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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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Abstract

Signaling through the canonical 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 (GC), 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.

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Keywords

B-cell; B-cell compartment; B-cell activation; follicle; marginal zone; germinal center; immune response; NF-κB transcription factors

INTRODUCTION

Signaling through the nuclear factor-κB (NF-κB) signal transduction pathway is critical for the generation of a normal mature B-cell compartment and for antigen-dependent B-cell activation.[1-3] The NF-κB pathway comprises two branches, a canonical (classical) and an alternative (non-canonical, non-classical) pathway, that transmit signals elicited by distinct cell surface receptors, eventually leading to the nuclear translocation of NF-κB transcription factors.[4,5] c-REL, RELA and NF-κB1/p50 are the subunits of the canonical pathway, while RELB and NF-κB2/p52 transduce signals of the alternative pathway. In B-cells, activation of the canonical NF-κB pathway predominantly results in the nuclear translocation of c-REL/p50 and RELA/p50 heterodimers and occurs during various stages of antigen-independent and antigen-dependent B-cell development.[1-3] Only c-REL and RELA are transcriptionally active as they contain transactivation domains (TAD).[4,5] A large body of work has provided insights into the role of the NF-κB pathway in lymphocyte biology and during the antibody response; however, the B-cell-intrinsic functions of RELA and c-REL in the generation and maintenance of mature B-cells and during antigen-dependent B-cell activation remains incompletely defined due to the lack of suitable experimental systems.

Studies in which upstream regulators of the canonical NF-κB pathway were deleted in mouse B-cells have firmly established that activation of this pathway is required for the generation and maintenance of mature B-cells.[6-9] However, the roles of the downstream transcription factors c-REL and RELA in this process are less well defined. Germ-line deletion of *rela* causes embryonic lethality at day 15 of gestation.[10,11] Transfer of $rela^{-/-}$ fetal liver cells into irradiated SCID mice indicated that mature B-cells developed in the recipient mice,[11,12] although analogous experiments with irradiated $Rag\text{-}1^{-/-}$ mice found only a partial reconstitution of splenic B-cells.[13] In contrast, mice with germ-line deletion of *rel* or that lack the TAD of c-REL ($rel^{\Delta TAD/\Delta TAD}$) are viable.[14-16] These mice showed normal generation of mature B-cells. Results from experiments performed with chimeric mice repopulated with $rel^{-/-}rela^{-/-}$ fetal liver hematopoietic stem cells suggested a requirement for both RELA and c-REL in this process.[17] 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 germinal center (GC) reaction where antigen-activated B-cells undergo somatic hypermutation and class switch recombination.[18-20] While nuclear translocation of NF-κB is observed rapidly upon B-cell activation.[21,22] NF-κB signaling is not activated in the majority of GC B-cells.[23,24] However, the canonical NF-κB subunits show nuclear translocation in a small subset of B-cells in the light zone of the GC.[24] We recently demonstrated that c-REL and

RELA exert distinct functions in these light zone B-cells.[25] 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.[25] However, *rela* deletion in GC B-cells impaired plasma cell development.[25] While 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[26] and in a later developmental stage for GC maintenance.[27] Similarly, interferon regulatory factor 4 (IRF4) controls the formation of the GC upon T-dependent immunization[28,29] and in the established GC is required for plasma cell differentiation.[30,31]

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*,[21] 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*.[12] In contrast, c-REL-deficient B-cells showed defects in activation and proliferation following mitogenic stimulation *in vitro*,[14-16] and $rel^{-/-}$ and $rel^{\Delta TAD/\Delta TAD}$ 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] Since $rel^{-/-}$ 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[25] to CD19-Cre mice that express Cre-recombinase in B-cells[33] 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.* *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre and CD19-Cre control mice (**Fig. 1A,B**). B220^{+} cells comprised ~31% of total splenocytes in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice *vs.* ~52% and ~48% in *rel*$^{fl/+}$*rela*$^{fl/+}$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 to ~24×10^{6} and ~29×10^{6} B220^{+} cells in the control mice. In the conditional mice, *rel* and *rela* deletion is concomitantly linked to the expression of an eGFP gene,[25] which allows the tracking of the *rel/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; **Fig. 1C**); in contrast, ~67% of B-cells of *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre mice were eGFP^{+} (**Fig. 1C**). This indicates that eGFP^{+} B-cells double-deficient for c-REL and RELA were outcompeted by eGFP^{-} B-cells that escaped Cre-deletion. *Rel/rela*-deleted eGFP^{+} B-cells therefore represented only ~14% of total splenocytes in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice compared to ~52% observed in the CD19-Cre control mice (total cell numbers, ~3×10^{6} *rel/rela*-deleted eGFP^{+} B-cells were present in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice *vs.* ~29×10^{6} B-cells in the control mice) (**Fig. 1A,B**).

Immunohistochemistry (IHC) analysis of spleen sections for IgM and CD3 revealed that, in line with the severely reduced B-cell fraction observed by flow cytometry, *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice had fewer B-cell follicles within the splenic white pulp relative to *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre and CD19-Cre control mice (**Fig. 2A**). The B-cell follicles in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice were also more heterogeneous in size compared to 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 *rel/rela*-deleted marginal zone (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 *rel*$^{fl/fl}$CD19-Cre, *rela*$^{fl/fl}$CD19-Cre and *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre and littermate controls to determine the fractions of FO B-cells (B220^{+}CD23^{+}CD21^{int}) and marginal zone (MZ) B-cells (B220^{+}CD23^{-}CD21^{hi}). With regard to the single conditional knockouts, the results revealed only minor (c-REL cohort) or no significant (RELA cohort) differences in the fractions of FO and MZ B-cell subsets (**Suppl. Fig. 1**), or in the fractions of IgM^{+}IgD^{hi} *vs.* IgM^{hi}IgD^{lo} B-cells (data not shown), among *rel*$^{fl/fl}$CD19-Cre or *rela*$^{fl/fl}$CD19-Cre mice and the corresponding heterozygous and wild-type controls. In accordance, analysis of H&E 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.[11,12,14-16]

In contrast to the single conditional knockouts, *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice displayed a reduced fraction of FO B-cells compared to *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre and CD19-Cre mice (~43% vs. ~77%), with no significance increase in the fraction of MZ B-cells (**Suppl. Fig.**

2). However, analysis of eGFP$^+$ *vs.* eGFP$^-$ cells in the MZ B-cell compartment of *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice revealed a strong counterselection against *rel/rela*-deleted MZ B-cells, which surpassed that observed in the FO B-cell compartment (**Fig. 2B**). Specifically, only ~26% of MZ B-cells in these mice were eGFP$^+$ (and thus *rel/rela*-deleted) compared with ~48% of FO B-cells. In accordance with the flow cytometry results, IHC analysis of splenic sections revealed that the majority of B-cells in the MZ of *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice were eGFP-negative, in contrast to B-cells in the follicular area that mostly stained for eGFP (**Fig. 2C**). This severe counterselection against *rel/rela*-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 *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre or CD19-Cre control mice with B-cell activating factor (BAFF), which is required for the survival of mature B-cells,[34] for three days. While 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 (~70% vs. ~27% by propidium iodide (PI) staining and ~ 60 % vs. ~15% by annexin V/7AAD staining) (**Fig. 3**). These findings provide additional evidence that the canonical NF-κB pathway transmits signals derived from BAFF-mediated activation[35-37] and may explain in part the importance of canonical NF-κB signaling for mature B-cell maintenance.[6,7]

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) on. 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 *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre *versus rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre and CD19-Cre mice by flow cytometric analysis for pro-B (CD93^{hi}B220^{int}IgM$^-$), pre-B (CD93^{hi}B220^{int}IgM$^+$) and mature (CD93^{lo}B220$^+$) B-cells. We observed that both the fraction and cell number of immature B-cells did not differ among the genotypes (**Suppl. Fig. 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 *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice compared to the controls (~6% compared to ~14% and ~11% in the control mice; and in total cell numbers ~0.6×10^6 compared to ~1.7×10^6 and ~1.5×10^6 in the controls; **Suppl. Fig. 3**). These results demonstrate that c-REL and RELA are redundant during the generation of B-cells in the BM in a B-cell intrinsic fashion, extending previous findings on chimeric mice repopulated with *rel*$^{-/-}$*rela*$^{-/-}$ fetal liver hematopoietic stem cells that concluded that the combined absence of c-REL and RELA did not perturb the development of B-cell progenitors in the BM.[17]

Mature B-cells are characterized by the expression of low levels of the CD24 (heat stable antigen, HSA) cell surface antigen.[38] Consistent with the strong reduction of mature B-cells in the spleen of *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice, these mice were characterized by a small fraction of B220^{+}CD21^{int}CD24^{lo} FO B-cells compared to the control mice (~23% compared to ~70% and ~58% in the control mice; **Suppl. Fig. 4**). Instead, most splenic B220^{+} cells in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice were CD24^{hi} immature B-cells (~48% compared to ~16% and ~26% in the control mice; **Suppl. Fig. 4**), a population that comprises transitional B-cells which 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.[39]

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 *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre and control mice for their expression of CD93^{+}IgM^{hi}CD23^{-} (T1), CD93^{+}IgM^{hi}CD23^{+} (T2), and CD93^{+}IgM^{lo}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 *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice compared to the CD19-Cre littermate controls (~40% *vs.* ~20% T1 B-cells, ~33% *vs.* ~52% T2 B-cells, and ~7% *vs.* ~15% T3 B-cells) (**Fig. 4A**). Of note, in contrast to what we observed for the MZ B-cells (**Fig. 2B**), there was no counterselection of *rel/rela*-deleted eGFP^{+} T1 cells in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre *vs.* *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre mice (~83% were eGFP^{+} in both genotypes; **Fig. 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] which 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 *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre control mice, which however was not observed for the *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice (**Suppl. Fig. 5A**). Also, since 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.[9] We found that *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice harbor a significantly reduced fraction of CD93^{lo} cells among T2 cells compared to the control mice (**Suppl. Fig. 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 *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre, *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre and CD19-Cre mice, respectively, **Fig. 4A**), indicating normal generation up to the T1 stage in *rel*$^{fl/fl}$*rela*$^{fl/fl}$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. Since 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 fashion

To determine the B-cell-intrinsic role of c-REL in the formation of GCs upon T-dependent B-cell activation, *rel*$^{fl/fl}$CD19-Cre, *rel*$^{fl/+}$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, i.e. 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} (**Fig. 5A**) and BCL6^{+} (**Fig. 5B**) GC B-cells in the spleens of *rel*$^{fl/fl}$CD19-Cre mice, with a concurrent reduction in *rel*-deleted (eGFP^{+}) GC B-cells compared to *rel*$^{fl/+}$CD19-Cre mice (**Fig. 5A**). Similarly, the generation of total and eGFP^{+} splenic plasma cells was strongly impaired at this time-point in *rel*$^{fl/fl}$CD19-Cre compared to *rel*$^{fl/+}$CD19-Cre and CD19-Cre control mice (**Fig. 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 RELA's B-cell-intrinsic function in antibody responses *in vivo*. To determine the role of RELA in T-dependent B-cell responses, we immunized *rela*$^{fl/fl}$CD19-Cre, *rela*$^{fl/+}$CD19-Cre and CD19-Cre control mice with SRBC. In contrast to what we observed upon *rel* deletion, *rela*$^{fl/fl}$CD19-Cre mice mounted a GC response similar to control mice 5 days after SRBC-immunization (**Fig. 5D,E**). Accordingly, the fraction of eGFP^{+} GC B-cells was similar in *rela*$^{fl/fl}$CD19-Cre and *rela*$^{fl/+}$CD19-Cre mice (**Fig. 5D**). Also, *rela*$^{fl/fl}$CD19-Cre mice generated equal amounts of splenic plasma cells compared to *rela*$^{fl/+}$CD19-Cre and CD19-Cre control mice (**Fig. 5F**), and showed similar fractions of eGFP-expressing plasma cells compared to *rela*$^{fl/+}$CD19-Cre mice (**Fig. 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 responses against polysaccharides, *rel*$^{fl/fl}$CD19-Cre or *rela*$^{fl/fl}$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 (AECM)-Ficoll and assessed for NP-specific IgM and IgG3 serum titers 7 days later. This analysis showed a strong reduction (5 to 10-fold) in both NP-specific IgM and IgG3 serum titers in *rel*$^{fl/fl}$CD19-Cre mice compared to *rel*$^{fl/+}$CD19-Cre and CD19-Cre mice (**Fig. 6A**). *rela*$^{fl/fl}$CD19-Cre mice showed a less severe (~2-fold) reduction in NP-specific IgM titers, and no significant changes in the corresponding IgG3 titers (**Fig. 6B**).

Type-I T-independent B-cell responses are characterized by the co-activation of antigen-specific B-cells through Toll-like receptors (TLRs) such as TLR4, the receptor for LPS. We

immunized *rel*$^{fl/fl}$CD19-Cre or *rela*$^{fl/fl}$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 *rel*$^{fl/fl}$CD19-Cre and *rel*$^{fl/+}$CD19-Cre mice were strongly reduced (8 to 12-fold) compared to CD19-Cre mice (**Fig. 6C**), implying that the previously observed reduction in NP-specific IgG3 serum titers upon NP-LPS immunization in *rel*$^{-/-}$ mice[14] is a B-cell intrinsic effect. In contrast, *rela*$^{fl/fl}$CD19-Cre mice showed only a 3-fold reduction in serum IgM levels compared to controls, but no significant changes in IgG3 levels were observed among the different genotypes (**Fig. 6D**). Together, the results indicate a more prominent role for c-REL in T-independent type-II and type-I antibody responses in comparison to 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 GC reaction and only modestly impacted T-independent antibody responses.

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.[6-9] A recent study by Derudder et al. has more precisely dissected the roles of the canonical pathway in these processes by deleting the upstream regulators *ikk2* or *nemo* in different B-cell developmental stages.[9] 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 (**Fig. 2**). Of note, Derudder et al. report that a *bcl2*-transgene rescued FO, but not MZ B-cells in mice with B-cell-specific deletion of *nemo*[9]. 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 ref.[45]). In accordance, we recently showed that the functional abolishment of the alternative 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.[46] 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 (**Fig. 1&2A** and ref.[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 (**Fig. 2B,C** and ref.[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[46] 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.[8,37] 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—albeit 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 which is supported by the observation that 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 proliferation[25] 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 here described 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 which 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 *nfkb1*$^{-/-}$ mice may lead to enhanced c-REL activity in B-cells, which 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, while 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 which 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,[28,29] 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,[25,27] 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 other[49,50] 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, which was reflected by a dramatic reduction in the serum levels of NP-specific IgG1 antibodies (>10-fold).[25] While we here observed that *rela*$^{fl/fl}$CD19-Cre mice showed only a ~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 antigen-secreting cells in the different arms of the humoral immune response.

Integrating the results of our present and published[25] 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 malignancies[51,52] 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 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 Columbia University's IACUC. 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 two-fold 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 fashion. No animals were excluded from analysis.

Immunization

For T-dependent immune responses, mice were immunized i.p. with 1×10^9 SRBCs in PBS. For T-independent type-I and type-II responses, mice were immunized i.p. with 20 μg NP-LPS or 30 μg of NP-AECM-FICOLL (both Biosearch Technologies), 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). Purified B cells from the respective genotypes were cultured in the presence of 25 ng/ml BAFF (R&D Systems) at a cell density of 1.5×10^6 cells/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: Jo2); IgM-APC (clone II/41); anti-IgD-PE (clone 11-26c.2a); and anti-CD23-PE (clone B3B4) (all BD Pharmingen); and anti-B220-PerCP (clone: RA3-6B2); anti-CD21-APC (clone: 7E9); anti-CD24 (HSA)-PE (clone: 30-F1), anti-CD93-PE (clone: AA4.1); and anti-CD23-Pacific Blue (clone: B3B4) (all Biolegend); and anti-CD19-CF594 (clone: 1D3) (BD Horizon); and PNA-Biotin (Vector Laboratories) followed by Streptavidin-APC (BD Pharmingen). Annexin V/7-AAD stainings were performed using the APC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend). For DNA content analysis, cells were lysed and stained with propidium iodide (PI). The cells were analyzed on a FACSCalibur or a LSRII (Becton Dickinson). Transitional B-cells were identified by gating on $B220^+CD93^+$ lymphocytes.[39] GC B-cells were identified by gating on $B220^+$ lymphocytes. $eGFP^+$ and $eGFP^-$ $CD138^{hi}$ plasma cells were identified through the lymphocyte gate. Data were analyzed using FlowJo software.

Enzyme-linked immunosorbent assay (ELISA)

For NP-LPS and NP-Ficoll immunization experiments, 96 well immune-plates (Thermo Fisher Scientific) were coated with NP_{25}-BSA (Biosearch Technologies). Mouse serum samples were incubated for 2h at RT. Standard curves were generated using mouse IgM and IgG3 (Southern Biotech). Bound antibodies were detected by 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 H&E for morphologic evaluation. Primary antibodies, rabbit anti-mouse CD3 (clone: SP7; Thermo Fisher Scientific), rabbit anti-GFP (Molecular Probes, Invitrogen), or rabbit anti-mouse BCL6 (clone: N-3; Santa Cruz) or alkaline peroxidase (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 HRP-labeled polymer (Dako) was performed for BCL6, CD3 and eGFP and developed in aminoethylcarbazole (AEC; Sigma), while AP-conjugated antibodies were developed in nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche). 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).

Statistical analysis

P values were obtained using unpaired Student's *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FO	follicular
MZ	marginal zone
GC	germinal center
NP	4-hydroxy-3-nitrophenyl-acetyl

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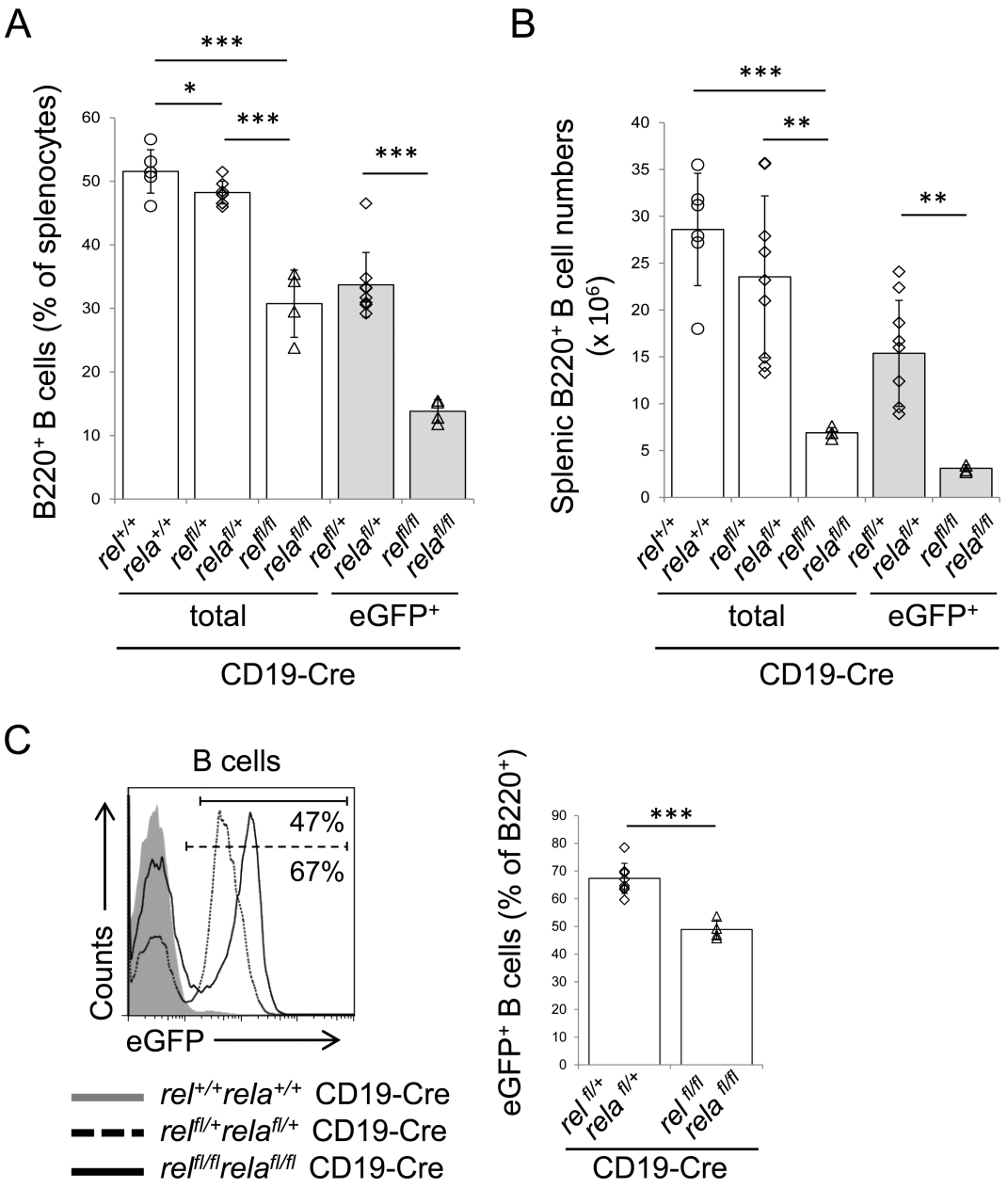

Figure 1. Combined c-REL and RELA deficiency leads to a severe reduction in splenic B-cells

(A) Percentage and **(B)** number of splenic B-cells in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre and the corresponding heterozygous and CD19-Cre control mice as determined by flow cytometry. **(C)** Flow cytometry of eGFP expression in splenic B-cells of the indicated genotypes. The numbers below the gate indicate the percentage of eGFP^{+} B-cells among of B220^{+} B-cells of *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre and *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre mice (*left*). Summary of the frequency of eGFP^{+} 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 ± standard deviation. Statistical significance was determined by Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

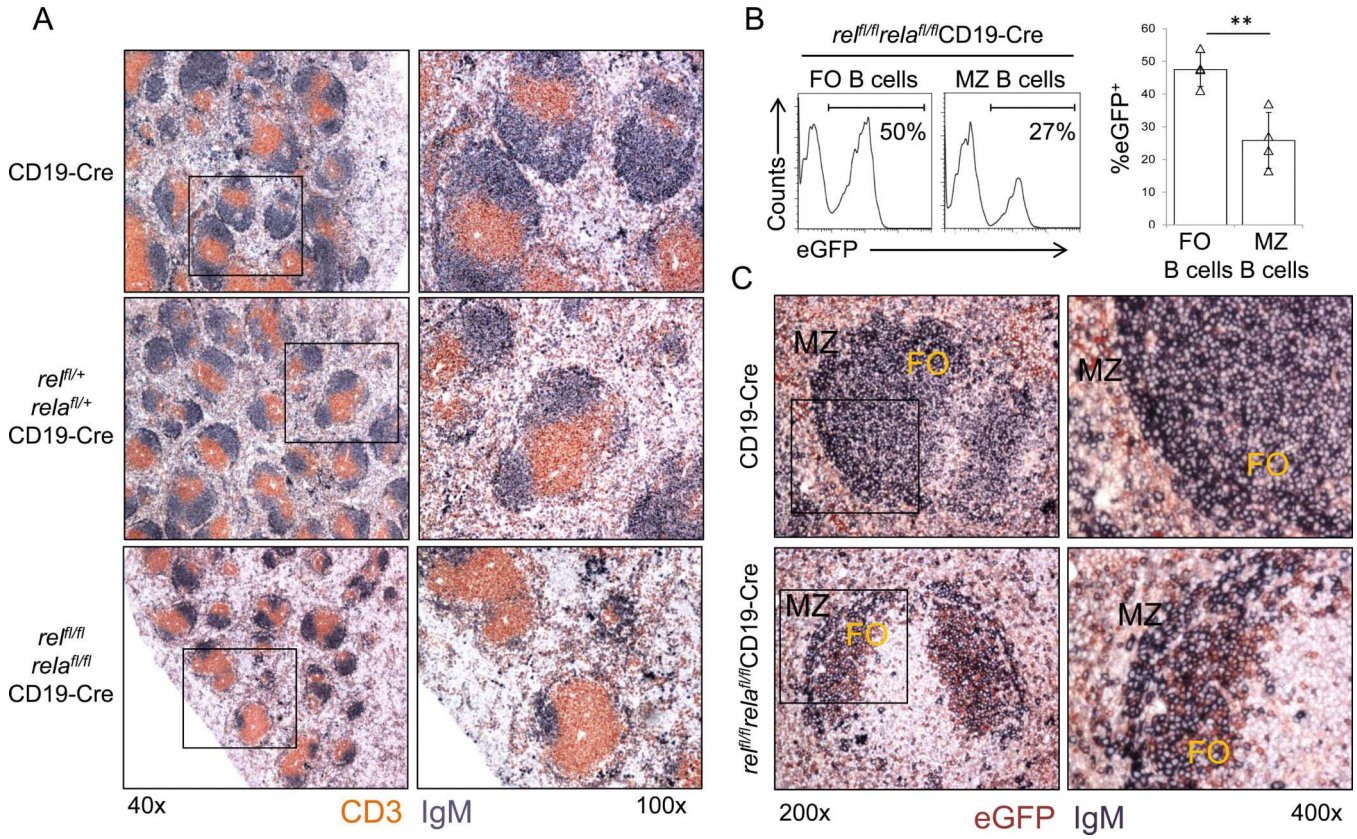

Figure 2. *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice display fewer B-cell follicles within the splenic white pulp compared to controls and are characterized by counter selection against *rel/rela*-deleted MZ B-cells

(A) Spleen sections from mice of the indicated genotypes were analyzed via 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^{+}CD21^{int}) and marginal zone (MZ; CD21^{hi}CD23^{-}) B-cells in *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre mice were determined by flow cytometry. 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 ± standard deviation. Statistical significance was determined by Student's *t* test (**, $P<0.01$). **(C)** Spleen sections from mice of the indicated genotypes were analyzed by IHC for the expression of eGFP and IgM. FO, follicular area; MZ, marginal zone area. One representative mouse of three per group is shown. Original magnification ×200 (*left*) and ×400 (*right*).

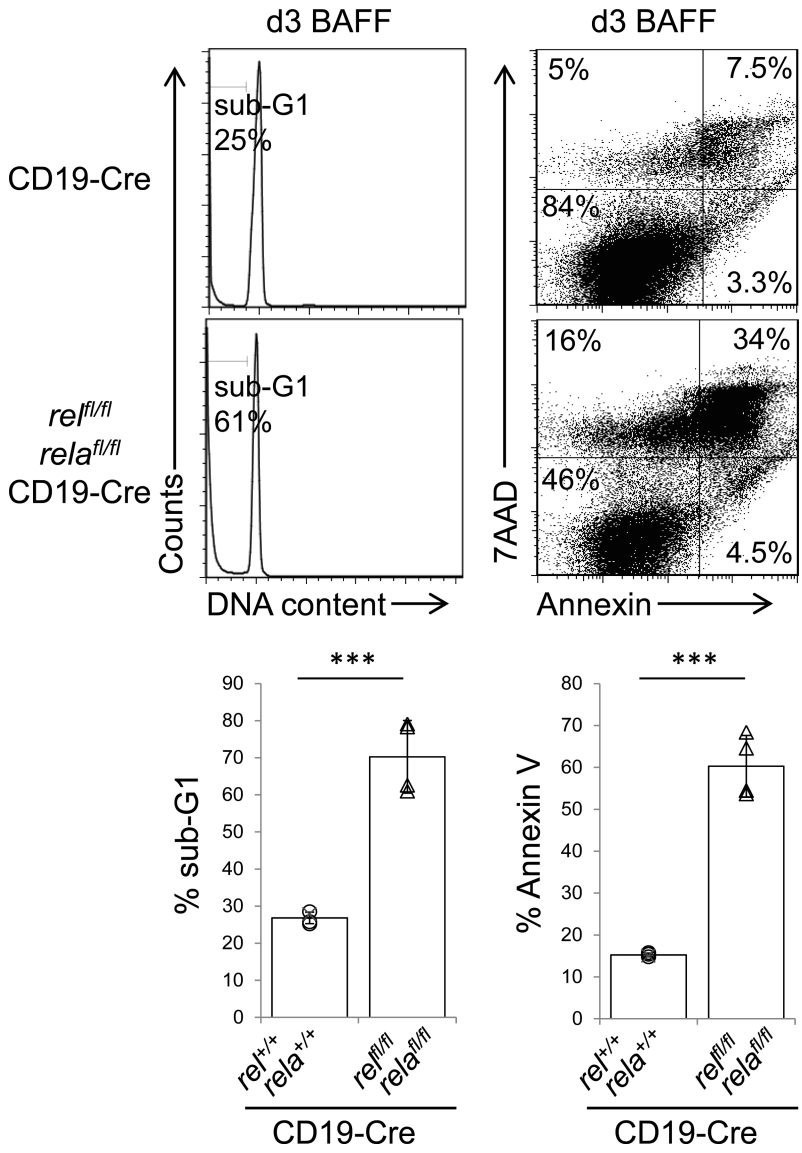

Figure 3. Combined c-REL and RELA deficiency impairs BAFF-mediated cell survival

(*left*) Flow cytometric analysis of BAFF-stimulated purified B-cells from *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre and CD19-Cre mice at d3 for DNA content by propidium iodide (PI) staining and summary of the corresponding percentage sub-G1 (*bottom*), and (*right*) for apoptotic/dead cells by annexin V/7AAD staining and summary of the corresponding percentage of annexin V/7AAD^{+} cells (bottom). Data are cumulative from independent experiments (n=4 per group), with each symbol representing a mouse. Data are shown as mean ± standard deviation. Statistical significance was determined by Student's *t* test (***, $P<0.001$).

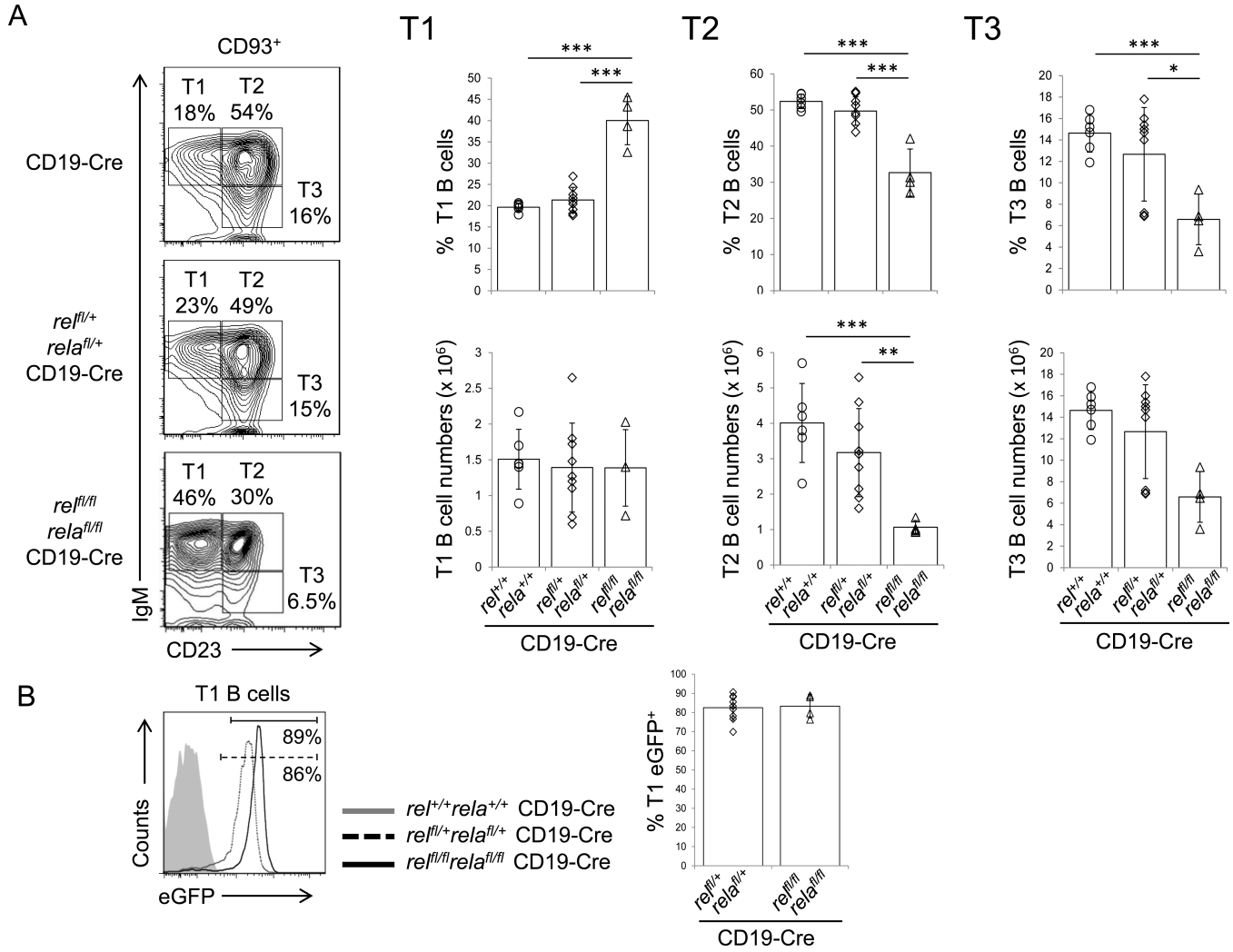

Figure 4. Combined c-REL and RELA deficiency leads to a developmental block at the T1 stage

(**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$^+$IgM^{hi}CD23$^-$), T2 (CD93$^+$IgM^{hi}CD23$^+$), and T3 (CD93$^+$IgM^{lo}CD23$^+$) 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 ± standard deviation. 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 *rel*$^{fl/fl}$*rela*$^{fl/fl}$CD19-Cre and *rel*$^{fl/+}$*rela*$^{fl/+}$CD19-Cre mice were determined by flow cytometry. Numbers below gates indicate the percentage of eGFP$^+$ B-cells among the indicated B-cell subsets (*left*). Data are cumulative from independent experiments (n=4-9 per group), with each symbol representing a mouse, showing the frequency of eGFP$^+$ cells among the corresponding B-cell subsets (*right*).

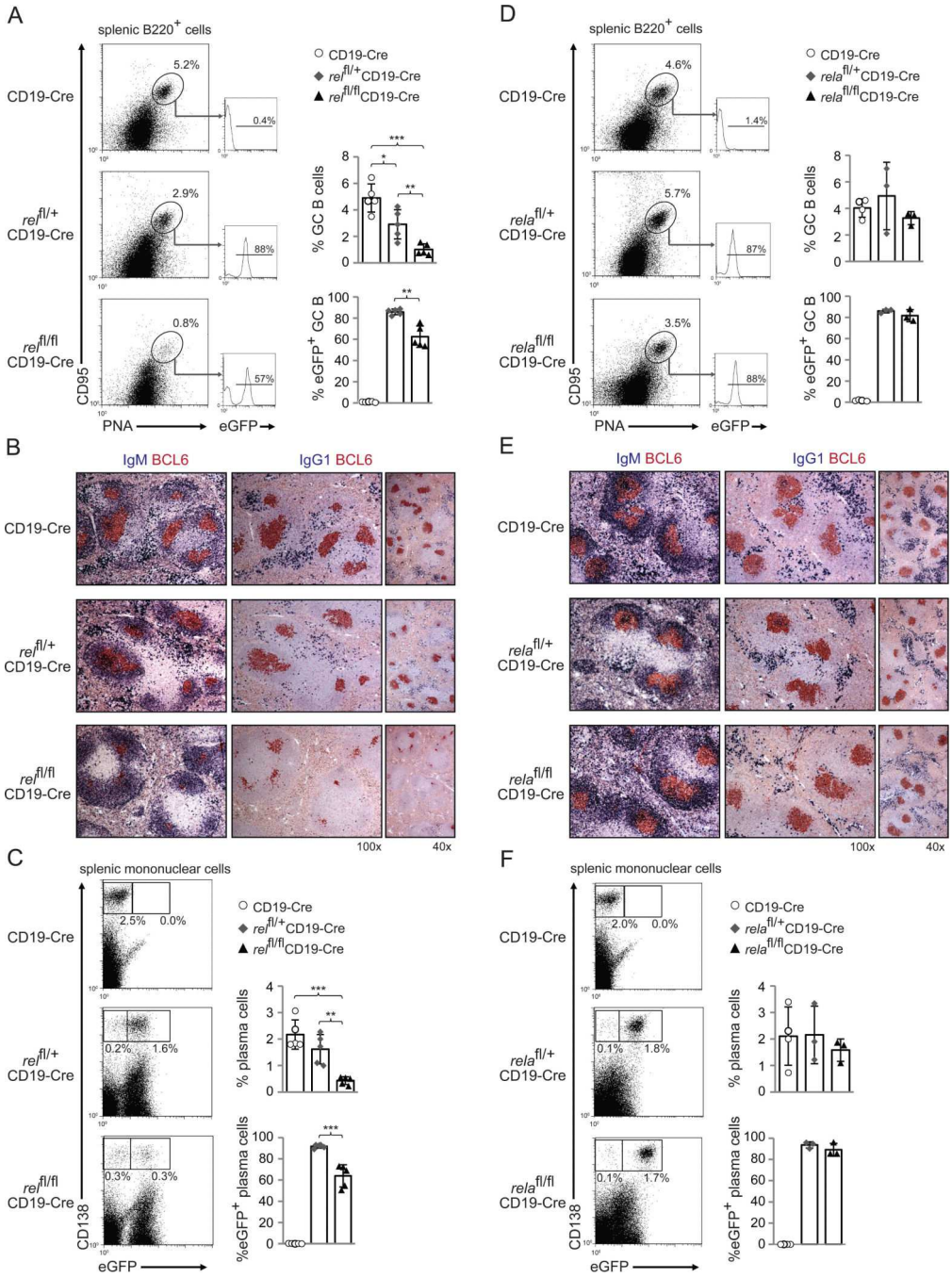

Figure 5. Impaired GC formation in mice with deletion of *rel*, but not *rela*, in B-cells

rel$^{fl/fl}$CD19-Cre, *rel*$^{fl/+}$CD19-Cre and CD19-Cre littermates (**A-C**), and *rela*$^{fl/fl}$CD19-Cre, *rela*$^{fl/+}$CD19-Cre and CD19-Cre littermates (**D-F**) were immunized with SRBC and analyzed 5 days later. (**A,D**) CD95, 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 haematoxylin. One representative mouse out

of 3 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 (n=3-5 per group), with each symbol representing a mouse. Data are shown as mean ± standard deviation. Statistical significance was determined by Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

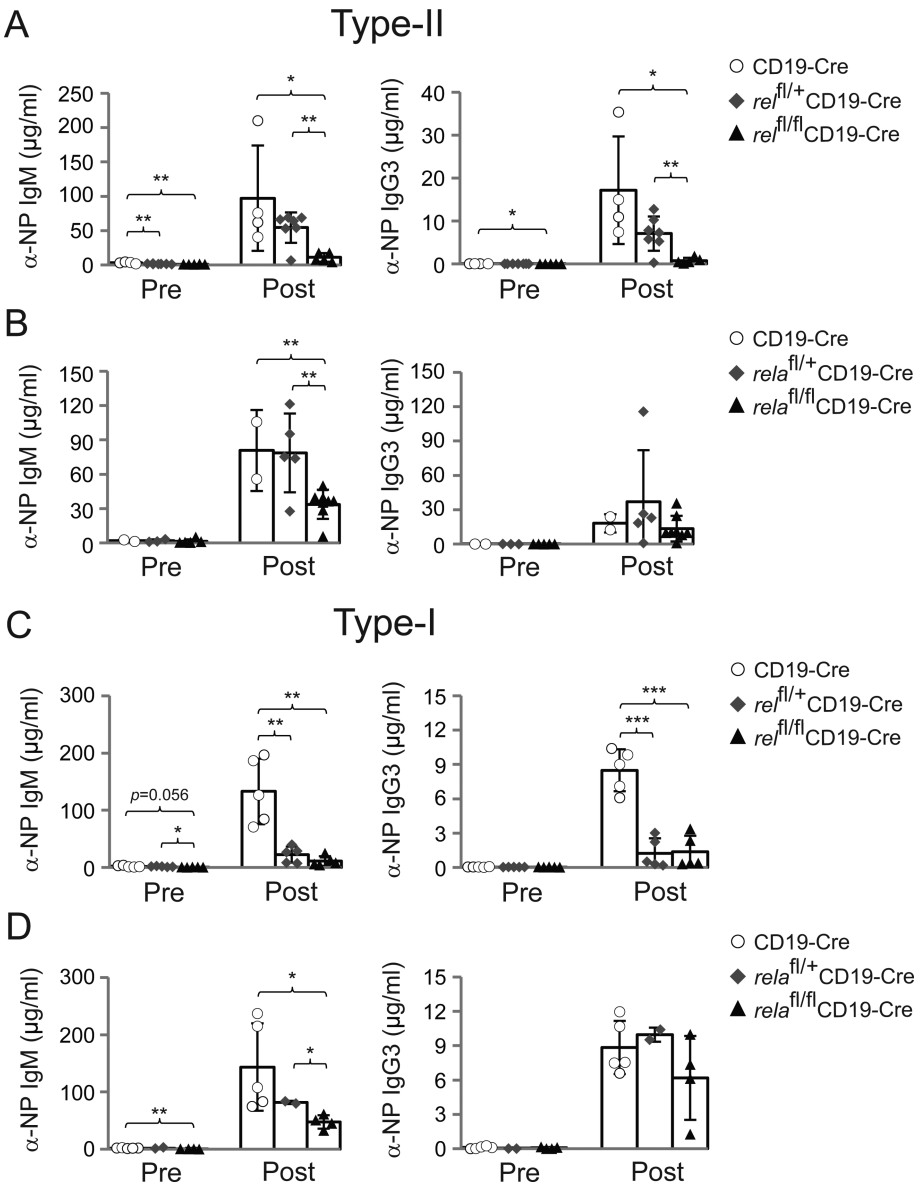

Figure 6. Mice with B-cell-specific deletion of c-REL show strong impairment of T-independent type-II and type-I antigen responses

(A) α-NP IgM (*left*) and α-NP IgG3 (*right*) serum response of *rel*$^{fl/fl}$CD19-Cre, *rel*$^{fl/+}$CD19-Cre and CD19-Cre mice to NP-ficoll immunization before (Pre), and 7d after immunization (Post) with NP-ficoll (*left*). **(B)** α-NP IgM (*left*) and α-NP IgG3 (*right*) serum response of *rela*$^{fl/fl}$CD19-Cre, *rela*$^{fl/+}$CD19-Cre and CD19-Cre mice to NP-ficoll immunization before (Pre), and 7d after immunization with NP-ficoll (*left*). **(C)** α-NP IgM (*left*) and α-NP IgG3 (*right*) serum response of *rel*$^{fl/fl}$CD19-Cre, *rel*$^{fl/+}$CD19-Cre and CD19-Cre mice to NP-LPS immunization before (Pre), and 7d after immunization with NP-LPS (*left*). **(D)** α-NP IgM (*left*) and α-NP IgG3 (*right*) serum response of *rela*$^{fl/fl}$CD19-Cre, *rela*$^{fl/+}$CD19-Cre and CD19-Cre mice to NP-LPS immunization before (Pre), and 7d after immunization with NP-LPS (*left*). **(A-D)** Data are cumulative from independent experiments (n=2-7 per group), with each symbol representing a mouse. Data are shown as mean ± standard deviation.

Statistical significance was determined by Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).