Developmental B-cell defects within a model of A20/Tnfaip3-deficient dendritic cells.

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ABSTRACT

Background: Dendritic cells (DCs) are central regulators of tolerance versus immunity. Mice with a DC-specific deficiency of deubiquitinating enzyme A20/*Tnfaip3* (*Tnfaip3*$^{CD11c\text{-}cre}$), a major negative regulator of NF-κB signaling, exhibit increased DC activation. Aged *Tnfaip3*$^{CD11c\text{-}cre}$ mice show activation of mature B cells and develop humoral autoimmunity resembling systemic lupus erythematosus (SLE). In addition, mature B cells are severely decreased in numbers.

Objective: To identify the arrest in B cell development in *Tnfaip3*$^{CD11c\text{-}cre}$ mice and to investigate whether mature peripheral B cells have functional defects.

Methods: B cell development was studied in bone marrow and spleen of 6-week-old and 24-week-old *Tnfaip3*$^{CD11c\text{-}cre}$ mice. Splenic naïve B cells were stimulated *in vitro* to measure cellular activation and immunoglobulin production capacity. *In vivo* B cell responses were determined.

Results: B cell development in the bone marrow was hampered at the immature B cell stage in 6-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice and at the pre-B cell stage in 24-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice. The observed age-dependent developmental arrest of B-lineage cells most likely reflected changes in the bone marrow micro-environment. Although development of B-1 cells in *Tnfaip3*$^{CD11c\text{-}KO}$ appeared impaired, the IgM and IgG3 B cell responses to T cell independent antigen DNP-Ficoll were unaffected. While serum immunoglobulin levels were increased, *Tnfaip3*$^{CD11c\text{-}KO}$ mice displayed a defective T cell-dependent B cell response *in vivo*. In addition, mature naïve peripheral B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice showed increased activation upon *in vitro* stimulation compared with control mice.

Conclusion: DC-specific deficiency of A20/*Tnfaip3* is associated with defective B cell development, reflecting a dysfunctional bone marrow micro-environment. The observed defects may contribute to defective immune homeostasis and autoimmunity in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifactorial disease[1]. Often malfunction of multiple innate and adaptive immune cell types, such as dendritic cells (DCs), T cells and B cells lies at the base of the autoimmune phenotype[1]. B cells can contribute to pathology by autoantigen presentation, secretion of pro-inflammatory cytokines, ectopic germinal center generation and autoantibody secretion[2]. DCs have a crucial role in the maintenance of tolerance[3]. Control of immune cell activation is essential to prevent autoimmunity. For example, transgenic removal of regulatory proteins such as the tyrosine kinase Lyn or Src homology region 2 domain–containing phosphatase-1 (SHP1) in B cells[4, 5] or DCs[6, 7] in mice results in the spontaneous development of SLE symptoms.

Proper signals within the NF-κB pathway are also essential to maintain tolerance[8]. Tumor necrosis factor alpha-induced protein 3 (TNFAIP3), also known as A20, is one of the major negative regulators of the NF-κB pathway[9]. Targeted deletion of the *Tnfaip3* gene in B cells or DCs in mice resulted in spontaneous autoimmunity resembling SLE and was characterized by increased germinal center (GC) responses, autoantibody production and glomerulonephritis[10, 11]. Interestingly, in mature 24-week-old mice in which the *Tnfaip3* gene was conditionally deleted in DCs, the numbers of B cells in the periphery were reduced, indicating that B cell development may be disturbed.

Development of autoreactive B cells in the bone marrow is prevented by various central tolerance mechanisms, including receptor editing[12, 13] and clonal deletion[14]. Tolerance mechanisms that function at later stages, such as B cell anergy[15], are referred to as peripheral tolerance. Because defects due to central tolerance can be observed in autoimmune patients with rheumatoid arthritis (RA)[16, 17], type 1 diabetes (T1D)[16] and SLE[18-20], as well as in corresponding mouse models[21, 22], we investigated B cell development in the bone marrow and peripheral B cell function in A20/*Tnfaip3*$^{CD11c\text{-}KO}$ mice.

In this study, we demonstrate that B cell development is hampered in the bone marrow of *Tnfaip3*$^{CD11c\text{-}KO}$ mice in an age-dependent fashion. Mature B cells that reach the periphery exhibit various abnormalities. They display a more activated phenotype upon activation *in vitro*, but fail to efficiently respond to a T cell-dependent antigen *in vivo*. Thus, hyperactivation of DCs, due to loss of A20/*Tnfaip3*, did not only deregulate mature T and B cells, but also disturbed B cell development in the bone marrow.

MATERIAL & METHODS

Mice

Male and female C57BL/6 mice harbouring a conditional *Tnfaip3* allele flanked by LoxP sites[23], were crossed onto a transgenic line expressing the Cre recombinase under the

control of the *CD11c* promotor[24], generating CD11c-Cre transgenic *Tnfaip3*$^{fl/fl}$ mice (*Tnfaip3*$^{CD11c\text{-}KO}$ mice). CD11c-Cre non-transgenic *Tnfaip3*$^{fl/fl}$ littermates (*Tnfaip3*$^{CD11c\text{-}WT}$ mice) served as controls and heterozygous CD11c-Cre transgenic *Tnfaip3*$^{fl/+}$ mice (*Tnfaip3*$^{CD11c\text{-}HZ}$) mice were also analyzed. Mice were sacrificed at ~6-8 weeks or ~24-26 weeks of age. Mice were housed under specific pathogen-free conditions and attained food and water ad libitum. All experiments were approved by the animal ethical committee of the Erasmus MC, Rotterdam, The Netherlands.

Cell suspension preparation

Spleen, bone marrow (BM) and mesenteric lymph node (MesLN) were obtained using standard procedures. The hind legs were crushed initially, and spleen and MesLN were homogenized through a 100-µm cell strainer to obtain single-cell suspensions. To remove erythrocytes, spleens and BM were lysed using an osmotic lysis buffer (8.3% NH_4CL, 1% $KHCO_3$, and 0.04% NA_2EDTA in Milli-Q).

Flow cytometry procedures

Flow cytometry surface and intracellular staining procedures have been described previously[25]. Monoclonal antibodies used for flow cytometric analyses are listed in **Supplementary Table 1**. For all experiments, dead cells were excluded using fixable AmCyan viability dye (eBioscience, San diego, CA, USA). To measure cytokine production (in the case of B cells), cells were stimulated with 10 ng/mL Phorbol 12-myristate 13-acetate PMA (Sigma-Aldrich, St. Louis, MI, USA) and 250 ng/mL ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences, San Jose, CA, USA) for 4 hrs at 37°C. To measure cytokine production in DCs, cells were kept in the presence of GolgiPlug (BD Biosciences) for 4 hrs at 37 °C. Data were acquired using an LSR II flow cytometer (BD Biosciences) with FACS Diva™ software and analysed by FlowJo version 9 (Tree Star Inc software, Ashland, OR, USA).

In vitro B cell stimulation

Via MACS separation (Miltenyi Biotec, Bergisch Gladbach, Germany) naïve B cells were isolated from spleen single-cell suspensions via negative selection. Cells were labeled with the biotin-conjugated markers NK1.1, CD4, CD8, Ter119, CD11c, GR-1, FcRI, CD5, CD43, CD138, CD11b, CD95 and Streptavidin Microbreads (Miltenyi Biotec).

For investigating B cell activation markers, naïve B cells were stimulated with 10 µg/ml anti-IgM F'ab fragments (Jackson Immunoresearch, West Grove, PA, USA), 5 ng/ml LPS (Enzo Life Sciences, Farmingdale, NY, USA), 1 µM CpG (Invitrogen, Carlsbad, CA, USA) or 20 µg/ml anti-CD40 (BD Biosciences, San Jose, CA, USA) overnight in RPMI medium (Thermo Fisher, Waltham, Massachusetts) supplemented with gentamycin (Thermo Fisher), β-mercapto-ethanol (Sigma) and 5% Fetal Bovine Serum (Capricorn Scientific,

Ebsdorfergrund, Germany). To investigate immunoglobulin production, naïve B cell fractions were stimulated for 4 days in context of LPS (Enzo Life Sciences) with/without IL-4 (Peprotech, Rocky Hill, NJ, USA).

IL-7 culture of small pre-B cells and immature B cells

Hind legs of *Tnfaip3*$^{CD11c\text{-}KO}$ and *Tnfaip3*$^{CD11c\text{-}WT}$ mice were crushed and erythrocytes were lysed. After labeling with anti-CD19 beads, B cells were positively separated from the CD19^{neg} fraction that also contains stromal cells, using a MACS column. In a 24-wells plate, 2 x 10^6 stroma cells and 300.000 B cells, from either *Tnfaip3*$^{CD11c\text{-}KO}$ or *Tnfaip3*$^{CD11c\text{-}WT}$ mice, were cultured in 1 ml IMDM medium supplemented with gentamycin (Thermo Fisher), β-mercaptoethanol (Sigma), 5% Fetal Bovine Serum (Capricorn Scientific, Ebsdorfergrund, Germany), Glutamine (Gibco) and 100U/ml recombinant IL-7 (Peprotech) per well. B cell and stromal cell suspensions were sex-matched. On day 4, cells were harvested, washed and cultured with or without the presence of IL-7 for 2 days. At day 7, cells were prepared for FACS analysis.

DNP-Ficoll and OVA-Alum immunization

For T cell–independent immunization, 50 μg of DNP(49)-Ficoll (Biosearch Technologies, Petaluma, CA, USA) in PBS was injected i.p. Serum was collected at baseline and 7 days after immunization.

For T cell-dependent immunization, mice received 10 μg OVA (Worthington Biochemical Corp, Lakewood, NJ, USA) i.p. in a saline solution containing 1:20 Imject-alum adjuvant (Thermo Scientific). The mixture was left stirring for 1hr, and then 500μl of OVA-Alum solution was injected per mouse. A booster was given after 1 week. Serum was collected at a pre-immune timepoint, at 8 days and 15 days after booster immunization.

Immunoglobulin levels

For quantification of total immunoglobulin (Ig) levels, Nunc Microwell plates (Life technologies, Carlsbad, CA, USA) were coated overnight at 4C° with 1 μg/ml goat-anti-mouse Ig_x (being the immunoglobulin isotype, Southern Biotech, Birmingham, AL, USA). Wells were blocked with 10% FCS/PBS for 1 hr. Subsequently, standards and serum (diluted in multiple series) were incubated for 3 hrs. Depending on the isotype, anti-mouse Ig_x biotin/streptavidin-HRP were used to develop the ELISA.

For DNP-specific sandwich ELISA assays, Nunc Microwell plates were coated with DNP-Ficoll in 0.1M carbonate buffer (pH 9.5); the standard curve wells were plated with purified unlabeled goat anti-IgM or IgG3 (Southern Biotech) in PBS overnight at 4C°. Wells were blocked with 1% BSA for 1 hr, followed by 3 hrs of incubation with serum samples (diluted in multiple series) and standard IgM or IgG3. Anti-mouse Ig_x biotin/streptavidin-HRP was used to develop the ELISA.

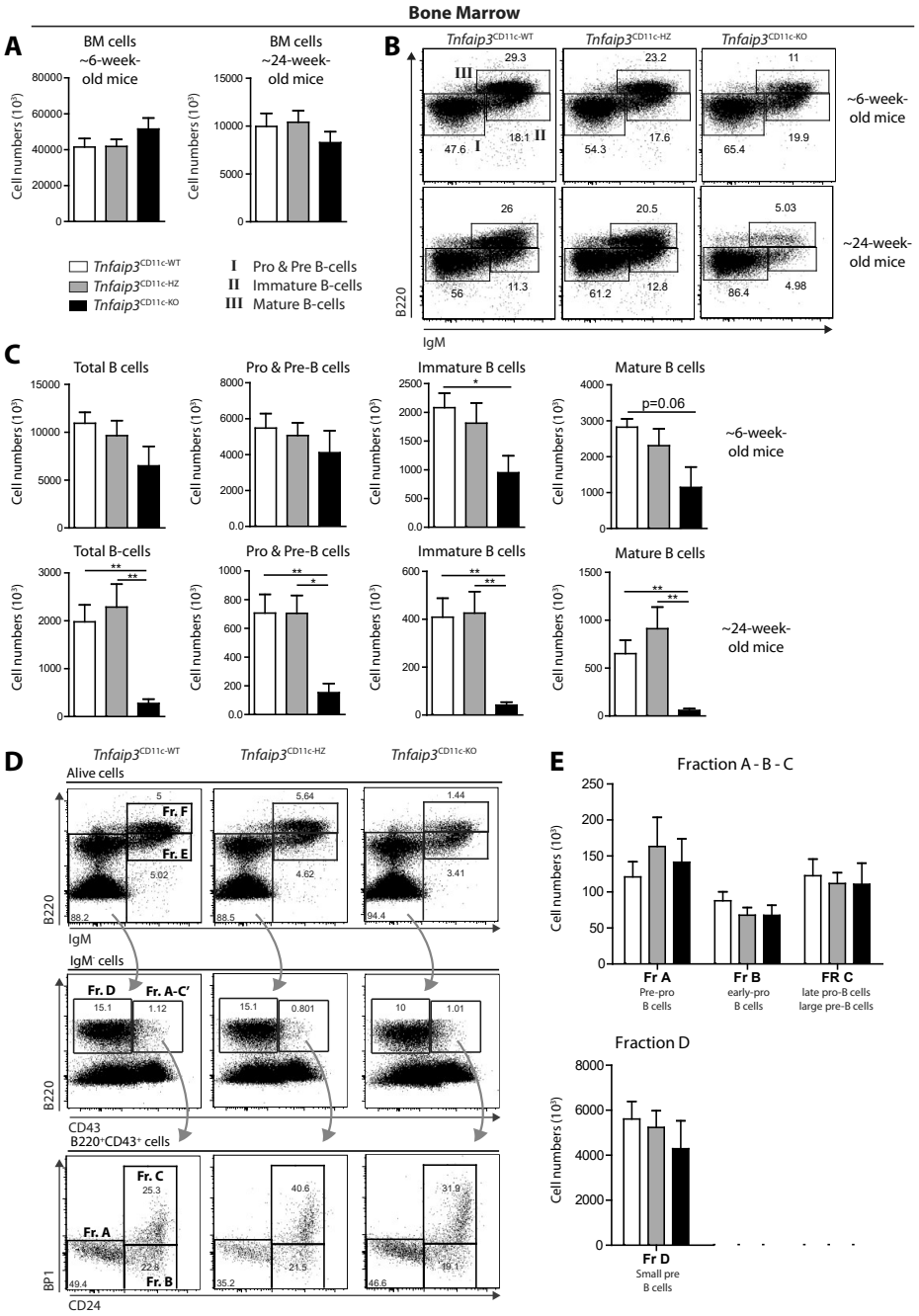

Figure 1: Age-dependent arrest in B cell development in the bone marrows of *Tnfaip3*$^{CD11c\text{-}KO}$ mice.
(**A**) Enumeration of total bone marrow cells in *Tnfaip3*$^{CD11c\text{-}WT}$, *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice at the indicated age, using flow cytometry. (**B**) Flow cytometric analysis of bone marrow Pro-/Pre-B cells ($CD19^+B220^{lo}IgM^-$), immature B cells ($CD19^+B220^{lo}IgM^+$) and mature B cells ($CD19^+B220^{hi}IgM^+$). Representa-

tive examples of gated CD19$^+$ lymphoid cells fractions are shown from *Tnfaip3*$^{CD11c\text{-}WT}$, *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice. (**C**) Quantification of total B cells, Pro-/Pre-B cells, immature B cells and mature B cells in bone marrow of mice (legend shown in panel A) at the indicated age. (**D**) Flow cytometric analysis of bone marrow total lymphoid cells, showing B cell stages according to Hardy et al.[26] in representative examples from three genotypes in 6-week-old mice. (**E**) Quantification of Hardy fraction A (B220^{lo}IgM$^-$CD43$^+$BP1$^-$CD24$^-$), Hardy fraction B (B220^{lo}IgM$^-$CD43$^+$BP1$^-$CD24$^+$), Hardy fraction C (B220^{lo}IgM$^-$CD43$^+$BP1$^+$CD24$^+$), Hardy fraction D (B220^{lo}IgM$^-$CD43$^-$), Hardy fraction E (B220^{lo}IgM$^+$) and Hardy fraction F (B220^{hi}IgM$^+$), using flow cytometry in 6-week-old mice. Results are representative of 3 independent experiments and are presented as mean values ± SEM of n = 4-6 per group. *P<0.05, **P < 0.01 using the Mann-whitney U statistical test.

Statistics

Statistical significance of data was calculated using the non-parametric Mann Whitney U test. P-values <0.05 were considered significant. All data were plotted as mean values with the standard error of the mean (SEM).

RESULTS

Age-dependent arrest in B cell development in the bone marrows of *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

To investigate how DC-specific A20/*Tnfaip3* deficiency affects B cell development, we analyzed bone marrows of ~6-week-old and ~24-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice, as well as *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}WT}$ controls. Bone marrow total cell counts did not differ between the three genotypes at either age (**Figure 1A**). We used flow cytometry to quantify the populations of developing B cells in the bone marrow and found that at the age of ~6 weeks the *Tnfaip3*$^{CD11c\text{-}KO}$ mice had significantly lower numbers of immature B cells compared to *Tnfaip3*$^{CD11c\text{-}WT}$ controls (**Figure 1B,1C**). These young mice showed a near-significant reduction of mature B cells. However, at the age of 24 weeks, the total numbers of bone marrow B cells, pro/pre B cells, immature B cells and mature B cells were significantly reduced in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to WT and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice (**Figure 1C**), indicating a progressive loss of mature B cell development over time.

Because we found evidence for a B cell development disorder already at ~6 weeks of age, we investigated mice at this age for the remaining experiments. To identify the individual stages of early B cell development, we used the classification according to Hardy et al[26]. The total numbers of pre/pro-B cells within Hardy fractions A, B or C were not different between the three genotypes (**Figure 1D,1E**). Accordingly, as proportions of these fractions from live cells, all these fractions showed no differences across the genotypes (**Supplementary Figure 1**). Although a significant decrease of the proportions of small pre-B cells (Hardy fraction D) was noticed in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to *Tnfaip3*$^{CD11c\text{-}WT}$ mice (**Supplementary Figure 1**), there was no significant reduction of the absolute cell numbers of fraction D small pre-B cells (**Figure 1E**). As a proportion

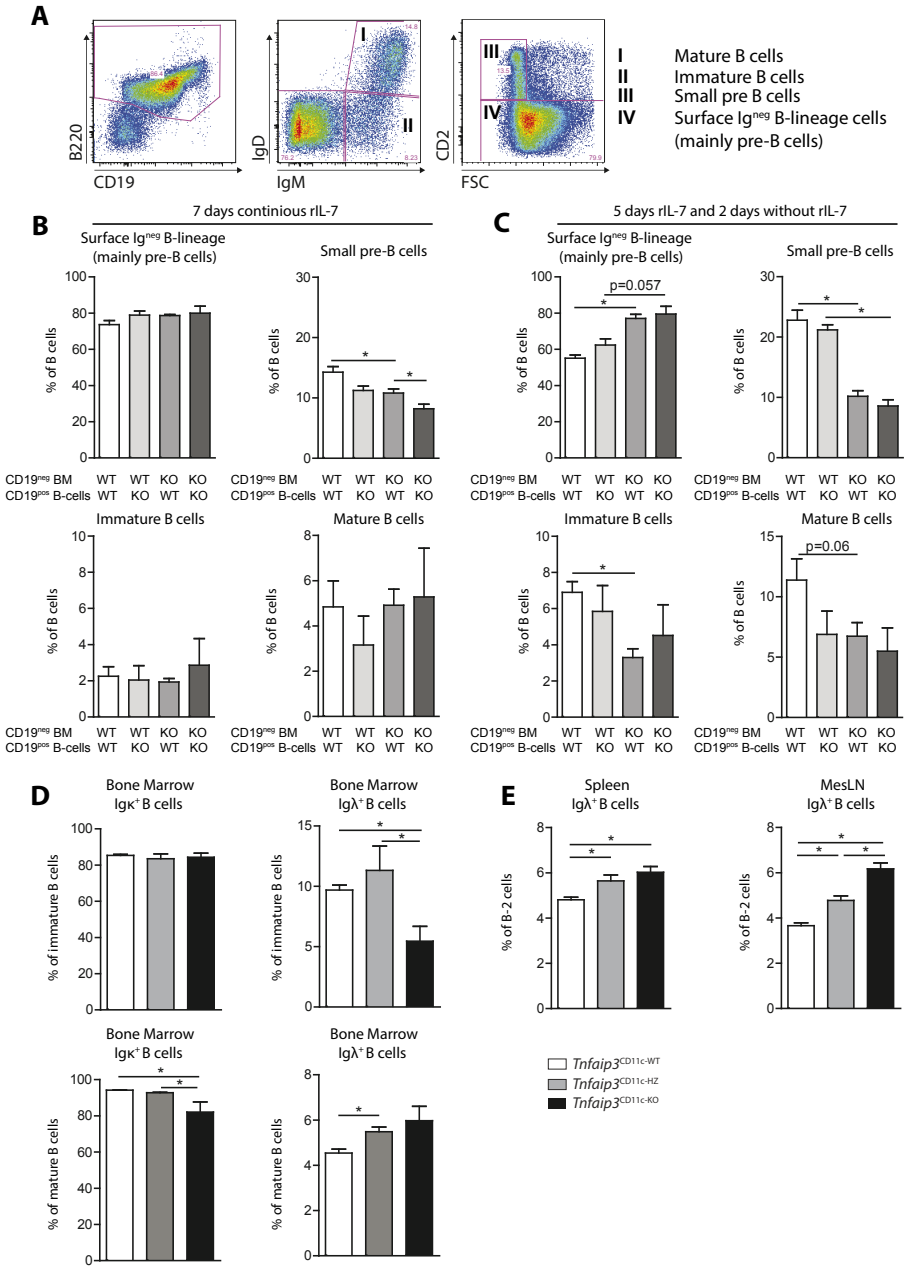

Figure 2: Cell non-intrinsic defect in B lineage cells and evidence for aberrant BCR repertoire selection of Igλ B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

Combinations of CD19^{pos} and CD19^{neg} fractions from bone marrows of *Tnfaip3*$^{CD11c\text{-}WT}$ mice (termed 'WT') and *Tnfaip3*$^{CD11c\text{-}KO}$ mice (termed 'KO') were cultured in vitro. (A) Flow cytometric analysis of *in vitro* Mature B cells (B220^{+}CD19^{+}IgD^{hi}IgM^{hi}), immature B cells (B220^{+}CD19^{+}IgD^{lo}IgM^{hi}), small pre-B cells (B220^{+}CD19^{+}IgD^{lo}IgM^{lo}CD2^{+}FSC^{lo}) and remaining surface Ig^{neg} B-lineage cells containing mainly large cy-

cling pre-B cells ($B220^+CD19^+IgD^{neg}IgM^{lo/neg}CD2^-$). (**B-C**) Quantification of previously described (in **A**) B cell proportions in continuous presence of IL-7 (**B**) and in absence of IL-7 during the last 2 days of culture (**C**) using flow cytometry. (**D**) Quantification of the proportion of $Ig\kappa^+$ and $Ig\lambda^+$ light chain of immature B cells and mature B cells in the bone marrow using flow cytometry. (**E**) The percentage $Ig\lambda^+$ B cells in spleen and Mesenteric Lymph node (MesLN) quantified using flow cytometry. Results are presented as mean values ± SEM of $n = 4$-6 per group. *$P<0.05$ using the Mann-whitney U statistical test.

of live cells, immature B cells and mature B cells (Fractions E and F, respectively), were decreased in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to *Tnfaip3*$^{CD11c\text{-}WT}$ mice (**Supplementary Figure 1**).

In conclusion, ~6-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice have a B cell development disorder at the transition of small pre-B cells to immature B cells. This progresses over time, resulting in a reduction of all B cell stages at 24 weeks of age.

Cell non-intrinsic defect in B lineage cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

Since the *Tnfaip3* gene was specifically deleted in DCs, it was highly unlikely that the observed B cell arrest in *Tnfaip3*$^{CD11c\text{-}KO}$ mice was due to an intrinsic B cell defect. Rather, the defective B cell development in these mice might be explained by changes in the cytokine milieu or cell-cell interactions in the bone marrow micro-environment. For example, it has been reported that cytokines such as IL-1α[27] or IL-1β[28], interferon **(**IFN) α or β [29, 30], IFNγ[31] or TNFα[32] can hamper B cell development in the bone marrow. It is therefore conceivable that high systemic levels of pro-inflammatory cytokines – associated with the autoimmune phenotype – affect B lymphopoiesis.

We utilized an *in vitro* co-culture system to distinguish whether the small pre-B cell to immature B cell transition was hampered due to local cytokines signals or due to anomalous cell-cell interactions between developing B cells and stromal components or myeloid cells, including DCs, present in the bone marrow. We assumed that in such an *in vitro* co-culture all inhibitory cytokines present that would have been present in the bone marrow *in vivo* were removed by the extensive washing procedures. As IL-7 is crucial for B cell development, we performed *in vitro* IL-7-driven bone marrow co-cultures of magnetically sorted $CD19^{pos}$ B-lineage cell fractions in combination with $CD19^{neg}$ fractions containing bone marrow stromal cells as well as myeloid cells. In these experiments, we used various combinations of $CD19^{pos}$ and $CD19^{neg}$ fractions of *Tnfaip3*$^{CD11c\text{-}KO}$ or *Tnfaip3*$^{CD11c\text{-}WT}$ bone marrows. Continuous IL-7 supplementation is known to support pre-B cell survival and proliferation and to limit the developmental progression of pre-B cells into IgM^+ immature B cells[33]. This condition resulted in a major population (~80%) of surface Ig^{neg} B cells that precede small pre-B cells, such as large pre-B cells (**Figure 2B**; for gating strategy see **Figure 2A**). This condition also results in a low percentage of small pre-B cells, immature B cells and mature B cells of about ~10%, ~2% and ~4%, respectively, irrespective of the genotypes of the $CD19^{pos}$ and $CD19^{neg}$ cell fractions (**Fig-**

ure 2B). A moderate, yet significant, reduction of the proportions of small pre-B cells was observed in the co-cultures of *Tnfaip3*$^{CD11c\text{-}WT}$ CD19^{pos} B cell fractions and *Tnfaip3*$^{CD11c\text{-}KO}$ CD19^{neg} supporting cells, in comparison to WT cultures of CD19^{pos} and CD19^{neg} fractions, and also in comparison to the condition with both CD19^{pos} and CD19^{neg} fractions from *Tnfaip3*$^{CD11c\text{-}KO}$ mice (**Figure 2B**).

Upon removal of IL-7 after 5 days of culture, pre-B cells stop cycling and a proportion of the pre-B cells generally proceeds to the IgM^{+} immature B cell stage[33]. Thus, the proportions of small pre-B cells were expected to rise, which however only occurred in conditions whereby B-lineage cells were cultured with *Tnfaip3*$^{CD11c\text{-}WT}$ CD19^{neg} supporting cells, irrespective of the *Tnfaip3*$^{CD11c\text{-}WT}$ or *Tnfaip3*$^{CD11c\text{-}KO}$ genotype of the B-lineage cell fractions (**Figure 2C**). In contrast, the CD19^{neg} fraction from *Tnfaip3*$^{CD11c\text{-}KO}$ bone marrow hampered the efficient generation of small pre-B cells, both from *Tnfaip3*$^{CD11c\text{-}KO}$ and *Tnfaip3*$^{CD11c\text{-}WT}$ CD19^{pos} B-lineage cells (**Figure 2C**). Consistently, the largest population cells that contained the pre-B cells, were reduced in the conditions that gave rise to high small pre-B cells (**Figure 2C**). After withdrawal of IL-7, the proportions of immature B cells were also higher in the presence of *Tnfaip3*$^{CD11c\text{-}WT}$ than *Tnfaip3*$^{CD11c\text{-}KO}$ CD19^{neg} supporting cells, which reached significance for B-lineage cells from *Tnfaip3*$^{CD11c\text{-}WT}$ bone marrow (**Figure 2C**).

Taken together, these *in vitro* experiments support a cell non-intrinsic defect in B lineage cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice. Our finding that the function of *Tnfaip3*$^{CD11c\text{-}KO}$ CD19^{neg} supporting cells *in vitro* was impaired, suggest that inhibitory signals from cytokines are not directly responsible for the defective B cell development in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

Evidence for aberrant BCR repertoire selection of Igλ B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

After successful rearrangement of the Ig heavy chain, immunoglobulin light (IgL) chain rearrangement is initiated at the transition from large to small pre-B cells[33]. The light chain can be either Igκ or Igλ[34], whereby Igλ^{+} B cells often contain non-productive Igκ rearrangements or productive Igκ rearrangements that are associated with autoreactivity[35]. We thus investigated Igκ and Igλ light chain usage in immature B cells and mature B cells within the bone marrow samples (For gating strategy according to Dingjan et al[36], see **Supplementary Figure 2A**). As expected, the majority of immature B cells was Igκ^{+}, irrespective of the genotype (**Figure 2D**). Importantly, we found that the proportions of Igλ^{+} immature B cells was significantly reduced in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to WT controls (**Figure 2D**). In contrast, mature B cells had significantly reduced Igκ^{+} proportions in *Tnfaip3*$^{CD11c\text{-}KO}$ compared to WT mice, while a non-significant rise of the proportions of Igλ^{+} cells in the population of recirculating mature B cells was observed (**Figure 2D**).

Mature B cell populations in peripheral organs such as spleen (For gating strategy according to Dingjan et al[36], see **Supplementary Figure 2B**) and mesenteric lymph node (MesLN) from *Tnfaip3*$^{CD11c\text{-}KO}$ mice and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice displayed significantly increased usage of Ig λ chain, compared to B cells from WT littermates (**Figure 2E**), in line with the observed (non-significant) rise of the proportions of Igλ^{+} recirculating mature B cells. Subpopulation analysis or the splenic B cell population indicated that IgD^{hi}IgM^{hi} and IgD^{hi}IgM^{lo} B cell splenic fractions were responsible for the elevated Igλ proportions (**Supplementary Figure 2B**). Likewise, we found elevated proportions of Igλ^{+} B cells in the IgD^{hi} B cell fraction in the MesLN of *Tnfaip3*$^{CD11c\text{-}KO}$ mice (data not shown).

Taken together, these data show that immature B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice had significantly decreased proportions of Igλ^{+} cells, compared to *Tnfaip3*$^{CD11c\text{-}HZ}$ or *Tnfaip3*$^{CD11c\text{-}KO}$ control mice. Remarkably, during B cell maturation in the spleen these Igλ^{+} B cell proportions increased in *Tnfaip3*$^{CD11c\text{-}KO}$ mice, resulting in elevated Igλ usage in IgD^{hi}IgM^{lo} B cells in spleen, MesLN and bone marrow. Although we cannot exclude that *Tnfaip3*$^{CD11c\text{-}KO}$ mice have a reduced Igλ locus accessibility affecting Igλ recombination, it is more likely that in these mice BCR repertoire selection is abnormal. This would then explain reduced receptor editing in the bone marrow and decreased selection against Igλ^{+} B cells in the periphery[37].

Defects in the B-1 cell compartment in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

Since in contrast to B-2 cell development, B-1 cell precursors do not abide in the bone marrow[38] and develop independently of IL-7[39], we studied B-1 cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

We investigated B-1 and B-2 cells in the peritoneal (**Figure 3A**) and pleural cavities. While total B cell numbers were unchanged, the proportions of B-1 cells (from total B cells) were reduced in *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared with WT controls, both in the peritoneal cavity (**Figure 3B**) and in the pleural cavity (**Figure 3C**). B-1 cells have a restricted B cell receptor (BCR) repertoire and a considerable fraction of these cells recognize neutral phospholipids such as phosphatidylcholine PtC[40, 41]. When we stained peritoneal B cells for PtC-specificity, we observed reduced PtC binding in *Tnfaip3*$^{CD11c\text{-}HZ}$ mice and *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared with *Tnfaip3*$^{CD11c\text{-}WT}$ controls (**Figure 3D,3E**). The frequency of intracellular IL-10^{+} B-1 and B-2 cells in the peritoneum of *Tnfaip3*$^{CD11c\text{-}KO}$ mice was decreased compared to *Tnfaip3*$^{CD11c\text{-}WT}$ mice and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice (**Figure 3F**).

Potential mechanisms for the observed abnormalities in peritoneal B-1-cells may involve changes in DC activity. We did not detect differences in surface expression of co-stimulatory molecules on DCs or in the capacity of T-cells to produce cytokines across the three genotypes (data not shown). However, we did find increased proportions of IL-6^{+} and IL-10^{+} DCs in the peritoneum of *Tnfaip3*$^{CD11c\text{-}HZ}$ mice and *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to controls (**Supplementary Figure 3**). Conversely, the frequencies of IFNγ^{+} DCs were decreased in the peritoneum of *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to WT controls (**Supplementary Figure 3**).

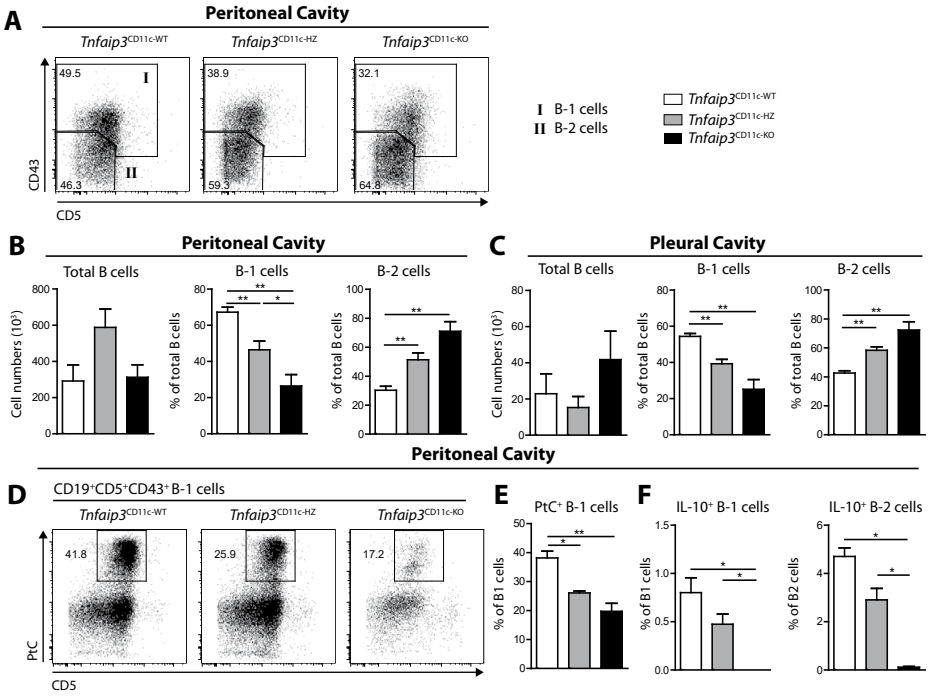

Figure 3: Defects in the B-1 cell compartment in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.
(**A**) Flow cytometric analysis of peritoneal cavity B-1 cells ($CD19^+B220^+CD43^+CD5^{int}$) and B-2 cells ($CD19^+B220^+CD43^-CD5^-$). FACS profiles of gated $CD19^+$ lymphoid cells fractions are shown for the indicated mice. (**B-C**) Enumeration of total B cells, B-1 cells and B-2 cells in peritoneal (**B**) and pleural (**C**) cavity, using flow cytometry. (**D**) Flow cytometry plots of peritoneal PtC^+ B-1 cells, identified by CD5/PtC profiles of $CD19^+CD43^+$ B cells. Representative examples are shown from *Tnfaip3*$^{CD11c\text{-}WT}$, *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice. (**E**) Quantification of PtC^+ B-1 cells as a proportion of B-1 cells in the peritoneal cavity, using flow cytometry. (**F**) Quantification intracellular IL-10^+ cells in the B-1 and B-2 cell population in peritoneal cavity from the three mouse groups, using flow cytometry. Legend for the individual mouse groups is shown in panel A. Results of (A-C) are representative of 3 independent experiments, and (D-F) are from 2 independent experiment and are presented as me an values ± SEM of $n = 4$-7 per group. $^*P<0.05$, $^{**}P < 0.01$ using the Mann-whitney U statistical test.

Summarizing, these data show that the absence of *Tnfaip3* in the DC-lineage affected the numbers of peritoneal B cells. The reduction in PtC-binding and the changes in cytokine profile furthermore suggest altered B-1 cell function.

Reduction of B cell numbers in the spleen of *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

Given that B cell development was disturbed in *Tnfaip3*$^{CD11c\text{-}KO}$ mice, we next characterized the B cell populations in peripheral lymphoid organs. Total splenic B-2 cell numbers were reduced in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to WT controls (**Figure 4A**). Splenic transitional B cells did not differ between the three genotypes (**Figure 4B**). Marginal zone (MZ) B cells were decreased in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to *Tnfaip3*$^{CD11c\text{-}HZ}$ and

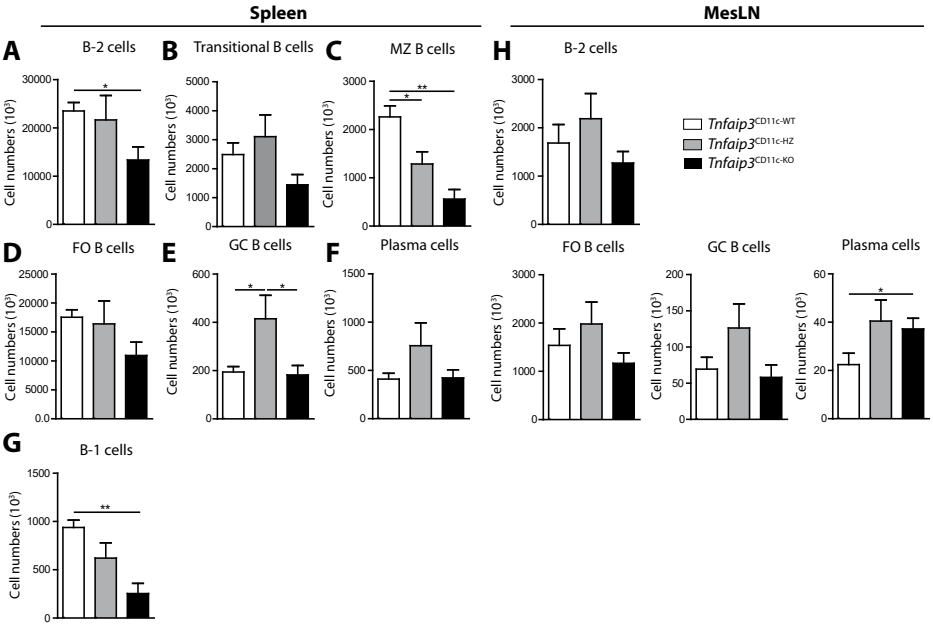

Figure 4: Reduction of B cell numbers in the spleen of *Tnfaip3*$^{CD11c\text{-}KO}$ mice.
(**A-G**) Enumeration of splenic B-2 cells ($CD19^+B220^+CD43^-CD5^-$) (**A**), Transitional B cells ($CD19^+B220^+CD21/35^-CD23^-$) (**B**), marginal zone B cells ($CD19^+B220^+CD21/35^+CD23^-$) (**C**), follicular B cells ($CD19^+B220^+CD21/35^-CD23^+$) (**D**), Germinal center B cells ($CD19^+B220^+CD95^+IgD^-$) (**E**), plasma cells ($B220^-CD138^+$) (**F**) and B-1 cells ($CD19^+B220^+CD43^+CD5^{int}$) (**G**) using flow cytometry. (**H**) Quantification of all B cell subsets from (**A-G**) except transitional B-cells, MZ and B-1 B cells within the MesLN, using flow cytometry. Mice were 6 weeks of age. Results are from representative of 2 independent experiments and are presented as mean values ± SEM of $n = 6$ per group. *P<0.05, **P < 0.01 using the Mann-whitney U statistical test.

Tnfaip3$^{CD11c\text{-}WT}$ mice (**Figure 4C**), but the numbers of total follicular (FO) B cells did not differ significantly between the three genotypes (**Figure 4D**). While the numbers of splenic germinal center (GC) B cells were highest in *Tnfaip3*$^{CD11c\text{-}HZ}$ mice, GC B cells and plasma cells were not different between *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice (**Figure 4E, 4F**). Parallel to our findings in the peritoneal cavity, also the number of splenic B-1 cells were reduced in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to WT littermate controls (**Figure 4G**). The numbers of B cell subpopulations in MesLN were similar between *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice, except for a small but significant increase in the total numbers of plasma cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice (**Figure 4H**).

Taken together, at ~6 weeks of age defective B cell development in the bone marrow of *Tnfaip3*$^{CD11c\text{-}KO}$ mice was associated with a reduction of the numbers of splenic B cells, in particular of the mature B-2 cell and MZ B cell fractions.

B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice show normal T-cell independent and defective T-cell dependent antibody responses.

Since supernatants of WT B cells that were co-cultured with *Tnfaip3*$^{CD11c\text{-}KO}$ DCs contained increased amounts of IgA and IgG1, when compared to co-cultures with *Tnfaip3*$^{CD11c\text{-}WT}$ DCs[10], we determined total Ig levels in serum of ~6-week-old mice. Total IgM was highest in *Tnfaip3*$^{CD11c\text{-}HZ}$ mice, compared to *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice (**Figure 5A**). Total IgA and IgG was increased in the serum of *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice, compared with *Tnfaip3*$^{CD11c\text{-}WT}$ controls (**Figure 5A**). Specifically, the IgG1, IgG2b and IgG2c isotypes were increased in these two genotypes (**Figure 5B**). In contrast, IgG3 serum levels were specifically elevated in *Tnfaip3*$^{CD11c\text{-}HZ}$ mice, paralleling our findings for serum IgM levels (**Figure 5B**).

We next examined the specific Ig response against the T-cell dependent antigen ovalbumin (OVA). While *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice elicited an anti-OVA IgG1 response at day 8 and day 15 after booster immunization, this response was severely reduced in *Tnfaip3*$^{CD11c\text{-}KO}$ mice (**Figure 5C**). On the other hand, a T-cell independent antigen such as DNP-Ficoll, elicited anti-DNP-Ficoll IgM and IgG3 responses in *Tnfaip3*$^{CD11c\text{-}KO}$ mice that were in the normal range (**Figure 5D**).

In summary, *Tnfaip3*$^{CD11c\text{-}KO}$ mice had increased serum levels of several Ig subclasses including IgG1, but showed a severely reduced T-cell dependent IgG1 response. In these mice IgM and IgG3 levels, as well as type II T cell-independent IgM and IgG3 responses, were not affected.

Splenic naïve mature B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice show enhanced responsiveness to activating stimuli.

Because spontaneous Ig production by B cells from aging *Tnfaip3*$^{CD11c\text{-}KO}$ mice was increased[10], we wondered whether mature naïve splenic B cells from 6-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice displayed an increased capacity to produce pro-inflammatory cytokines. Indeed, splenic B cells in *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice showed an increased capacity to produce IFNγ, compared with *Tnfaip3*$^{CD11c\text{-}WT}$ mice, as measured by intracellular cytokine staining upon short *in vitro* activation with PMA and ionomycin (**Figure 6A**).

This prompted us to investigate whether naïve mature B cells in the periphery from *Tnfaip3*$^{CD11c\text{-}KO}$ mice showed evidence for enhanced activation by stimuli such as lipopolysaccharide (LPS) in comparison to B cells from control mice. Indeed, stimulating naïve B cells *in vitro* with LPS or the TLR9 ligand CpG, resulted in higher surface expression levels of CD86 and CD80 on B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice, compared to *Tnfaip3*$^{CD11c\text{-}WT}$ controls (**Figure 6B-6D**). The GC marker Fas Ligand or CD95 was more readily induced upon anti-IgM, LPS, CpG or anti-CD40 stimulation of naïve splenic B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice, compared with B cells from WT control mice (**Figure 6E**). The stimulation-induced expression of CD69 did not differ across the genotypes (**Figure 6F**).

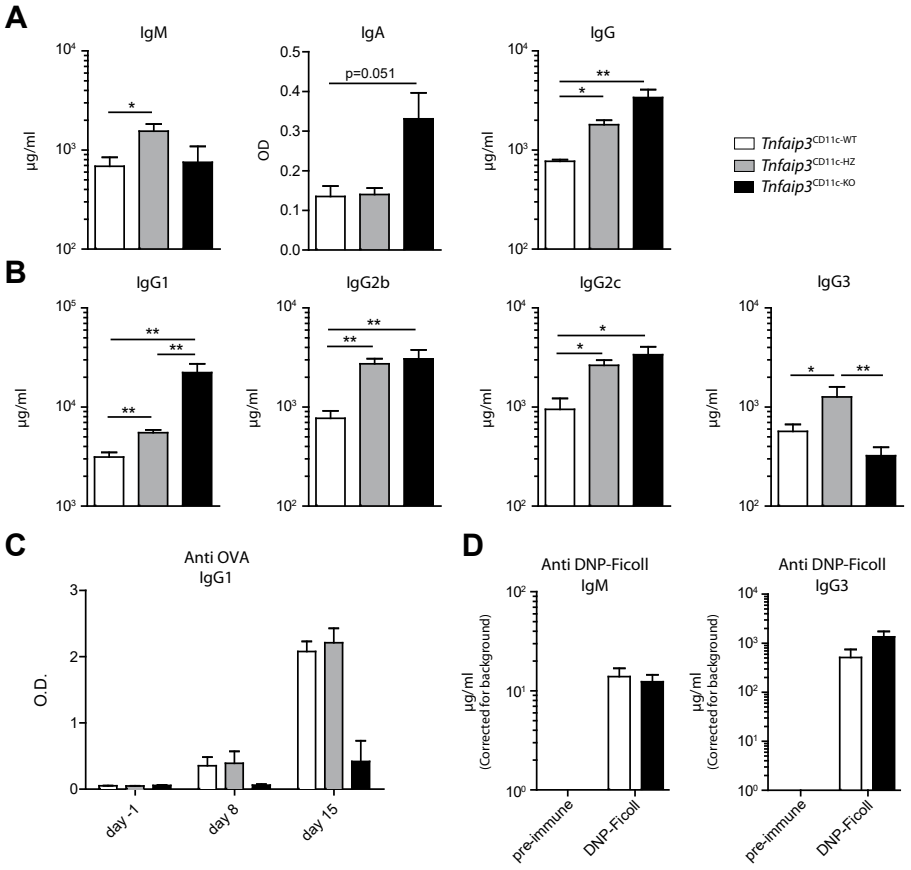

Figure 5: B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice show normal T-cell independent and defective T-cell dependent antibody responses.

(**A-B**) Measurement of total serum levels of IgM, IgA and IgG (**A**) and subclasses IgG1, IgG2b, IgG2c and IgG3 (**B**) using ELISA. (**C**) T-cell dependent B cell response: pre-immune and post-OVA immunisation assessment of OVA-specific IgG1 serum levels using ELISA. (**D**) T-cell independent type II B cell response: pre-immune and post-immunisation assessment of DNP-specific IgM and IgG3 serum levels, using ELISA. Results are presented as mean values ± SEM of n = 3-10 per group. *P<0.05, **P < 0.01 using the Mann-whitney U statistical test.

We next evaluated *in vitro* plasma cell differentiation upon LPS stimulation in the presence or absence of IL-4. Increased proportions of IgG1^{+}CD138^{+} plasmablasts were found upon LPS/IL-4 stimulation of purified B cell fractions from *Tnfaip3*$^{CD11c\text{-}KO}$ mice, compared with WT mice (**Figure 6G**). After LPS stimulation alone, the proportions of IgG3^{+} B cells or plasmablasts were not different between cultures from *Tnfaip3*$^{CD11c\text{-}KO}$ or *Tnfaip3*$^{CD11c\text{-}WT}$ mice (**Figure 5H**).

In conclusion, these data illustrate that following *in vitro* activation naïve splenic B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice display a more activated phenotype and an increased differentiation capacity towards IgG1^{+} plasmablasts, compared to B cells from *Tnfaip3*$^{CD11c\text{-}WT}$ mice.

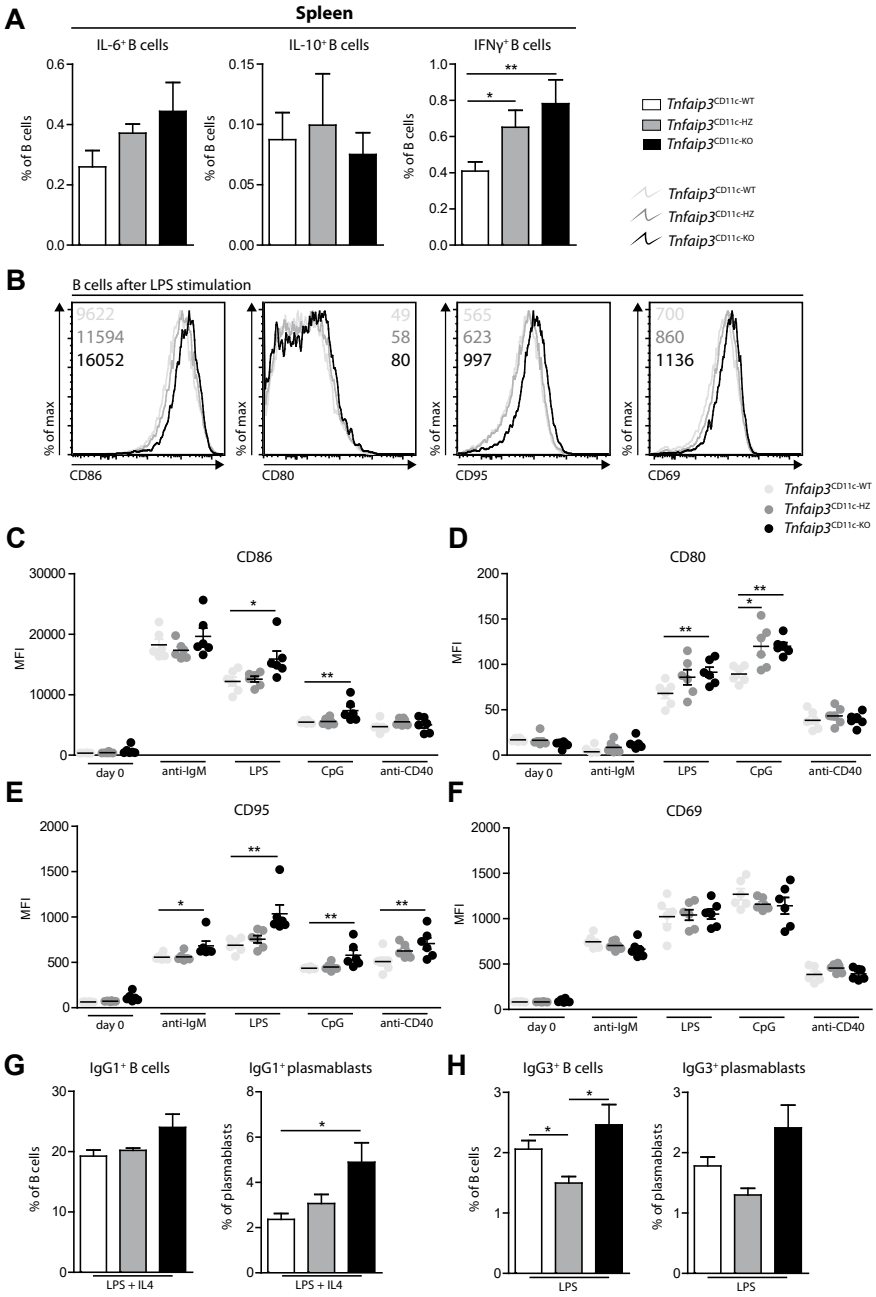

Figure 6: Splenic naïve mature B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice show enhanced responsiveness to activating stimuli.

(**A**) Quantification of the proportions of IL-6$^+$, IL-10$^+$ and IFNγ$^+$ splenic B cells in the indicated mice. (**B-F**) Purified naïve B cells (CD19$^+$B220$^+$NK1.1$^-$CD4$^-$CD8$^-$Ter119$^-$CD11c$^-$GR1$^-$FCRla$^-$CD5$^-$CD43$^-$CD138$^-$CD11b$^-$CD95$^-$) were *in vitro* stimulated using LPS (**B**) and assessed for the indicated activation markers. Data are shown as

histogram overlays (**B**) or quantifications of expression of the activation markers CD86 (**C**), CD80 (**D**), CD95 (**E**) and CD69 (**F**), as determined by flow cytometry, on B cells that were cultured in the presence of the indicated activating stimuli. (**G-H**) Naïve B cells were cultured *in vitro* with LPS in the presence or absence of IL-4, as indicated, and assessed for $IgG1^+$ (**G**) and $IgG3^+$ (**H**) B cells or plasmablasts ($CD19^+B220^+CD138^+$), using flow cytometry. Results are representative of 2 independent experiments and are presented as mean values ± SEM of $n = 6$ per group. $^*P<0.05$, $^{**}P < 0.01$ using the Mann-whitney U statistical test.

DISCUSSION

B cells are crucial in autoimmune diseases[2, 42] and therefore the immune system has several checkpoints to remove autoreactive B cells from the repertoire. In a lupus mouse model of DC-specific deletion of A20/*Tnfaip3* (*Tnfaip3*$^{CD11c\text{-}cre}$), we previously demonstrated that B cells are more activated and engaged in autoreactive Ig production associated with glomerulo-nephritis[10]. Because aged *Tnfaip3*$^{CD11c\text{-}cre}$ mice displayed severely reduced numbers of peripheral B cells and B cell tolerance is initiated in the bone marrow[12-15], we studied bone marrow B cell development in *Tnfaip3*$^{CD11c\text{-}cre}$ mice.

We observed an age-dependent developmental arrest of B-lineage cells: B cell development was hampered at the immature B cell stage in 6-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice and at the pre-B cell stage in 24-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice. Various molecular mechanisms may explain these findings. An intrinsic defect in B cell differentiation affecting the pre-B cell stage is not very likely, since deletion of the *Tnfaip3* gene was specifically targeted to the DC-lineage. Secondly, B cell development might be hampered as a result of the circulating pro-inflammatory cytokines arising during autoimmune pathology. For example, IFN α/β[30] and IFNγ[31] can directly induce apoptosis of IL-7 dependent B cell precursors. In addition, cytokines such as IL-4[43], IL-1(α/β)[27, 28] and transforming growth factor (TGF)-β[44, 45] can exert inhibitory effects on B cell development. Thirdly, B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice might be subjected to improper cell-cell interaction signals, because of an altered activation status of DCs or because activated DCs affected stromal cells or other supportive cells in the bone marrow environment. Finally, a possible scenario could be that B-lineage cells have a reduced migration capacity into $IL\text{-}7R^{lo}$ niches in the bone marrow, e.g. due to reduced expression of CXCL12[32].

To address this issue, we performed IL-7-driven bone marrow cultures *in vitro* using combinations of *Tnfaip3*$^{CD11c\text{-}KO}$ and *Tnfaip3*$^{CD11c\text{-}WT}$ B cells and supporting stromal/myeloid cells. We found that *Tnfaip3*$^{CD11c\text{-}KO}$ stromal/myeloid cells were less beneficial for developmental progression of pre-B cells, indicating that cell-cell contact rather than external cytokines or chemokine gradients hampered B cell development in *Tnfaip3*$^{CD11c\text{-}KO}$ mice. With this *in vitro* experiment, however, we cannot formally exclude the possibility that *Tnfaip3*$^{CD11c\text{-}KO}$ stromal/myeloid cells in our culture system produced soluble factors that hamper B cell development.

Small pre-B cells rearrange their Ig κ or λ light chains, with a 95:5 ratio respectively in the peripheral B cell population in mice[46]. We found that immature B cells in the bone marrow of *Tnfaip3*$^{CD11c\text{-}KO}$ mice had significantly decreased proportions of Igλ^{+} cells, whereas Igλ usage was elevated in the most mature IgD^{hi}IgM^{lo} B cells in peripheral immune compartments. It is possible that *Tnfaip3*$^{CD11c\text{-}KO}$ mice have a reduced Igλ locus accessibility which would subsequently affect Igλ recombination and receptor editing events. However, it appears much more likely that in *Tnfaip3*$^{CD11c\text{-}KO}$ mice the BCR repertoire selection is abnormal, leading to reduced receptor editing in the bone marrow and decreased selection against Igλ^{+} B cells in the periphery[37]. This could e.g. be related to reduced survival of B cells in these mice.

Further experiments, e.g. involving crosses with transgenic mice expressing autoreactive and non-autoreactive BCRs are likely to show whether or not deletion of the *Tnfaip3* gene in DCs affects selection of the BCR repertoire in mice.

B-1 cell progenitors develop independently of IL-7[39] and the bone marrow[38]. Although in *Tnfaip3*$^{CD11c\text{-}KO}$ mice B-1 cells were reduced in numbers and had an altered BCR repertoire, as evidenced by reduced PtC-binding, we found that serum IgM and IgG3 levels were normal and that B-1 cells were able to mount a normal response to the type II T cell-independent antigen DNP-Ficoll. In contrast to this relatively unaffected B-1 cell compartment, we detected severe defects in B-2 cells. Serum levels of IgG1, IgG2b and IgG2c were increased, but B cells were unable to mount a specific T cell-dependent antibody response towards OVA *in vivo,* suggesting that B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice received aberrant stimulatory signals from T-cells. This might well be related to the disturbed micro-architecture of the spleen observed in *Tnfaip3*$^{CD11c\text{-}KO}$ mice[10]. Although we did not check for autoreactive IgG in 6-week-old mice, it was already demonstrated that 12-week-old mice had enhanced anti-dsDNA IgG and one 10-week-old mice had positivity for self-antigen RNP-A[10].

Naïve splenic B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice showed an enhanced response to activating stimuli. A minor proportion of B cells might have been targeted by CD11c-cre-mediated deletion[47], because CD11c can be expressed by a small fraction of B cells, associated with aging and autoimmunity[48]. However, such CD11c^{+} B cells were hardly present in the B cell fractions of our *in vitro* experiments, because we negatively selected for CD11c during our magnetic B cell purification procedure. More likely, the enhanced responsiveness of B cells was a result of activating signals *in vivo*, which appear to be present in a very early phase of the developing autoimmune pathology. Similar co-stimulatory molecule enhancements was also seen in autoimmune disease, as CD80 MFI values were higher in unstimulated naïve peripheral B cells of multiple sclerosis patients[49] and CD80/CD86 MFI values were elevated in CD40-stimulated naïve B cells from SLE patients[50], compared to healthy controls. Although increased MZ B cells[51] and memory B cells[52] could explain rapid and enhanced activation or class switched immu-

noglobulin production, this is not likely to underlie the increased responsiveness of B cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice: these mice have reduced MZ B cell numbers and no presence of memory B cells, as suggested by equal memory B cell marker CD80[53] levels at baseline as WT controls.

To conclude, we demonstrated that loss of A20/*Tnfaip3* in DCs mainly affects B cell development, BCR repertoire selection and function. Improper signals from the bone marrow microenvironment are likely responsible for the age-dependent arrest of B cell development. B cells that reach the periphery act differently to stimuli, indicating that either (i) B cell selection in bone marrow might alter the activation threshold of B cells, or (ii) that the activation status of DCs and T cells in lymphoid organs have a large impact on B cell activation and function. These findings in mice indicate that humoral B cell-driven diseases including SLE, may develop from primary defects in immune cells from a different compartment, such as DCs.

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Conflict of interest

The authors declare no conflict of interest.

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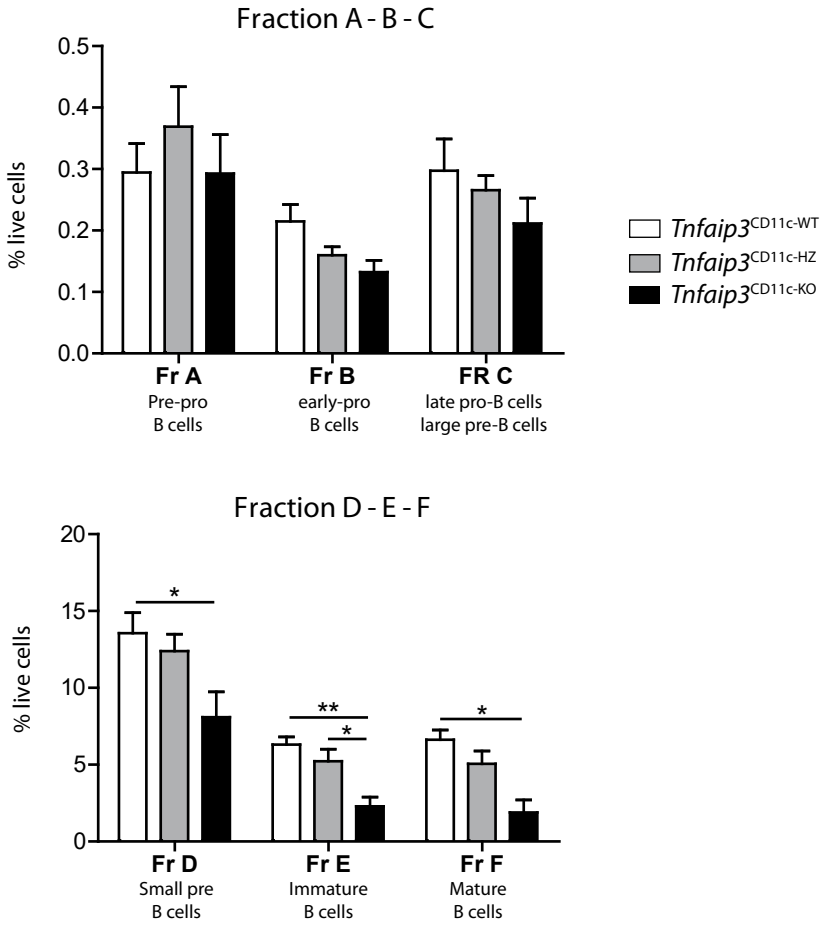
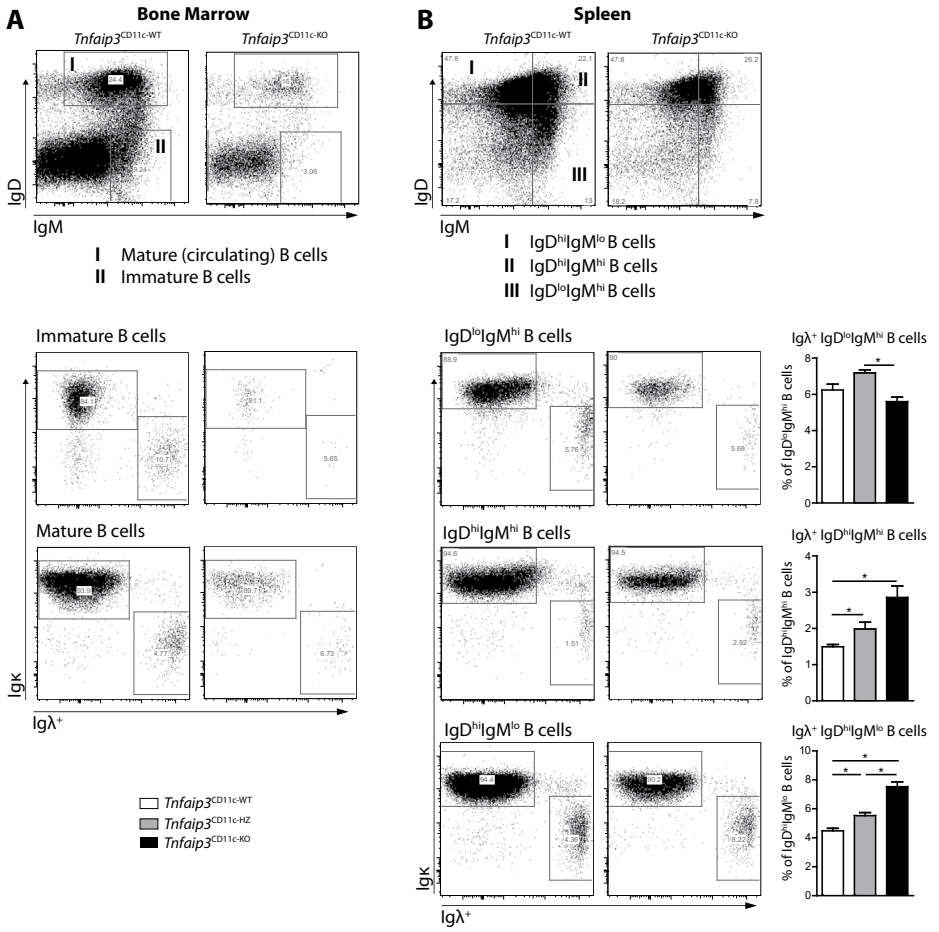
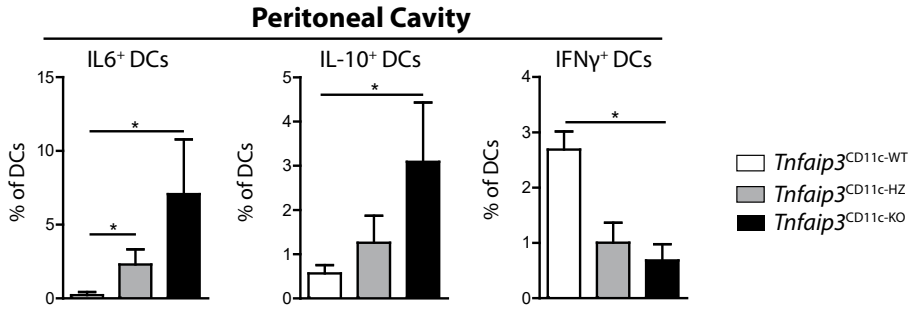


Supplementary Figure 1: Pro B cell stages are unaffected in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.
The indicated developmental B-lineage fractions, according to the Hardy nomenclature[26] in the bone marrow of the three mouse groups, calculated as a percentage of living cells. Results are representative of 3 independent experiments and are presented as mean values ± SEM of $n = 6$ per group. *P<0.05, **P < 0.01 using the Mann-whitney U statistical test.



Supplementary Figure 2: Igκ$^+$ and Igλ$^+$ light chains in bone marrow and spleens in *Tnfaip3*$^{CD11c\text{-}KO}$ mice. (**A**) Flow cytometric analysis Igκ$^+$ and Igλ$^+$ light chains in bone marrow immature B cells (B220$^+$CD19$^+$IgD^{lo}IgM^{hi}) and mature B cells (B220$^+$CD19$^+$IgD^{hi}IgM^{hi}), in the indicated mice. Gating strategy according to Dingjan et al[36]. (**B**) Flow cytometric analysis Igκ$^+$ and Igλ$^+$ light chains in splenic immature B cells (B220$^+$CD19$^+$IgD^{lo}IgM^{hi}), IgD^{hi}IgM^{hi} B cells (B220$^+$CD19$^+$IgD^{hi}IgM^{hi}) and mature B cells (B220$^+$CD19$^+$IgD^{hi}IgM^{lo}), with enumeration of the proportion Igλ$^+$ light chain across all indicated genotypes.



Supplementary Figure 3: Peritoneal cavity DCs from *Tnfaip3*$^{CD11c\text{-}KO}$ mice express increased IL-6, IL-10 and reduced IFN-γ.

6-week-old *Tnfaip3*^{CD11c} mice were analyzed for peritoneal cavity DCs. Quantification of DC (CD11c^{+}MHC-II^{+}) that expressed IL-6, IL-10 and IFN-γ, using flow cytometry. Results are from a single experiment and are presented as mean values ± SEM of $n = 6$ per group. *$P<0.05$ using the Mann-whitney U statistical test.