Transcription factor c-Rel mediates communication between commensal bacteria and mucosal lymphocytes

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Abstract
The NF-κB transcription factor c-Rel plays a crucial role in promoting and regulating immune responses and inflammation. However, the function of c-Rel in modulating the mucosal immune system is poorly understood. T follicular helper (Tfh) cells and IgA production in gut-associated lymphoid tissues (GALT) such as Peyer’s patches (PPs) are important for maintaining the intestinal homeostasis. Here, c-Rel was identified as an essential factor regulating intestinal IgA generation and function of Tfh cells. Genetic deletion of c-Rel resulted in the aberrant formation of germinal centers (GCs) in PPs, significantly reduced IgA generation and defective Tfh cell differentiation. Supporting these findings, the Ag-specific IgA response to Citrobacter rodentium was strongly impaired in c-Rel-deficient mice. Interestingly, an excessive expansion of segmented filamentous bacteria (SFB) was observed in the small intestine of animals lacking c-Rel. Yet, the production of IL-17A, IgA, and IL-21, which are induced by SFB, was impaired due to the lack of transcriptional control by c-Rel. Collectively, the transcriptional activity of c-Rel regulates Tfh cell function and IgA production in the gut, thus preserving the intestinal homeostasis.

Keywords: commensal bacteria, c-Rel, cytokines, IgA, Peyer’s patches

1 | INTRODUCTION

The NF-κB family of transcription factors comprises 5 subunits, belonging either to canonical or alternative NF-κB pathways. RelA/p50 and c-Rel/p50 dimers compose the most common subunits of the canonical pathway, while RelB/p52 is the heterodimeric form of the alternative NF-κB pathway.\textsuperscript{1,2} Only RelA, RelB, and c-Rel contain a transactivation domain being capable of triggering the transcription of target genes following the nuclear translocation due to activation of NF-κB signaling.\textsuperscript{3} In naïve lymphocytes, NF-κB proteins are localized in the cytoplasm bound to the inhibitor proteins belonging to the family of the inhibitor of NF-κB (IκB).\textsuperscript{4} Upon Ag-specific activation of T and B cells, or stimulation of innate immune cells via TLRs, the upstream protein kinase IKK2 rapidly catalyzes the phosphorylation of IκB, resulting in ubiquitination and degradation of this protein and subsequent

Abbreviations:
GALT, gut-associated lymphoid tissue; GC, germinal center; IκB, inhibitor of NF-κB; ILF, isolated lymphoid follicle; mLN, mesenteric lymph node; PP, Peyer’s patch; SFB, segmented filamentous bacteria; Tfh, T follicular helper; Treg, regulatory T cell

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translocation of active NF-κB dimers into the nucleus. Functional analysis of NF-κB proteins in vivo revealed that both, T cell-dependent B cells responses and the germinal center (GC) B cell development are dependent on the activity of NF-κB, although the contribution of individual subunits is still unclear. Interestingly, the NF-κB transcription factor c-Rel was shown to be indispensable for IgG, but not for IgA responses, suggesting that c-Rel is not required for the class switch to IgA. However, it has to be noted that the serum IgA levels, but not intestinal IgA concentrations were measured in this study.

IgA-producing B lymphocytes and plasma cells are mainly located in the gut-associated lymphoid tissues including Peyer’s patches (PPs), isolated lymphoid follicles (ILFs), cryptopatches, and the cecal patch. IgA derived from the small intestine and colon is secreted as a dimer into the intestinal lumen. Secretory IgA is not only important for preventing invasion of pathogens into the intestinal tissue, but it is also involved in maintaining gut homeostasis. Previous studies revealed that the majority of bacterial species in the gut lumen are bound to IgA and that commensal bacteria utilize secretory IgA for gut colonization thus directly contributing to the host-microbial symbiosis.

Recent observations have indicated the specific role of follicular helper T cells (Tfh), responsible for IgA selection and synthesis in PPs. Defective secretion of intestinal IgA, smaller PPs, and reduced frequencies of Tfh cells have been described for germ-free (GF) mice devoid of microbiota. Thus, intestinal commensals promote IgA production and GC formation in PPs, with Tfh cells essentially supporting this process.

NF-κB activity is associated with B cell development, proliferation, and IgG production. However, whether the NF-κB canonical pathway containing hetero- and homodimers of c-Rel also play an important role in IgA generation has not been investigated in detail yet. We thus examined whether c-Rel is required for regulating the interactions between Tfh cells and Ig-secreting B and plasma cells in the intestine at steady-state conditions.

2 MATERIALS AND METHODS

2.1 Mice

C57BL/6 WT mice were purchased from the Jackson Laboratory. Both WT and rel−/− mice on C57BL/6 background were maintained under specific pathogen-free conditions at the animal facility of BMFZ Marburg, Germany. 8-12-week-old female mice were used for experiments. All experiments were performed in accordance with the protocols approved by the local authorities (Study Nr. Ex-22-2020, RP Giessen, Germany).

2.2 Flow cytometry

The single-cell suspensions derived from PPs, mesenteric lymph nodes (mLN), or intestinal lamina propria (approximately 2 × 10⁶ cells) were stained with FITC-, PE-, PerCP-, or APC-conjugated Abs for 20 min at 4°C, washed, and analyzed using a FACSCalibur flow cytometry analyzer. For intracellular staining of cytokines, the cells were fixed for 20 min with 2% paraformaldehyde, subsequently permeabilized in 0.3% saponin, and stained using anti-IFN-γ-APC and anti-IL-17A-PE (both from eBioscience). For analysis of Foxp3+ Tregs, the commercially available Foxp3 staining kit was used (anti-Foxp3-PE, FJK, FJK-16s, eBioscience). Abs used for phenotypic characterization of B and T lymphocytes include: anti-B220 (RA3-6B2, BD Bioscience), anti-IgA (11-44-2, Southern Biotech), PNA-biotin (Vector Laboratories), biotin conjugates were visualized with PE-streptavidin, eBioscience), anti-CD4 (RM4-5, eBioscience), anti-CD62L (MEL-14), anti-CD44 (IM7, BD Biosciences), anti–CXCR5 (2G8, BD Biosciences), anti–PD-1 (J43, eBioscience), anti–IL-21 (12-7213-82, eBioscience), anti–IL-17A (eBio17B7, eBioscience), and anti–IFN-γ (XMG1.2, eBioscience). All results were analyzed with FlowJo software (Tree Star).

2.3 Immunofluorescence analysis

PP tissue was snap-frozen in OCT Tissue-Tek (Sakura Finetek). 5 μm thick cryosections were generated using the Leica 2800E Frigocut cryostat microtome. The samples were air-dried and incubated with blocking solution for 10 min at 37°C, washed, and analyzed using a FACSCalibur flow cytometry analyzer. For intracellular staining of cytokines, the cells were fixed for 20 min with 2% paraformaldehyde, subsequently permeabilized in 0.3% saponin, and stained using anti-IFN-γ-APC and anti-IL-17A-PE (both from eBioscience). For analysis of Foxp3+ Tregs, the commercially available Foxp3 staining kit was used (anti-Foxp3-PE, FJK, FJK-16s, eBioscience). Abs used for phenotypic characterization of B and T lymphocytes include: anti-B220 (RA3-6B2, BD Bioscience), anti-IgA (11-44-2, Southern Biotech), PNA-biotin (Vector Laboratories), biotin conjugates were visualized with PE-streptavidin, eBioscience), anti-CD4 (RM4-5, eBioscience), anti-CD62L (MEL-14), anti-CD44 (IM7, BD Biosciences), anti–CXCR5 (2G8, BD Biosciences), anti–PD-1 (J43, eBioscience), anti–IL-21 (12-7213-82, eBioscience), anti–IL-17A (eBio17B7, eBioscience), and anti–IFN-γ (XMG1.2, eBioscience). All results were analyzed with FlowJo software (Tree Star).

2.4 Infection with Citrobacter rodentium

All mice used in the experiments were 8–12 weeks old females. Animals were orally gavaged with 1 × 10¹⁰ colony forming units (CFU/ml) of naldixid acid-resistant C. rodentium in a total volume of 200 μl of PBS. For analysis of bacterial burden, colonic feces from infected WT and c-Rel-deficient mice were collected into tubes containing LB medium. Serial dilutions were made from the collected samples and the number of colonies was determined at day 14 post-infection.

2.5 Fluorescence in situ hybridization

Small intestinal tissues were fixed overnight using the methacarn fixative solution prior to embedding into paraffin. For FISH analysis, 3–5 μm thin tissue sections derived from the terminal ileum were deparaffinized and treated with 100 μl 4% lysozyme solution for 45 min at 37°C. After washing, 100 μl hybridization solution containing DNA probes (0.5 pMol/μl) were added and incubated for 3.5 h at 52°C. Tissue sections were washed several times in FISH washing buffer and dried at RT before being mounted with ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). The samples were documented at a Leica DM 5500 wide-field microscope (Leica) and images were analyzed with ImageJ (National Institutes of Health). The
following labeled fluorescence DNA probes were used: FITC-labeled DNA probe 5′-GCTGCTCCCTAGGAGT-3′ for all bacteria, and Cy3-labeled DNA probe 5′-GGTACTTATTGCGTTTGCGACGCAC-3′ for SFB.

### 2.6 Enzyme-linked immunosorbent assay

For detection of fecal IgA small intestinal feces were homogenized in PBS and centrifuged at 12,000 rpm for 5 min. IgA in fecal supernatants was measured using a primary rabbit-anti-mouse IgA Ab (Rockland), and a secondary rabbit-anti-mouse IgA-HRP Ab and TMB solution (R&D Systems). Reaction was stopped by adding H₂SO₄ into plate wells. To detect _C. rodentium_-specific IgA in feces, ELISA plates were coated with 50 μl of _C. rodentium_ lysate (10 μg/ml) and incubated overnight. The plates were washed with washing buffer and blocked with 3% BSA for 2 h. Afterward, the supernatants of feces were added to the plate. After incubation overnight, the plates were washed and incubated with anti-mouse IgA-HRP (SouthernBiotech). The IgA concentration was measured by absorbance at 450 nm with an FLUOstar Omega ELISA Reader (BMG Labtech).

### 2.7 Statistical analysis

Statistical analysis was performed using Student’s t-test. All results are expressed as mean± SEM, unless otherwise indicated. A _P_ < 0.05 was
FIGURE 2 Reduced generation of IgA in the intestine of rel−/− mice. (A and B) Flow cytometry analysis showing the percentage of IgA+ B cells in PPs of WT and rel−/− mice (n = 5 mice per group). (C) Total IgA concentration in feces of the small intestine (n = 5 mice per group). IgA amounts were measured by ELISA. (D) C. rodentium burden in colonic feces of WT and rel−/− mice on day 14 post-infection. Animals were infected with 1 × 10^{10} CFU/ml of C. rodentium (n = 5 mice per group). (E) Ag-specific IgA production upon infection with mucosal pathogen C. rodentium in feces of the small intestine was measured on day 14 post infection (n = 5 mice per group). The Student’s t-test was used for comparison of groups (**P < 0.01; data are shown as mean ± SEM).

considered statistically significant. The calculations were performed using GraphPad Prism 8.

3 | RESULTS AND DISCUSSION

3.1 | Mucosal T and B lymphocytes from c-Rel-deficient mice exhibit several functional defects

The analysis of lymphocytes derived from rel−/− mice revealed that both T and B cells require c-Rel for various aspects of their activity. Although mucosal and systemic immune systems contain the same T cell subsets, only intestinal T lymphocytes are constitutively activated by Ags and molecules originating from commensal microbiota and food. Previously, we and others showed that the development of thymic Foxp3+CD4+ regulatory T cells (Tregs) is regulated by the activity of NF-κB subunit c-Rel. We accordingly observed lower frequencies of Foxp3+ Tregs in mucosa-associated lymphoid tissue (MALT) such as PPs and mLNs in the absence of c-Rel (Supplementary Fig. S1A,B). Interestingly, despite low Treg percentages in mice lacking c-Rel, these animals did not develop overt autoimmune or inflammatory responses mediated by effector CD4+ T or B cells. When we searched for activated and memory T cells using the activation marker CD44 (CD44+CD62L− CD4+ T lymphocytes), we observed significantly decreased frequencies of effector/memory CD4+ T cells in mLNs and PPs of c-Rel-deficient mice as compared to WT counterparts, which might explain the lack of autoimmunity and inflammation in aged rel−/− mice (Supplementary Fig. S1C,D).

Interestingly, despite the normal formation of PPs and a comparable absolute number of PP lymphocytes, as well as a similar ratio of T and B cells in WT and c-Rel-deficient mice, a strong reduction in frequencies of Tfh cells in PPs of rel−/− mice was observed (Fig. 1A-D; Supplementary Fig. S2A, B). Tfh cells are CD4+ T cells favoring and mediating the Ab isotype switching to the IgA isotype in germinal centers of PP due to continuous microbial Ag exposure. To determine whether Tfh-dependent GC development and the instruction of GC B cells to produce IgA is affected in c-Rel-deficient mice, we examined GC formation by fluorescence microscopy, as well as the percentages of GC-resident activated B lymphocytes and the frequency of IgA+ B cells by flow cytometry. Transcription factor c-Rel was previously shown to regulate proliferation of B lymphocytes and humoral immunity. Defective Ab production in naive and immunized mice was particularly demonstrated for IgG1 and IgG2a but not for IgG3 and IgM in the absence of c-Rel. GCs within PPs generate high-affinity IgA against food and bacterial antigens. We here demonstrate that both, the formation of GCs as well as that of highly activated GC PNA+ B220+ B cells were significantly impaired in c-Rel-deficient mice (Fig. 1E, F). Furthermore, flow cytometry analysis revealed that mice lacking c-Rel have low levels of membrane-bound IgA on PP-derived B cells indicating that the generation of IgA within GCs is crucially dependent on the activity of...
**FIGURE 3** Expansion of segmented filamenotous bacteria (SFB) in the gut of c-Rel-deficient mice. (A) The distribution of SFB in the small intestine of WT and c-Rel-deficient mice was analyzed by FISH, hybridizing with a SFB-specific probe (red). All bacteria are detected with a FITC-labeled probe (green). Cell morphology was visualized by DAPI staining. The quantification of fluorescence intensity for SFB at the villus tip ($n = 10$ animals per group) is shown in the right panel. (B and C) Analysis of percentages of IFN-γ$^+$ and IL-17A$^+$ CD4$^+$ T cells in PPs and small intestine (SI) of WT and c-Rel-deficient mice ($n = 4$ mice per group). (D) Representative FACS histogram showing the expression of IL-21 in PP T cells (left panel). The frequency of IL-21$^+$ cells within PP CD4$^+$ T lymphocytes is presented in the right panel ($n = 4$ mice per group). The Student's t-test was applied for statistical analysis ($^{**}P = 0.001-0.01; ^{***}P < 0.001; data are shown as mean ± SEM$)
c-Rel (Fig. 2A, B). Moreover, mice lacking this transcription factor had also substantially decreased percentages of IgA+ B220− plasma cells in the intestinal lamina propria (Supplementary Fig. S3A,B). Compared to WT mice, IgA concentrations in feces were decreased in animals lacking c-Rel (Fig. 2C). To further examine the impact of c-Rel on generating Ag-specific IgA responses in vivo, we orally infected c-Rel-Deficient and WT mice with the murine intestinal pathogen C. rodentium. Of note, c-Rel-deficient mice showed an increased susceptibility to C. rodentium infection than WT mice (Fig. 2D). Consistent with this, the infection with C. rodentium induced significantly higher amounts of pathogen-specific IgA antibodies in the intestine of WT mice as compared to animals lacking c-Rel (Fig. 2E). Taken together, the function of intestinal T and B lymphocytes that sense Ags of commensals and pathogens is strongly dependent on the activity of the transcription factor c-Rel.

3.2 Aberrant growth of segmented filamentous bacteria in the intestine of c-Rel deficient mice

Previously, it was shown that AID−/− mice lacking the enzyme activation-induced cytidine deaminase (AID) display defective intestinal IgA production together with the dominant and persistent outgrowth of segmented filamentous bacteria (SFB) in the small intestine.21 Conversely, reconstitution of AID−/− mice with IgA resulted in significantly decreased SFB population and reestablishment of the bacterial homeostasis. Recently, it was shown that IgA controls the composition, metabolic function, and selection of microbial communities in the intestinal lumen.22 Because both, rel−/− and AID−/− mice, have a similar reduced capacity to produce intestinal IgA, we wondered if mice lacking c-Rel exhibit a higher intestinal abundance of SFB than WT animals. We applied fluorescent in situ hybridization (FISH) to monitor the distribution of SFB in the small intestine of c-Rel-deficient mice. Visualization by FISH revealed a presence of SFB in the intestinal lumen of c-Rel-deficient animals. Indeed, we detected a significant amount of SFB tightly bound to the ileal epithelium in mice lacking c-Rel, whereas these bacteria were rarely observed in the gut of WT animals (Fig. 3A). SFB are bacteria known to continuously shape the intestinal immune responses by inducing Th17 and Tfh cell differentiation, as well as intestinal IgA production. Germ-free (GF) mice mono-colonized with SFB have a similar generation of IL-17A and IgA as mice with a normal microbiota, indicating the central role for this bacterium in shaping the small intestinal immune system.23,24 Thus, we next investigated the IL-17A production by Th17 cells in both WT and rel−/− animals during intestinal homeostasis. Interestingly, in spite of the extensive SFB expansion in c-Rel-deficient animals, we observed significantly reduced levels of IL-17A in PPs and small intestinal lamina propria, indicating the lack of direct or indirect transcriptional control of this cytokine (Fig. 3B and C). We and others have recently described a defective secretion of IL-23 in the gut of c-Rel-deficient mice, which might explain the reduced IL-17A production.25,26 Similarly, we found a defective expression of IL-21 (Fig. 3D), a Tfh and Th17 cell-derived cytokine, which is also induced following the colonization of GF mice with SFB.25 Previously, a direct binding of c-Rel to the Il21 promoter sequence was described.26 Notably, although the lack of intestinal IgA production in c-Rel-deficient mice was associated with a dominant expansion of SFB, IL-17A, and IL-21 cytokines, which are normally induced by this bacterium, were reduced in the absence of c-Rel. Together, these findings indicate that c-Rel is essential for the appropriate communication of the mucosal immune system with commensal bacteria, resulting in a stable composition of gut microbiota, formation, and secretion of protective IgA and cytokines IL-21 and IL-17A.

4 CONCLUDING REMARKS

In conclusion, our data suggest that the transcription factor c-Rel regulates several aspects of bidirectional communications between commensal bacteria and the gut immune system. The PPs are inductive sites crucial for Tfh cell-dependent production of IgA, which is the most important antibody isotype for gut homeostasis and regulation of immune responses to commensals. While the size and number of PPs distributed along the small intestinal tissues were not altered in c-Rel-deficient mice, we observed the reduced formation of GCs in PPs, with consequently impaired differentiation of Tfh and decreased IgA secretion in the absence of c-Rel. These homeostatic defects led to an aberrant outgrowth of SFB without subsequent activation of the mucosal immune system due to the lack of transcriptional regulation of IgA, IL-17A, and IL-21.

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DATA AVAILABILITY STATEMENT

All results generated and examined in this study are available from the corresponding author on reasonable request.

AUTHORSHIP

A.V., M.L., B.S., and U.S. designed the study. M.L., S.H., and M.K. performed the experiments with B and T cells, J.B. and R.R. performed the infection experiments. K.B. and B.S. performed and evaluated the FISH experiments. All authors approved the final version of the manuscript.

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DISCLOSURE

The authors declare no conflict of interest.

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