Differential requirements for the canonical NF-κB transcription factors c-REL and RELA during the generation and activation of mature B cells

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Signaling through the canonical nuclear factor-κB (NF-κB) pathway is critical for the generation and maintenance of mature B cells and for antigen-dependent B-cell activation. c-REL (rel) and RELA (rela) are the downstream transcriptional activators of the canonical NF-κB pathway. Studies of B cells derived from constitutional rel knockout mice and chimeric mice repopulated with rela–/– fetal liver cells provided evidence that the subunits can have distinct roles during B-cell development. However, the B cell-intrinsic functions of c-REL and RELA during B-cell generation and antigen-dependent B-cell activation have not been determined in vivo. To clarify this issue, we crossed mice with conditional rel and rela alleles individually or in combination to mice that express Cre-recombinase in B cells. We here report that, whereas single deletion of rel or rela did not impair mature B-cell generation and maintenance, their simultaneous deletion led to a dramatic reduction of follicular and marginal zone B cells. Upon T cell-dependent immunization, B cell-specific deletion of the c-REL subunit alone abrogated the formation of germinal centers (GCs), whereas rela deletion did not affect GC formation. T-independent responses were strongly impaired in mice with B cell-specific deletion of rel, and only modestly in mice with RELA-deficient B cells. Our findings identify differential requirements for the canonical NF-κB subunits c-REL and RELA at distinct stages of mature B-cell development. The subunits are jointly required for the generation of mature B cells. During antigen-dependent B-cell activation, c-REL is the critical subunit required for the initiation of the GC reaction and for optimal T-independent antibody responses, with RELA being largely dispensable at this stage.

Immunology and Cell Biology (2017) 95, 261–271; doi:10.1038/icb.2016.95

Received 10 June 2016; revised 29 August 2016; accepted 13 September 2016; article preview online 21 September 2016; advance online publication, 18 October 2016

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However, the extent to which c-REL and RELA, either individually or in combination, contribute to mature B-cell development is unclear.

With regard to antigen-dependent B-cell development, the canonical NF-κB pathway shows a biphasic activation pattern during T cell-dependent B-cell activation and the germinatal center (GC) reaction where antigen-activated B cells undergo somatic hypermutation and class switch recombination. However, nuclear translocation of NF-κB is observed rapidly upon B-cell activation. NF-κB signaling is not activated in the majority of GC B cells. However, the canonical NF-κB subunits show nuclear translocation in a small subset of B cells in the light zone of the GC. We recently demonstrated that c-REL and RELA exert distinct functions in these light zone B cells. Thus, conditional deletion of rel in GC B cells revealed that c-REL is required for the maintenance of the GC reaction, whereas RELA was found to be dispensable at this stage. Although it is clear that c-REL and RELA have specific roles in late B-cell development, the B cell-intrinsic functions of the separate subunits during the antigen-dependent B-cell activation and the initiation of the GC reaction have not been determined in vivo. However, this is a particularly relevant issue in the light of recent studies that demonstrated distinct functions for the same transcription factors in the early initiation of the GC reaction and later in the established GC. For example, c-MYC is required for the formation of the GC reaction and in a later developmental stage for GC maintenance. Similarly, interferon regulatory factor 4 (IRF4) controls the formation of the GC upon T-dependent immunization and in the established GC is required for plasma cell differentiation.

Several observations imply critical roles for c-REL and RELA during B-cell activation. A recent study demonstrated that the two subunits follow a different activation pattern in B cells stimulated with anti-IgM in vitro, suggesting that RELA and c-REL may exert unique functions upon antigen encounter also in vivo. RELA-deficient fetal liver-derived B cells proliferated similarly to wild-type B cells upon mitogenic stimulation in vitro. In contrast, c-REL-deficient B cells showed defects in activation and proliferation following mitogenic stimulation.

**Figure 1** Combined c-REL and RELA deficiency leads to a severe reduction in splenic B cells. (a) Percentage and (b) number of splenic B cells in rel<sup>fl/fl</sup>rela<sup>fl/fl</sup>CD19-Cre and the corresponding heterozygous and CD19-Cre control mice as determined by flow cytometry. (c) Flow cytometry of GFP expression in splenic B cells of the indicated genotypes. The numbers below the gate indicate the percentage of GFP<sup>+ </sup>B cells of among of B220<sup>+</sup> B cells of rel<sup>fl/fl</sup>rela<sup>fl/fl</sup>CD19-Cre and rel<sup>fl/+</sup>rela<sup>fl/+</sup>CD19-Cre mice (left). Summary of the frequency of GFP<sup>+</sup> cells among the corresponding B-cell subsets (right). (a–c) Data are cumulative from independent experiments (n=4–9 per group), with each symbol representing a mouse. Data are shown as mean ± s.d. Statistical significance was determined by Student’s t-test (*P<0.05; **P<0.01; ***P<0.001).
The study of chimeric mice repopulated with splenic B cells and also led to a strong reduction of activated B cells dramatically impaired the formation of GCs upon T-dependent immunization and also led to a strong reduction of activated B cells dramatically impaired the formation of GCs upon T-dependent immunization. c-REL deficiency in B cells also have impaired activation and proliferation upon T-cell receptor stimulation. Numbers below gates indicate the percentage of eGFP+ B cells among the indicated B-cell subsets (left). Data are cumulative from independent experiments (n=4 per group), with each symbol representing a mouse, showing the frequency of eGFP+ cells among the corresponding B-cell subsets (right). Each symbol represents a mouse. Data are shown as mean ± s.d. Statistical significance was determined by Student’s t-test (**P<0.01).

Figure 2 re^{fl/fl} rel^{fl/fl} CD19-Cre mice display fewer B-cell foci within the splenic white pulp compared with controls and are characterized by counter selection against rel/rela-deleted MZ B cells. (a) Spleen sections from mice of the indicated genotypes were analyzed via immunohistochemistry (IHC) for the expression of CD3 and IgM. One representative mouse of three per group is shown. Original magnification ×40 (left) and ×100 (right). (b) The fractions of eGFP+ cells among splenic follicular (FO; CD23+CD21int) and marginal zone (MZ; CD21hiCD23-) B cells in re^{fl/fl} rel^{fl/fl} CD19-Cre mice were determined by flow cytometry. (c) Spleen sections from mice of the indicated genotypes were analyzed by IHC for the expression of eGFP and IgM. One representative mouse of three per group is shown. Original magnification ×200 (left) and ×400 (right).

in vitro,14-16 and rel^{−/−} and rel^{ΔTAD/ΔTAD} mice displayed an impaired T-dependent B-cell response in vivo. Accordingly, constitutional rel knockout mice were characterized by the appearance of smaller GCs relative to control mice.15,32 As rel^{−/−} T cells also have impaired activation and proliferation upon T-cell receptor stimulation in vitro,14 it is unclear to what extent the defective T-dependent B-cell response and GC formation are due to the loss of c-REL function in B cells.

We here deleted rel and/or rela conditionally in B cells in order to unequivocally identify the specific, B cell-autonomous roles of c-REL and RELA in the generation and maintenance of mature B cells and in T-dependent and T-independent immune responses in vivo. We found that whereas c-REL and RELA were functionally redundant during B-cell generation and maintenance, c-REL deficiency in antigen-activated B cells dramatically impaired the formation of GCs upon T-dependent immunization and also led to a strong reduction of T-independent responses. Conversely, RELA deficiency in B cells did not impede GC formation and led to only a modest reduction in serum immunoglobulin (Ig) levels upon T-independent immunizations.

RESULTS
Combined c-REL and RELA deficiency leads to a severe reduction in splenic B cells
The study of chimeric mice repopulated with rel^{−/−} rela^{−/−} fetal liver hematopoietic stem cells implicated a role for both c-REL and RELA in the generation of mature B cells.17 To determine the extent of the B cell-intrinsic requirement of c-REL and RELA in the generation and maintenance of mature B cells in vivo, we crossed mice with conditional rel and rela alleles to CD19-Cre mice that express Cre recombinase in B cells to jointly ablate the canonical NF-κB subunits in B cells. We observed a marked reduction in the fraction and cell number of splenic B cells in rel^{fl/fl} rela^{fl/fl} CD19-Cre mice vs rela^{fl/fl} CD19-Cre and CD19-Cre control mice (Figures 1a and b). B220+ cells comprised ~31% of total splenocytes in rel^{fl/fl} rela^{fl/fl} CD19-Cre mice vs ~52% and ~48% in rel^{fl/fl} rela^{fl/fl} CD19-Cre and CD19-Cre control mice, respectively, and, in total cell numbers, ~7 × 10^6 × B220+ cells were present in rel^{fl/fl} rela^{fl/fl} CD19-Cre mice compared with ~24 × 10^6 and ~27 × 10^6 B220+ cells in the control mice. In the conditional mice, rel and rela deletion is concomitantly linked to the expression of an enhanced green fluorescent protein (eGFP) gene that allows the tracking of the rela-deleted B cells in the tissues. Analysis for eGFP expression among B cells of rel^{fl/fl} rela^{fl/fl} CD19-Cre mice revealed distinct eGFP+ and eGFP− peaks of equal proportions (~49% eGFP+ vs ~51% eGFP− B cells; Figure 1c); in contrast, ~67% of B cells of rel^{fl/fl} rela^{fl/fl} CD19-Cre mice were eGFP+ (Figure 1c). This indicates that eGFP+ B cells double deficient for c-REL and RELA were outcompeted by eGFP− B cells that escaped Cre deletion. Rela-deleted eGFP+ B cells therefore represented only ~14% of total splenocytes in rel^{fl/fl} rela^{fl/fl} CD19-Cre mice compared with ~52%
observed in the CD19-Cre control mice (total cell numbers, \( \sim 3 \times 10^6 \) rela-fl/fl CD19-Cre B cells were present in rela-fl/flCD19-Cre mice vs \( \sim 29 \times 10^6 \) B cells in the control mice; Figures 1a and b).

Immunohistochemistry analysis of spleen sections for IgM and CD3 revealed that, in line with the severely reduced B-cell fraction observed by flow cytometry, rela-fl/flCD19-Cre mice had fewer B-cell follicles within the splenic white pulp relative to rela-fl/flCD19-Cre and CD19-Cre control mice (Figure 2a).

The B-cell follicles in rela-fl/flCD19-Cre mice were also more heterogeneous in size compared with the controls. Together, the simultaneous, B cell-specific deletion of rel and rela leads to a severe reduction in the number of splenic B cells that is reflected by abnormalities in the architecture of the white pulp.

Counterselection against rela-fl/fl-deleted MZ B cells

To investigate whether B cell-specific ablation of c-REL or RELA individually or in combination affects the development of mature B-cell subsets in the spleen, we performed flow cytometry analysis for CD23 and CD21 expression on splenocytes from rela-fl/flCD19-Cre, rela-fl/flCD19-Cre and rela-fl/flCD19-Cre litterate controls to determine the fractions of follicular (FO) B cells (B220^CD23^CD21^hi) and marginal zone (MZ) B cells (B220^CD23^CD21^int). With regard to the single conditional knockout, the results revealed only minor (c-REL cohort) or no significant (RELA cohort) differences in the fractions of FO and MZ B-cell subsets (Supplementary Figure 1), or in the fractions of IgM^+IgD^hi vs IgM^+IgD^lo B cells (data not shown), among rela-fl/flCD19-Cre or rela-fl/flCD19-Cre mice and the corresponding heterozygous and wild-type controls. In accordance, analysis of hematoxylin and eosin-stained sections showed normal splenic architecture in both mouse models (data not shown). These results are in line with earlier observations based on the analysis of constitutional knockout mice that c-REL and RELA have redundant functions in the generation and maintenance of mature B cells.1,2,14-16

In contrast to the single conditional knockouts, rela-fl/flCD19-Cre mice displayed a reduced fraction of FO B cells compared with rela-fl/flCD19-Cre and CD19-Cre mice (\( \sim 43\% \) vs \( \sim 77\% \)), with no significant increase in the fraction of MZ B cells (Supplementary Figure 2). However, analysis of eGFP^+ cells in the MZ B-cell compartment of rela-fl/flCD19-Cre mice revealed a strong counter-selection against rela-fl/fl-deleted MZ B cells that surpassed that observed in the FO B-cell compartment (Figure 2b). Specifically, only \( \sim 26\% \) of MZ B cells in these mice were eGFP^+ (and thus rela-fl/fl-deleted) compared with \( \sim 48\% \) of FO B cells. In accordance with the flow cytometry results, immunohistochemistry analysis of splenic sections revealed that the majority of B cells in the MZ of rela-fl/flCD19-Cre mice were eGFP negative, in contrast to B cells in the FO area that mostly stained for eGFP (Figure 2c). This severe counterselection against rela-fl/fl-deleted MZ B cells is consistent with the published observation that deletion of upstream regulators of the canonical NF-κB pathway causes impaired development and/or persistence of MZ B cells.1,2,9

Mature B cells require c-REL and RELA for their survival

To determine whether c-REL/RELA deficiency in mature B cells impairs survival in vitro, we stimulated B cells from rela-fl/flCD19-Cre or CD19-Cre control mice with B cell-activating factor (BAFF), which is required for the survival of mature B cells,34 for 3 days. Although BAFF is a strong activator of the alternative NF-κB pathway, it partly signals also through the canonical pathway,35,36 and it was recently shown that c-REL and RELA indeed undergo nuclear translocation in B cells following BAFF stimulation.37 The results showed significantly enhanced cell death in the cultures of c-REL/RELA-deficient vs control B cells in response to BAFF stimulation (\( \sim 70\% \) vs \( \sim 27\% \)) by propidium iodide (PI) staining and \( \sim 60 \% \) vs \( \sim 15\% \) by annexin V7-AAD staining (7-AAD) staining; Figure 3). These findings provide additional evidence that the canonical NF-κB pathway transmits signals derived from BAFF-mediated activation35-37 and may explain in part the importance of canonical NF-κB signaling for mature B-cell maintenance.5,7

Combined c-REL and RELA deficiency does not impair B-cell maturation in the bone marrow

In CD19-Cre mice, the Cre recombinase is expressed from the pre-B-cell stage in the bone marrow (BM). Therefore, we were able to determine whether the reduction in mature B cells in the spleen may be the result of a defect during B-cell maturation in the BM of rela-fl/flCD19-Cre versus rela-fl/flCD19-Cre and CD19-Cre mice by flow cytometric analysis for pro-B (CD93+B220^hiIgM^+),
pre-B (CD93hiB220intIgM+) and mature (CD93loB220+) B cells. We observed that both the fraction and cell number of immature B cells did not differ among the genotypes (Supplementary Figure 3). However, in accordance with the observed reduction of mature B cells in the periphery, mature B cells were significantly reduced in the BM of relafl/flCD19-Cre mice compared with the control mice (~23% compared with ~70% and ~58% in the control mice; Supplementary Figure 4). Instead, most splenic B220+ cells in relafl/flCD19-Cre mice were CD24low immature B cells (~48% compared with ~16% and ~26% in the control mice; Supplementary Figure 4), a population that comprises transitional B cells that are the precursors of mature B cells. Transitional (T) B cells are further distinguished into recent immigrants from the BM (transitional 1 (T1)) that differentiate into T2 and T3 B cells.17

Combined c-REL and RELA deficiency leads to a developmental block at the T1 stage

To determine whether the canonical NF-κB subunits are functionally required during the B-cell transitional phase (T1 to T3) development, we analyzed splenic B cells from relafl/flCD19-Cre and control

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**Figure 4** Combined c-REL and RELA deficiency leads to a developmental block at the T1 stage. (a) IgM and CD23 expression of CD93+ (AA4.1+) splenic B cells from mice of the indicated genotypes were analyzed by flow cytometry. Numbers beside gates indicate the percentage of T1 (CD93+IgMhiCD23−), T2 (CD93+IgMhiCD23+) and T3 (CD93+IgMloCD23−) B cells (left). Summary of the frequencies of T1–T3 B cells (right). Data are cumulative from independent experiments (n = 4–9 per group), with each symbol representing a mouse. Data are shown as mean ± s.d. Statistical significance was determined by Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001). (b) The fractions of eGFP+ cells among splenic T1 B cells in relafl/flCD19-Cre and relafl/flCD19-Cre mice were determined by flow cytometry. Numbers beside gates indicate the percentage of T1 (CD93+IgMhiCD23−) B cells (left). Summary of the frequencies of T1–T3 B cells (right). Data are cumulative from independent experiments (n = 4–9 per group), with each symbol representing a mouse, showing the frequency of eGFP+ cells among the corresponding B-cell subsets (right).
Figure 5 Impaired GC formation in mice with deletion of rel, but not rela, in B cells. \textit{rel}^{fl/fl}CD19-Cre, \textit{rel}^{fl/+}CD19-Cre and CD19-Cre littermates (a–c), and \textit{rela}^{fl/fl}CD19-Cre, \textit{rela}^{fl/+}CD19-Cre and CD19-Cre littermates (d–f) were immunized with SRBC and analyzed 5 days later. (a, d) CD95, peanut agglutinin (PNA) and eGFP expression by splenic B cells were analyzed by flow cytometry. Numbers above gates indicate the percentage of CD95^{hi}PNA^{hi} (dot plots) or eGFP^{+}CD95^{hi}PNA^{hi} (histograms) GC B cells. (b, e) Spleen sections from mice of the corresponding genotypes were analyzed for the expression of BCL6 and IgG1 or IgM; IgG1 stainings were counterstained with hematoxylin. One representative mouse out of three per group is shown. (c, f) CD138 and eGFP expression by splenic mononuclear cells from mice of the corresponding genotypes were analyzed by flow cytometry. Numbers below gates indicate the percentage of CD138^{hi}eGFP^{+} or CD138^{hi}eGFP^{−} cells. (a, c, d, f) Data are cumulative from independent experiments (\textit{n}=3–5 per group), with each symbol representing a mouse. Data are shown as mean ± s.d. Statistical significance was determined by Student’s \textit{t}-test (*\textit{P}<0.05; **\textit{P}<0.01; ***\textit{P}<0.001).
mice for their expression of CD93\(^-\)IgM\(^{hi}\)CD23\(^-\) (T1), CD93\(^+\)IgM\(^{hi}\)CD23\(^+\) (T2) and CD93\(^+\)IgM\(^{hi}\)CD23\(^+\) (T3) B cells.\(^{39}\) We found a significantly increased fraction of T1 B cells with a concomitant reduction in the populations of T2 and T3 B cells in ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre mice compared with the CD19-Cre littermate controls (~40% vs ~20% T1 B cells, ~33% vs ~52% T2 B cells and ~7% vs ~15% T3 B cells) (Figure 4a). Of note, in contrast to what we observed for the MZ B cells (Figure 2b), there was no counterselection of rela/rela-deleted eGFP\(^+\) T1 cells in ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre vs ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre mice (~83% were eGFP\(^+\) in both genotypes; Figure 4b). To further characterize the block in the T1 to T2 transition, we determined the T1/T2 correlation as described by Derudder et al\(^{9}\) that in normal mice is positive as T2 cells arise from T1 cells.\(^{39}\) As expected, we observed a positive T1/T2 correlation for the CD19-Cre and ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre control mice that however was not observed for the ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre mice (Supplementary Figure 5A). In addition, as CD23—which is used along with other markers to identify transitional B cells—is a potential NF-κB target gene,\(^{40}\) we used CD93 (AA4.1), which is expressed at lower levels on T2 cells,\(^{41,42}\) as an independent marker to confirm the identity of the T1 and T2 B-cell subsets.\(^{39}\) We found that ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre mice harbor a significantly reduced fraction of CD93\(^{lo}\) cells among T2 cells compared with the control mice (Supplementary Figure 5B). In addition, we observed that T1 B cells occurred at equal numbers across all genotypes (~1.4 × 10\(^6\), ~1.4 × 10\(^6\) and ~1.5 × 10\(^6\) in ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre, ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre and CD19-Cre mice, respectively, Figure 4a), indicating normal generation up to the T1 stage in ref\(^{\text{fl}}\)/rela\(^{\text{fl}}\)/CD19-Cre mice. Together, these findings reveal the importance of a c-REL/RELA-controlled biological program at this B-cell developmental stage, in agreement with the results from studies in which the deletion of upstream components of the canonical NF-κB pathway caused a block in the T1 stage of B-cell development.\(^{8,42}\) The underlying mechanism for the T1 to T2 block upon combined c-REL and RELA deficiency in B cells remains to be determined. As T1 cells do not proliferate,\(^{39}\) the T1 to T2 block is unlikely to be associated with cell cycle control. Interestingly, the recent finding that a bcl2-transgene was unable to rescue the T1 to T2 block observed in NEMO-deficient mice\(^{9}\) (NEMO is an upstream regulator of the canonical pathway ultimately resulting in c-REL and RELA nuclear translocation) suggests that activation of the canonical NF-κB pathway may be required for a developmental transition rather than cell survival.

**c-REL is required for the formation of GCs in a B cell-intrinsic manner**

To determine the B cell-intrinsic role of c-REL in the formation of GCs upon T-dependent B-cell activation, ref\(^{\text{fl}}\)/CD19-Cre, rela\(^{\text{fl}}\)/CD19-Cre and CD19-Cre control mice were immunized with the T-dependent antigen sheep red blood cells (SRBC) to induce GC formation, and analyzed 5 days later, that is, when the initiation phase of the GC reaction has been completed and the early GC has formed.\(^{43,44}\) The results showed a marked reduction of CD95\(^hi\)/PNA\(^{hi}\) (Figure 5a) and BCL6\(^hi\) (Figure 5b) GC B cells in the spleens of ref\(^{\text{fl}}\)/CD19-Cre mice, with a concurrent reduction in rel-deleted (eGFP\(^+\)) GC B cells compared with ref\(^{\text{fl}}\)/CD19-Cre mice (Figure 5a). Similarly, the generation of total and eGFP\(^+\) splenic plasma cells was strongly impaired at this time point in ref\(^{\text{fl}}\)/CD19-Cre compared with ref\(^{\text{fl}}\)/CD19-Cre and CD19-Cre control mice (Figure 5c). These findings indicate that c-REL is required for the formation of GCs and for the extrafollicular plasma cell response upon T-dependent immunization.

**RELA is dispensable for the formation of GCs**

The embryonic lethality of rela\(^{-/-}\) mice\(^{10}\) practically impeded the study of the B-cell-intrinsic function of RELA in antibody responses in vivo. To determine the role of RELA in T-dependent B-cell responses, we immunized rela\(^{-/-}\)/CD19-Cre, rela\(^{fl/+}\)/CD19-Cre and CD19-Cre control mice with SRBC. In contrast to what we observed upon rel deletion, rela\(^{-/-}\)/CD19-Cre mice mounted a GC response similar to control mice 5 days after SRBC immunization (Figures 5d and e). Accordingly, the fraction of eGFP\(^+\) GC B cells was similar in rela\(^{-/-}\)/CD19-Cre and rela\(^{fl/+}\)/CD19-Cre mice (Figure 5d). In addition, rela\(^{-/-}\)/CD19-Cre mice generated equal amounts of splenic plasma cells compared with rela\(^{fl/+}\)/CD19-Cre and CD19-Cre control mice (Figure 5f), and showed similar fractions of eGFP-expressing plasma cells compared with rela\(^{-/-}\)/CD19-Cre mice (Figure 5f). Thus, RELA seems to be dispensable for both the formation of GCs and the generation of extrafollicular plasma cells during the T-dependent immune response.

**Mice with B cell-specific deletion of c-REL show strong impairment of T-independent type-II and type-I antibody responses**

To investigate the roles of c-REL and RELA in T-independent type-II and type-I antibody responses against polysaccharides, ref\(^{\text{fl}}\)/CD19-Cre or rela\(^{fl/+}\)/CD19-Cre and the corresponding heterozygous and CD19-Cre control mice were immunized with the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) conjugated to aminoethyl carboxymethyl-Ficoll and assessed for NP-specific IgM and IgG3 serum titers 7 days later. This analysis showed a strong reduction (5–10-fold) in both NP-specific IgM and IgG3 serum titers in ref\(^{\text{fl}}\)/CD19-Cre mice compared with ref\(^{\text{fl}}\)/CD19-Cre and CD19-Cre mice (Figure 6a). rela\(^{-/-}\)/CD19-Cre mice showed a less severe (2-fold) reduction in NP-specific IgM titers, and no significant changes in the corresponding IgG3 titers (Figure 6b).

Type-I T-independent B-cell responses are characterized by the co-activation of antigen-specific B cells through Toll-like receptors such as Toll-like receptor-4, the receptor for lipopolysaccharide (LPS). We immunized ref\(^{\text{fl}}\)/CD19-Cre or rela\(^{fl/+}\)/CD19-Cre and the corresponding heterozygous and CD19-Cre control mice with NP-LPS and analyzed for NP-specific IgM and IgG3 serum titers 7 days later. NP-specific IgM and IgG3 serum titers in ref\(^{\text{fl}}\)/CD19-Cre and rela\(^{fl/+}\)/CD19-Cre mice were strongly reduced (8–12-fold) compared with CD19-Cre mice (Figure 6c), implying that the previously observed reduction in NP-specific IgG3 serum titers upon NP-LPS immunization in ref\(^{-/-}\) mice\(^{13}\) is a B cell-intrinsic effect. In contrast, rela\(^{-/-}\)/CD19-Cre mice showed only a threefold reduction in serum IgM levels compared with controls, but no significant changes in IgG3 levels were observed among the different genotypes (Figure 6d).

Together, the results indicate a more prominent role for c-REL in T-independent type-II and type-I antibody responses in comparison with RELA.

**DISCUSSION**

The B cell-intrinsic roles of the canonical NF-κB transcription factors c-REL and RELA in mature B-cell development and activation are incompletely understood. Our studies revealed differential roles of these subunits during distinct B-cell developmental stages. We found that combined, but not individual, ablation of c-REL and RELA strongly impaired the generation of mature B cells, indicating redundancy of the canonical subunits during this developmental stage. Conversely, the subunits were not functionally redundant during antigen-activation of B cells, as c-REL deficiency alone strongly impaired the formation of GCs and T-independent antibody responses, whereas RELA was dispensable for the initiation of the
The observation that the B cell-specific combined deletion of rel and rela strongly impaired B-cell development is in keeping with previous publications that demonstrated a crucial role for canonical NF-κB signaling in the generation and maintenance of mature B cells.\(^6^\)\(^,^7\)

A recent study by Derudder et al.\(^9\) has more precisely dissected the roles of the canonical pathway in these processes by deleting the upstream regulators \(i k k 2\) or \(n e m o\) in different B-cell developmental stages. Thus, activation through this pathway is required at the T1 stage of development, and—for those cells that have overcome this developmental block—also later in MZ B cells for maintenance and in FO B cells for long-term persistence.\(^9\)

In agreement with these findings, rel/rela-deletion led to a block in the T1 to T2 transition in the spleen. Moreover, the spleens of rel\(^{fl/fl}\)/rela\(^{fl/fl}\)/CD19-Cre mice were characterized by fewer and smaller B-cell follicles (composed predominantly of FO B cells) and a dramatic counterselection against c-REL/RELA-deficient MZ B cells (Figure 2). Of note, Derudder et al.\(^9\) report that a \(b c l 2\)-transgene rescued FO but not MZ B cells in mice with B cell-specific deletion of \(n e m o\). It therefore appears that whereas c-REL and RELA may contribute to the maintenance of FO B cells by upregulating BCL2 expression, in MZ B cells, c-REL and RELA may be required for the establishment of a biological program beyond the control of cell survival.

The generation of a normal mature B-cell compartment requires activation through the alternative NF-κB pathway in addition to the canonical pathway (reviewed in Gardam and Brink\(^{45}\)). In accordance, we recently showed that the functional abolishment of the alternative

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Figure 6 Mice with B cell-specific deletion of c-REL show strong impairment of T-independent type-II and type-I antigen responses. (a) \(\alpha\)-NP IgM (left) and \(\alpha\)-NP IgG3 (right) serum response of rel\(^{fl/fl}\)/CD19-Cre, rel\(^{fl/fl}\)/CD19-Cre and CD19-Cre mice to NP-Ficoll immunization before (Pre) and 7 days after immunization (Post) with NP-Ficoll (left). (b) \(\alpha\)-NP IgM (left) and \(\alpha\)-NP IgG3 (right) serum response of rel\(^{fl/fl}\)/CD19-Cre, rel\(^{fl/fl}\)/CD19-Cre and CD19-Cre mice to NP-Ficoll immunization before (Pre) and 7 days after immunization with NP-LPS (left). (c) \(\alpha\)-NP IgM (left) and \(\alpha\)-NP IgG3 (right) serum response of rel\(^{fl/fl}\)/CD19-Cre, rel\(^{fl/fl}\)/CD19-Cre and CD19-Cre mice to NP-LPS immunization before (Pre) and 7 days after immunization with NP-LPS (left). (a-d) Data are cumulative from independent experiments \((n=2–7 \text{ per group})\), with each symbol representing a mouse. Data are shown as mean±s.d. Statistical significance was determined by Student’s \(t\)-test \((^{*}P<0.05; \, \, ^{**}P<0.01; \, \, ^{***}P<0.001)\).
pathway in B cells via combined deletion of the downstream transcription factors RELB and NF-κB2 strongly impaired the generation and maintenance of mature B cells. How do the B-cell phenotypes observed upon inactivation of the canonical vs the alternative pathway compare qualitatively and quantitatively? By using a similar experimental strategy to conditionally delete the downstream transcription factors of the separate NF-κB pathways, we were able to directly compare the consequences of their inactivation on mature B-cell development. Three observations are evident from this comparison. First, the effects of the ablation of the separate pathways on the size and composition of the mature B-cell compartment were virtually the same (Figures 1 and 2a and De Silva et al.46). In addition, relb/nfkb2-deleted and rel/rela-deleted MZ B-cells were strongly counterselected, comprising only ~25% of MZ B cells in mice of both genotypes (Figures 2b and c and De Silva et al.46). These findings suggest that there is some level of complementation among the canonical and the alternative NF-κB pathways during the generation and maintenance of mature B cells. Second, our observation that relb/nfkb2-deleted and rel/rela-deleted B cells show the same predisposition to undergo apoptosis when cultured with BAFF provides further evidence for the functional requirements of both NF-κB pathways in BAFF signaling.8,67 Third, whereas c-REL and RELA were functionally redundant during the generation and maintenance of mature B cells, single ablation of RELB or NF-κB2 in B cells did impair B-cell development, although to a lesser extent than the combined deletion.46

In contrast to its redundant role during B-cell generation, c-REL was uniquely required for the formation of GCs upon antigen activation in the T-dependent immune response. c-REL is known to be crucial for normal B-cell activation in vitro, as c-REL-deficient B cells showed impaired proliferation in response to several mitogenic stimuli,14-16 a finding that is supported by the observation that B-cell receptor (BCR) stimulation led to a fast nuclear translocation of c-REL during B-cell activation.21 Our previous observation that CD40+IgM-stimulated c-REL-deficient B cells in vitro showed defects in the establishment of a metabolic program that precedes proliferation25 suggests that also upon antigen activation in vivo, c-REL may be required for cell growth and optimal proliferation.

A recent publication demonstrated that inhibition of IKK-induced proteolysis of p105, the precursor of p50, in murine B cells impaired the antigen-induced formation of both GCs and extrafollicular plasmablasts,47 similar to what we described here for rel deletion in B cells. It is therefore possible that the observed phenotype in the p105 mutant mice is due to the inability of these mice to process p105 that thereby prevents the formation and thus nuclear translocation of c-REL/p50 heterodimers. Conversely, the loss of p105 (which acts as an inhibitory κB protein for c-REL and RELA) in nfkbl-/- mice may lead to enhanced c-REL activity in B cells that might contribute to the increased formation of spontaneous GCs in aging mice lacking NF-κB1.48

c-REL shows a biphasic activation pattern during T-dependent B-cell activation and the GC response. c-REL undergoes rapid nuclear translocation upon B-cell activation,21,22 and although there is no active NF-κB signaling in the majority of GC B cells,23,24 nuclear translocation of c-REL is detectable within a small subset of light zone B cells,24 and we have previously shown that it is functionally required for the maintenance of the GC reaction.25 Together with our present observations, this indicates that c-REL is required at two stages of the GC reaction, first during the initial antigen activation phase and later in the fully established GC, presumably during the selection of high-affinity B cells. Thus, our study adds c-REL to a growing list of transcriptional regulators that have critical functions during the formation of GCs and also in a later stage of GC development that include IRF4 and c-MYC.26-29 Similar to rel ablation in all B cells (as opposed to GC-specific ablation), B cell-specific deletion of irf4 dramatically impaired GC formation upon T-dependent immunization,30,31 and c-MYC was found to be required for the initial expansion of GC dark zone cells within the follicle.26 Later, in the established GC, c-REL and c-MYC are required for the maintenance of the GC reaction,35,27 and IRF4 is essential for optimal class switch recombination and plasma cell differentiation.30,31 It will be interesting to determine in future studies to what extent these transcription factors crosstalk among each other49,50 in the different GC B-cell developmental stages.

By deleting rela specifically in GC B cells, we have recently demonstrated that RELA is required for the generation of GC-derived plasma cells that was reflected by a dramatic reduction in the serum levels of NP-specific IgG1 antibodies (>10-fold).25 Whereas we here observed that relb/nfkb2-deleted46 and rel/rela-deleted B cells show only an ~2-fold reduction in NP-specific antibodies upon T-independent immunization, we also found that these mice generated equal fractions of plasma cells early in the extrafollicular response upon T-dependent immunization. These results suggest that RELA deficiency does not affect all types of B-cell activation that lead to the generation of antibody-secreting cells to the same extent. One possible explanation is that c-REL may partly compensate for RELA in the T-dependent extrafollicular and T-independent antibody response, but not during the GC response. It will be interesting to identify the specific transcription factor networks involved in the generation of antibody-secreting cells in the different arms of the humoral immune response.

Integrating the results of our present and published25 studies from the conditional deletion of rel and/or rela in all B cells and GC B cells, the following picture emerges: c-REL and RELA are jointly required during the generation of the mature B-cell compartment and appear to have non-redundant and entirely distinct functions during antigen-dependent B-cell development. c-REL was found to be the critical subunit for the formation and maintenance of GCs, whereas RELA was dispensable for these processes but instead crucial for the differentiation or physiology of GC-derived plasma cells.25 The canonical NF-κB signaling pathway can be aberrantly activated in B-cell malignancies31,52 and in diseases with chronic B-cell activation.53,54 The differential requirements of c-REL and RELA in B-cell activation and differentiation may be exploited for the development of more specific and thus less toxic therapies aimed at inhibiting pathogenic NF-κB signaling in malignant or chronically activated B cells at the level of NF-κB subunits, the feasibility of which has recently been demonstrated for a small-molecule inhibitor of c-REL.55 Our findings provide additional examples for the diverse roles of separate NF-κB subunits that may be relevant for disease therapies.56

MATERIALS AND METHODS

Mice

Conditional rel, rela and CD19-Cre mice have been previously described.25,33 All mice were on a C57BL/6 background, male or female, with an age between 2 and 4 months. Mice were housed and treated in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and according to the guidelines of the Institute of Comparative Medicine at Columbia University. The animal protocol was approved by the institutional animal care and use committee of the Columbia University. To minimize the number of animals for ethical reasons, experiments used a number of mice per group required to provide the power
to detect a twofold difference between groups. Each experiment was performed multiple times. Littermates were randomly assigned to experimental groups according to genotype. Experiments were not performed in a blinded manner. No animals were excluded from analysis.

**Immunization**

For T-dependent immune responses, mice were immunized intraperitoneally with 1 x 10^5 SRBCs in PBS. For T-independent type-I and type-II responses, mice were immunized intraperitoneally with 20 µg NP-LPS or 30 µg of NP-aminoethyl carboxymethyl-Ficoll (both from LGC Biosearch Technologies, Petaluma, CA, USA), respectively. Peripheral blood and spleens were removed at the indicated time points for analysis.

**B-cell isolation and culture**

Single-cell suspensions of murine spleen were subjected to hypotonic lysis and 'untouched' B cells were purified by magnetic cell separation using the MACS B-cell isolation kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany). Purified B cells from the respective genotypes were cultured in the presence of 25 ng ml^-1 BAFF (R&D Systems Inc., Minneapolis, MN, USA) at a cell density of 1.5 x 10^6 cells per ml.

**Flow cytometry**

Spleen cell suspensions or cultured B cells were stained with the following antibodies as described:25,46 anti-CD138-PE (clone: 281-2); anti-CD95-PE (clone: J02); IgM-APC (clone II/41); IgG3-APC (clone 11-26c.2a); and anti-CD23-PE (clone B3B4) (all from BD Pharmingen, BD Biosciences, San Jose, CA, USA); anti-B20-PerCP (clone: RA3-6B2); anti-CD21-APC (clone: 7E9); anti-CD24 (HSA)-PE (clone: 30-F1); anti-CD93-PE (clone: 1D3) (BD Horizon, BD Biosciences); and PNA-Biotin (Vector Laboratories Inc., Burlingame, CA, USA) followed by Streptavidin-APC (BD Pharmingen). Annexin V-7-AAD stainings followed by Streptavidin-APC (BD Pharmingen). Annexin V-AAD stainings were performed using the APC Annexin V Apoptosis Detection Kit with 7-AAD (Biolag). For DNA content analysis, cells were lysed and stained with propidium iodide. The cells were analyzed on a FACSCalibur or a LSRII (Becton Dickinson, Franklin Lakes, NJ, USA). Transitional B cells were identified by gating on B220^+CD93^+ lymphocytes.39 GC B cells were identified by gating on B220^+eGFP^- and eGFP^-CD138^-CD5^+ plasma cells were identified through the lymphocyte gate. Data were analyzed using FlowJo software (FlowJo, Ashland, OR, USA).

**Enzyme-linked immunosorbent assay**

For NP-LPS and NP-Ficoll immunization experiments, 96-well immune-plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with NP25-BSA (Biosearch Technologies). Mouse serum samples were incubated for 2 h at room temperature. Standard curves were generated using mouse IgM and IgG3 (Southern Biotech, Birmingham, AL, USA). Bound antibodies were detected by alkaline peroxidase (AP)-conjugated anti-mouse IgM and IgG3-antibodies (Southern Biotech). Plates were developed with p-nitrophenylphosphate (Southern Biotech) dissolved in substrate buffer.

**Histology and immunohistochemistry**

Sections of splenic tissue (3 µm) were prepared after overnight fixation in 10% formalin and embedding in paraffin. Sections were stained with hematoxylin and eosin for morphologic evaluation. Primary antibodies, rabbit anti-mouse CD3 (clone: SP7; Thermo Fisher Scientific), rabbit anti-GFP (Molecular Probes, Invitrogen, Carlsbad, CA, USA) or rabbit anti-mouse BCL6 (clone: N-3; Santa Cruz Biotechnology Inc., Dallas, TX, USA) or AP-conjugated anti-mouse IgM and IgG1-antibodies (Southern Biotech) were applied to tissue sections and incubated overnight at 4 °C. Secondary staining with anti-rabbit horseradish peroxidase-labeled polymer (Dako North America Inc., Carpinteria, CA, USA) was performed for BCL6, CD3 and eGFP and developed in aminoethylcarbazole (Sigma-Aldrich, St Louis, MO, USA), whereas AP-conjugated antibodies were developed in nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate (Roche, Basel, Switzerland). Sections stained for BCL6/IgG1 were counterstained with hematoxylin. Images were acquired via a Digital Sight camera mounted to a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan).

**Statistical analysis**

P-values were obtained using unpaired Student’s t-test.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

We thank Laura Pasqualucci for critically reading the manuscript and David Dominguez-Sola for discussion. This work was supported by NCI/NIH Grant R01-CA157660 to UK, a grant from the Stewart Trust Foundation (USA), the HICCC and through fellowships of the German Research Council (DFG) to NH and MM and a Cancer Biology Training Program fellowship (NCI/NIH Grant 5T32-CA009503-26) to NSD.

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