimulation on mTECs and that Cd40- and LTr<sup>−/−</sup> stimulation contribute secondarily, since mTECs are not completely absent in Rankl<sup>−/−</sup> mice. Whether Rankl is also needed for activation of alternative NF-κB signaling in mTECs remains to be addressed.

We found that activation of NF-κB and high expression of Relb was predominantly confined to CD80<sup>hi</sup> mature mTECs. This was quite surprising taking into consideration that Relb<sup>TEC-KO</sup>, as well as Relab<sup>−/−</sup> Relb<sup>−/−</sup> and Traf6<sup>TEC-KO</sup> mice not only loose CD80<sup>hi</sup> mature mTECs, but also their presumed CD80<sup>hi</sup> immature progenitors. While Relb is dispensable for the development and maintenance of the mTEC stem cells [46], it cannot be excluded that low Relb levels in CD80<sup>hi</sup> mTECs are essential for the differentiation of the whole mTEC lineage and high levels of Relb in CD80<sup>hi</sup> mTECs fulfill an additional and different role. For example, high expression of Relb in mature DCs has been shown to be important for upregulation of the surface proteins CD80, MHC II and CD40 [41], which are also expressed on mature mTECs. However, alternatively, high Relb levels in mature mTECs might be essential for the expression of so far unknown mediators, which directly or indirectly control the differentiation of early progenitors toward the mTEC lineage in a feedback mechanism. One such factor among others might be CCL21, a target gene of alternative NF-κB signaling [17]. It is expressed by mTECs which either directly derive from Aire<sup>+</sup> mTECs or are promoted by them in their development [47]. CCL21 is involved in medulla recruitment of positively selected thymocytes, which have been shown to also induce differentiation of mTECs [13,48]. Furthermore, the findings that CCL21-expressing mTECs as well as terminally differentiated involucrin<sup>+</sup> Aire<sup>+</sup> mTECs, which also derive from Aire<sup>+</sup> mature cells, have low expression of CD80, suggest that a proportion of CD80<sup>hi</sup> mTECs rather derive from CD80<sup>+</sup> mature mTECs than being their predecessors [11,47]. Thus, the loss of at least some CD80<sup>hi</sup> cells in our mutant mice might be due to a developmental block at the CD80<sup>hi</sup> mature mTEC stage.

Collectively, our findings reveal an mTEC-intrinsic role for classical NF-κB signaling in establishment of self-tolerance. Its crosstalk with alternative NF-κB signaling by inducing the expression of Relb turned out to be essential for development of mature mTECs. We showed that these were important for PTA expression, nTreg induction, iNKT cell development and presence of Sirpα<sup>−/−</sup> migratory DC in thymus and for prevention of autoimmunity in peripheral organs.

Author contributions

M.R. and F.W. conceived of the project; M.R., N.A., M.F., E.M., D.W., H.F. and F.W. designed and performed the research and interpreted the data; R.S.-U., U.K. and Z.-Q.W. facilitated the research; M.R. prepared the figures and wrote the manuscript.

Disclosures

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