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Dickkopf-3 Acts as a Modulator of B Cell Fate and Function

Julia Ludwig,* Giuseppina Federico,† Sandra Prokosch,* Günter Kühlbeck,* Sabine Schmitt,* Alexandra Klevenz,* Hermann-Josef Gröne,† Lars Nitschke,‡ and Bernd Arnold*‡

The mechanisms responsible for the generation of a mature B1 and B2 cell compartment are still poorly understood. In this study, we demonstrated that absence of Dickkopf-3 (DKK3) led to changes in the composition of the B cell compartment, which were due to an altered development and maintenance program of B cells. Development of B2 cells was impaired at the pre- and immature B cell stage, resulting in decreased numbers of follicular B cells in adult DKK3-deficient mice. Furthermore, DKK3 limited B1 cell self-maintenance in the periphery, by decreasing the survival and proliferation behavior of B1 cells. DKK3 may act via the BCR signaling pathway, as Ca²⁺ influx upon BCR stimulation was increased and SiglecG, a molecule shown to inhibit Calcium signaling, was downregulated in the absence of DKK3. DKK3-deficient mice exhibited altered Ab responses and an increased secretion of the cytokine IL-10. Additionally, DKK3 limited autoimmunity in a model of systemic lupus erythematosus. In summary, we identified DKK3 as a novel modulator interfering with B cell fate as well as the maintenance program of B cells, leading to changes in B cell immune responses. The Journal of Immunology, 2015, 194: 000–000.

B cells are subdivided into B1 and B2 cells. The names were chosen due to the observation that B1 cells develop earlier than B2 cells during ontogeny (1, 2). B1 and B2 cells are found in different anatomical locations, have different surface markers, and fulfill their functions in different types of immune responses (3). B2 cells are generally responsible for the adaptive Ab response, whereas B1 cells serve as a first line of defense as part of the innate immune system, by secreting most of natural IgM (4, 5). B1 cells are commonly subdivided into B1a and B1b B cells according to the expression of CD5, which is expressed on B1a cells, but not on B1b cells. Both B1 cell subsets are present at a high proportion of 30–70% of the total B cells, depending on the mouse strain, in the pleural and peritoneal cavity (3). B2 cells are divided into follicular (FO) and marginal zone (MZ) B cells. FO B cells present the majority of B cells in the body, with >95% of B cells in lymph nodes (LN) and >70% of B cells in the spleen (3). MZ B cells are only found in the MZ of the spleen and have a distinct function in comparison with FO B cells. Similar to B1 cells, MZ B cells respond fast to blood-borne pathogens with T cell–independent Ab production (6).

*Division of Molecular Immunology, German Cancer Research Center, 69120 Heidelberg, Germany; †Division of Cellular and Molecular Pathology, German Cancer Research Center, 69120 Heidelberg, Germany; and ‡Division of Genetics, Department of Biology, University of Erlangen, 91058 Erlangen, Germany. Received for publication September 3, 2014. Accepted for publication January 7, 2015.

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Abbreviations used in this article: BM, bone marrow; DKK3, Dickkopf-3; dPBS, Dulbecco’s PBS; FO, follicular; IPA, Ingenuity Pathway Analysis; LN, lymph node; MZ, marginal zone; PerC, peritoneal cavity; SLE, systemic lupus erythematosus; TNP, 2,4,6-trinitrophenol; wt, wild-type.

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Materials and Methods

**Mice**

C57BL/6 (wild-type [wt]), Dkk3^{−/−} (C57BL/6 background), wt.Rag2^{−/−} (C57BL/6 background), Dkk3^{−/−} Rag2^{−/−} (C57BL/6 background), CD45.1, C57BL/6, Dkk3^{−/−} RA/EG^{+/−} (30) (C57BL/6 background), and MRL/LPR mice were bred at the central animal facility of the German Cancer Research Center (Heidelberg, Germany). All animals were held under specific pathogen-free conditions. Experiments were approved by the Regierungsrätsäidium Karlsruhe and conducted according to governmental and institutional guidelines.

**Flow cytometry**

Single-cell suspensions of BM, spleen, lymph nodes, or peritoneal cavity lavage were incubated 15 min at 4°C with anti-CD16/CD32 Fc-receptor block and washed, followed by 30 min at 4°C incubation with varying combinations of the following Abs: anti-CD19 (BioLegend), anti-B220 (BD Biosciences), anti-CD8 (BD Biosciences), anti-CD43 (BD Biosciences). Cells were fixed in Perm/Wash solution (BD Biosciences), and followed by staining with Hoechst in Perm/Wash solution for 30 min on ice. Cells were washed, resuspended in dPBS containing 2% FCS and 0.1% sodium azide and measured with the FACS Canto II.

**Adoptive transfer**

To generate BM chimeras, 2 × 10^6 BM cells from wt mice (CD45.1^+^) and Dkk3^{−/−} RA/EG^{+/−} mice (eGFP^+^) were mixed at a ratio of 1:1 and transferred i.v. into either wt.Rag2^{−/−} or Dkk3^{−/−} Rag2^{−/−} mice. Donor BM cells had been treated with anti-CD4-biotin, anti-CD8-biotin, anti-B220-biotin, and anti-CD19-biotin (all from BioLegend) and depleted negatively with streptavidin beads (Dynal). Five weeks after transfer, cells from the BM, spleen, and peritoneal cavity (PerC) were analyzed by FACS.

To generate B1 cell chimeras, B1 cells were purified from PerC of wt (CD45.1^+^) and Dkk3^{−/−} (CD45.2^+^) mice by anti-CD19 microbeads (Miltenyi Biotec), followed by a subsequent treatment with anti-CD23-biotin (BioLegend) and streptavidin beads to deplete CD23^+^ cells. Cells were mixed at a ratio of 1:1, and 1 × 10^6 B1 cells were transferred i.v. into either wt.Rag2^{−/−} or Dkk3^{−/−} Rag2^{−/−} mice. Four weeks after transfer, cells from spleen and PerC were analyzed by FACS.

**BrdU proliferation analysis**

Eight- to 12-wk-old mice were provided drinking water containing 0.8 mg/ml BrdU (Sigma-Aldrich) and 1% (w/v) sucrose (Roth). After 9 d, cells were isolated and stained with surface Abs and the BrdU Flow Kit (BD Pharmingen), according to manufacturer’s instructions. Samples were measured by FACS.

**Calcium measurements in primary cells**

Splenocytes and PerC cells were loaded with Indo-1 (Invitrogen) and stained extracellularly with CD5-PE (BioLegend), CD43-allophycocyanin (BD Biosciences), CD23 PE-Cy7 (BioLegend), B220 allophycocyanin-Cy7 (BD Biosciences), and CD19-FTTC (BioLegend). Calcium influx was assessed 1 min before until 5 min after stimulation with F(ab')2 anti-IgM (Dianova) in gated B cell population on a LSRII (BD Biosciences). Raw data files were transferred to FlowJo software, and mean of calcium influx (ratio of Ca^{2+}-unbound Indo-1 to Ca^{2+}-bound Indo-1) is presented in a comparative overlay analysis. Extracellular Ab staining had no influence on calcium measurements, as assessed by unstained cells.

**Sub-G₁ apoptosis analysis**

B1 cells from PerC were purified by anti-CD19 microbeads (Miltenyi Biotec), followed by a subsequent treatment with anti-CD23-biotin (BioLegend) and streptavidin beads to deplete CD23^+^ cells. B2 cells from spleen were purified by anti-CD19 microbeads. B1 and B2 cells were plated to 96-well plates in triplicates for each analysis time point. To assess apoptosis, cells from cultures were stained extracellular for surface markers. Then cells were fixed 10 min at room temperature with 2% (w/v) paraformaldehyde, washed with dPBS, permeabilized with Perm/Wash solution (BD Biosciences), and followed by staining with Hoechst in Perm/Wash solution for 30 min on ice. Cells were washed, resuspended in dPBS containing 2% FCS and 0.1% sodium azide, and measured with the FACS Canto II.

**Immunization**

C57BL/6N and C57BL/6N Dkk3^{−/−} mice were injected s.c. with 50 μg 2,4,6-trinitrophenol (TNP)-BSA or 100 μg phosphorylcholine–keyhole limpet hemocyanin in CFA or treated i.p. with 50 μg TNP-Ficoll or 4 mg/g weight LPS in PBS. Serum was isolated before immunization, at days 7, 14, and 21, and analyzed by ELISA for concentration of Ag-specific or total Ab titers of relevant isotypes.

**Ig ELISA**

Ag-specific Ig detection was carried out according to standard procedures. Costar polystyrene microplates (Sigma-Aldrich) were coated with 10 μg/ml respective Ags, and samples were incubated at room temperature for 2 h. HRP-conjugated Abs against the respective isotypes were added, and the following color reactions were measured at 492 nm at the ELISA reader (Victor 1420; PerkinElmer).

**Anti-DKK3 treatment and analysis of MRL/LPR mice**

Five-week-old MRL/LPR mice were treated with 0.7 mg/mouse anti-DKK3-4.22 Ab or MOPC21 isotype control i.p. twice per week for 6 wk. Afterward, mice were analyzed for symptoms of SLE.

**Statistical analysis**

Results are shown as mean ± SEM. For statistical analysis, the Student t test was applied to obtain p values. The p values <0.05 were considered significant.

**Results**

Dkk3^{−/−} mice exhibit decreased FO and increased B1 cell numbers

To analyze whether DKK3 affects B cell composition in general, we compared different B cell subpopulations in wild-type (wt) and DKK3-deficient mice. Decreased numbers of FO B cells were found in the spleen and mesenchymal LN of adult (8- to 12-wk-old) and younger (2- and 4-wk-old) Dkk3^{−/−} mice (Fig. 1A, Supplemental Fig. 1). MZ B cells were present at equal numbers in wt and Dkk3^{−/−} mice. In contrast, the B1 cell population was increased in spleen, peripheral lymph node (inguinal and axillary), and PerC of adult mice (Fig. 1A, Supplementary Fig. 1). We found that both B1a and B1b cells were increased in Dkk3^{−/−} adult mice (Fig. 1B). Interestingly, a third population being CD5^+ but CD43^{low} was visible in Dkk3^{−/−} mice, which was not present in wt animals (Fig. 1B).

B2 cell development is impaired in absence of DKK3

To analyze whether the observed B cell phenotype is due to an altered B cell developmental program, we analyzed the B cell compartment at different developmental stages. We started to analyze neonatal mice, as at this early time B2 cell development is rising and B1 cell development is still taking place. The total B cell number was decreased in spleen and PerC of Dkk3^{−/−} neonatal mice (Fig. 2A, Supplemental Fig. 2A). In the spleen, this was due to a reduction of the transitional (immature) B cell population and other progenitor B cells (IgM^{−} CD93^{−} B cells). Interestingly, at this time point the mature FO and the B1 populations were not affected by DKK3, indicating that DKK3 acts on earlier B cell stages (Fig. 2A). Also, in the PerC, the mature B1 and B2 populations had similar numbers compared with wt mice (Supplemental Fig. 2A).

To investigate all stages of B cell development, BM of 2 wk (data not shown), 4 wk, and adult mice was analyzed (Fig. 2B). Pre- and immature B cells were drastically reduced in Dkk3^{−/−} BM, whereas prepro^+^ pro-B cells were unaltered. Also, in this study, the B1 population was not affected by DKK3 deficiency. The mature B2 population in the BM of Dkk3^{−/−} mice was only reduced in 8- to 12-wk-old mice, but not yet in 4-wk-old mice. This observation suggests that the developmental step from the immature to the mature B cell seems not to be influenced by DKK3 (Fig. 2B). To confirm that DKK3 impact starts at the pre-B cell step, in which
As control populations, we analyzed T and B1 cells (Supplemental Fig. 2C). Alternatively, the increased number of B1 cells in DKK3 recipients, as observed in the steady state Dkk3−/− mice (Supplemental Fig. 2C). We found mainly cells expressing higher B220 levels and lower CD5 levels, representing rather B1b cells, than B1a cells, which would normally present the major B cell population of the PerC in the steady state (compare Supplemental Fig. 2C with Fig. 3A).

**B1 cells have a better survival and proliferation capacity in the absence of DKK3**

B1 cells were increased in Dkk3−/− mice in the steady state. This observation is not recognizably based on an altered B1 cell development, as numbers of B1 cells were unchanged in neonatal as well as in 2- and 4-wk-old Dkk3−/− mice and in the BM at all analyzed time points (Fig. 2A, 2B, Supplemental Fig. 1). Furthermore, transfer of BM cells to Rag2−/− recipient mice did not give rise to an increased B1 cell population in Dkk3−/− recipients (Supplemental Fig. 2C). Alternatively, the increased number of Dkk3−/− host B1 cells could be explained by a stronger self-maintenance program of the natural B1 cell population.

To address this point, we transferred purified mature B1 cells from the peritoneal cavity into Rag2−/− mice, which were either wt or Dkk3−/−. Analogous to the BM chimera experiment, we used a 1:1 mixture of wt (CD45.1) and Dkk3−/− (CD45.2) B1 cells. After the pre-BCR is expressed upon H chain recombination, RAG2-deficient (Rag2−/−) mice were analyzed, which are not able to express any form of pre-BCR or BCR. In these mice, no difference in B cell progenitor numbers was found (Supplemental Fig. 2B), confirming that B cell development is only impaired after BCR recombination started.

Dkk3 expression was found at low levels in B cells of the BM and to a higher extent in progenitor B cells of Rag2−/− mice, but not in mature B cells of the spleen (Fig. 2C). To analyze whether the intrinsic, autochthonous DKK3 expression is responsible or contributes to B cell development or whether only environmental, non-B cell–derived DKK3 plays a role, BM cells of wt (CD45.1) and Dkk3−/− (eGFP+) hosts depleted of all B and T cells were mixed in a 1:1 ratio and transferred into Rag2−/− (CD45.2) mice. (Fig. 2C). Gating of different B cell subsets from PerC leukocytes and absolute numbers of these subsets in individual mice. (B) Gating of different B1 cell subsets from PerC leukocytes and absolute numbers of these subsets. B1 cells were gated as CD19+CD5−, B1a cells as CD43+CD5+, B1b cells as CD43+CD5−, and a third B1 population being CD43lowCD5+ was also analyzed. Summary data of three independent experiments. One dot represents cell numbers of one mouse. Mean is indicated for each group. Groups were compared using Student's t test. **p < 0.005, ***p < 0.0005. ns, not significant.

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4 wk, B1 cells were analyzed in recipient mice. B1 cells of Dkk3<sup>−/−</sup> origin (CD45.2) exceeded the wt (CD45.1) B1 cells in all recipient mice (Fig. 3A). Hence, the self-renewal program is increased in Dkk3<sup>−/−</sup> B1 cells. In addition, the number of B1 cells in Dkk3<sup>−/−</sup> recipients was higher than in wt recipients (Fig. 3A). Therefore, environmental DKK3 is also of importance for the self-renewal program of B1 cells. However, in competition, B1 cells from Dkk3<sup>−/−</sup> mice exhibit a stronger self-maintenance capacity than B1 cells isolated from wt mice independent of environmental Dkk3.

The self-renewal process consists of proliferation and survival. To investigate these two processes, we fed wt and Dkk3<sup>−/−</sup> mice with drinking water containing BrdU. After 9 d, BrdU incorporation was similar in the different B1 cell populations in wt and Dkk3<sup>−/−</sup> mice in the PerC. However, in the spleen, in which BrdU incorporation of B1 cells was in general higher than in the PerC, we found an increased BrdU incorporation, indicating increased proliferation, of B1 cells in Dkk3<sup>−/−</sup> mice (Fig. 3B).

To analyze the survival capacity, B1 cells were purified from PerC of wt and Dkk3<sup>−/−</sup> mice and cultured without any stimuli for 8 d. Apoptosis was assessed at different time points by sub-G1 analysis. In contrast to B2 control cells, B1 cells from Dkk3<sup>−/−</sup> mice had a clearly reduced apoptosis rate compared with wt cells (Fig. 3C).

Thus, Dkk3<sup>−/−</sup> B1 cells exhibit both an increased proliferation and survival capacity, which lead to higher numbers of B1 cells in Dkk3<sup>−/−</sup> mice.
DKK3 may contribute to the control of BCR signaling

It has been shown that altered BCR signaling strength can lead to differences in B cell composition and numbers (12). Therefore, we addressed the question as to whether BCR signaling is affected by loss of DKK3 and compared calcium influx as part of the BCR signaling in B cells of wt and Dkk32/2 mice. We found that FO B cells of Dkk32/2 mice possess an increased calcium signaling upon BCR stimulation compared with wt controls, whereas B1 cells of Dkk32/2 mice showed only a slight, but reproducible tendency of increased BCR signaling at the lowest stimulation concentration (Fig. 4A).

To assess the molecular function of DKK3 in more detail, we performed a microarray gene analysis of purified B2 and B1 cells from wt and Dkk32/2 mice (data are accessible at Array Express [http://www.ebi.ac.uk/arrayexpress] under accession number E-MTAB-2850). The analysis of differentially expressed genes between wt and Dkk32/2 mice by Ingenuity Pathway Analysis (IPA) software pointed out genes responsible for the observed decrease in B2 cell—and increase in B1 cell numbers in absence of DKK3 (Supplemental Table I).

The L chain of the BCR was identified by IPA as one of the genes downregulated in Dkk32/2 mice with possible connection to the observed phenotype (Supplemental Table I). Moreover, an increased BCR signaling, as shown above for Dkk32/2 B cells, could lead to altered B cell development, including an altered BCR editing and therewith an altered expression of the L chain (32, 33). Therefore, we analyzed whether the ratio of k to L chain-expressing B cells is changed in Dkk32/2 mice on the protein level.
**Immune and cytokine responses are altered in the absence of DKK3**

To investigate whether the altered B cell compartment in absence of DKK3 may lead to functional changes of B cell responses, we analyzed Ab and cytokine secretion of B cells.

No difference in the Ab response between wt and Dkk3−/− mice was found upon immunization with TNP-Ficoll (Supplemental Fig. 3A), a thymus-independent type II Ag stimulating mainly MZ B cells (34, 35). TNP-BSA, a typical thymus-dependent Ag (36), led to slightly increased levels of TNP-specific IgG1 Abs in Dkk3−/− mice (Fig. 5A). TNP-IgM levels were increased only in the steady state in Dkk3−/− mice, which reflects a part of the overall increased levels of IgM in Dkk3−/− mice, as already shown by Barrantes et al. (29). The most significant alteration of Ab levels upon immunization in the absence of DKK3 was observed when mice were immunized with thymus-independent Ags stimulating B1 cells (6, 37–39), such as LPS (Supplemental Fig. 3B) or phosphorylcholine–keyhole limpet hemocyanin (Fig. 6B).

To analyze whether cytokine secretion is altered in the absence of DKK3, we purified B cells from spleen and PerC and stimulated them in vitro. Cytokine secretion was measured 24 h later by cytometric bead array and/or ELISA. From the analyzed cytokines (IL-6, TNF-α, IFN-γ, IL-4, and IL-10), only IL-10 secretion was changed significantly (data not shown). As B1 cells have been shown to secrete large amounts of IL-10 (40), we analyzed IL-10 secretion on a single-cell level by FACS. Indeed, only the B1 cell population was able to secrete high levels of IL-10, but not the B2 population. Nevertheless, the increased IL-10 level observed in Dkk3−/− mice was not exclusively due to the increased number of B1 cells. In addition, a higher proportion of the B1 cell population secreted IL-10 in Dkk3−/− mice (Fig. 5C).

**Autoimmune symptoms in MRL/LPR mice are increased upon treatment with a neutralizing anti-DKK3 Ab**

The different changes in B cell subset distribution and function in Dkk3−/− mice enticed us to investigate whether autoimmunity caused by B cells would be affected in the absence of DKK3. MRL/LPR mice, which present a mouse model of the human disease SLE, were treated 6 wk with an anti-DKK3 Ab with neutralizing activity, as demonstrated previously (28), or isotype control, as depicted in Fig. 6A. MRL/LPR mice suffer generally from lymphadenopathy. At the time of analysis, all LN of anti-DKK3–treated mice were even more increased than LN of isotype control-treated mice (Fig. 6B). The kidney, which is the organ typically inflamed in MRL/LPR mice, also revealed increased weight in the anti-DKK3–treated mice, indicating a higher degree of inflammation. Microscopic analysis confirmed that indeed increased numbers of mononuclear cells infiltrated the kidney and also the lung (Fig. 6C). In both organs, infiltration was observed predominantly in the perivascular space. Analyzing autoantibody levels, such as anti-DNA Abs, typically observed in SLE patients, we found increased amounts of IgG anti-ssDNA and anti-dsDNA Abs in anti-DKK3–treated mice (Fig. 6D). Similarly to DKK3-deficient C57BL/6 mice, we found an increased population of B1 cells in anti-DKK3–treated MRL/LPR mice.

**Discussion**

Our data establish a previously unrecognized function of DKK3 as a novel modulator of B cell fate and maintenance, thereby leading to changes in B cell responses. Loss of DKK3 function affected the following: 1) the development of B2 cells at the pre- and immature B cell stage; 2) B1 cell self-maintenance in the periphery, by increasing their survival and proliferation behavior; 3) Ab and cytokine production by B cells; and 4) B cell autoreactivity in a model of SLE.
A detailed analysis of B cell subsets in different sites of the body and at different ages of mice revealed that B cell development is impaired in the absence of DKK3, starting directly after birth, leading to reduced FO B cell numbers in adult spleen of DKK3-deficient mice. The maturation step from the immature to the mature B cell did not seem to be impaired, as in younger mice, mature FO B cell numbers were equal in wt and Dkk3<sup>2/2</sup> mice. B cell numbers were reduced at the pre- and immature B cell step in the absence of DKK3. In these two steps, pre-BCR and BCR signals are crucial. The transiently expressed pre-BCR provides feedback to the cell about the success of H chain recombination and induces proliferation (41, 42). After recombination of the L chain, the entire BCR is expressed in immature B cells. In this step, B cells are first tested for reactivity to self-Ags (43). Because we observed a reduction of B cell numbers in DKK3-deficient mice at the time of BCR expression and no difference in B cell numbers in absence of BCR signaling in Dkk3<sup>2/2</sup>Rag2<sup>2/2</sup> mice, it may be possible that DKK3 regulates BCR signaling. This aspect will be further discussed below.

Adoptive transfer experiments of BM progenitor cells confirmed that DKK3 is indeed necessary for the development of a normal B cell compartment. Not only environmental DKK3, but also DKK3 expressed by B cell progenitors controlled B cell development. Independent of the presence of DKK3 in the environment, BM progenitors from wt animals developed stronger into mature B cells than progenitor cells coming from DKK3-deficient hosts, in which the only cells expressing Dkk3 were B cell progenitor cells. Hence, Dkk3 expressed by B cell progenitors themselves is able to support B cell development.

It has been shown that transfer of adult BM into immune-deficient mice resulted mainly in the occurrence of B2 and B1b cells, but not B1a cells (44). In some transgenic systems, the generation of B1a cells was possible. However, these cells differed from the natural B1a cell population, with an altered IgH chain repertoire containing N nucleotide insertions, and no expression of the VH11 gene family (45). We observed B1 cells complying more with the B1b than the B1a cell phenotype in BM recipients. Interestingly, the development of B1 cells in our experiments was similarly supported by environmental DKK3 as one of the B2 cells. In contrast, the maintenance of B1a cells in adoptive transfer experiments, as discussed below, was limited in the presence of DKK3. This is in line with recent findings showing that B1b cells are more similar to B2 cells than B1a cells in terms of development (46).
FIGURE 6. DKK3 blockage leads to more severe symptoms of SLE in autoimmune MRL/LPR mice. (A) Experimental setup for anti-DKK3 Ab treatment of MRL/LPR mice. MRL/LPR mice were treated with anti-DKK3 Ab ($n = 12$) or isotype control Ab ($n = 13$) for 6 wk. At an age of 12 wk, mice were analyzed for symptoms of SLE (B–E). Blood was collected several times for serum extraction. (B) Weight of spleen, axillary LN, inguinal LN, mesenteric lymph nodes (MLN), heart, and kidney was determined. Summary data of three independent experiments are shown. One dot represents weight of one organ. (C) Mice were sacrificed, and tissues were collected in formalin. Paraffin tissue sections of lung were stained with H&E, and paraffin tissue sections of kidney were stained with periodic acid–Schiff stain. Original magnification $\times 20$. Representative pictures of each group are depicted. Kidneys of Dkk3$^{−/−}$ mice show focal arteritis and strong inflammatory infiltrates predominantly in the perivascular space. In lungs of Dkk3$^{−/−}$ mice, pronounced perivascular inflammatory infiltrates of mainly mononuclear cells were observed. (D) Concentration of anti-ssDNA–specific IgM and IgG as well as anti-dsDNA–specific IgM and IgG Abs was determined in sera taken at the start of treatment (Day 0) and 1 wk after treatment was finished (7 wk) by ELISA. Serum of 4- to 5-mo-old MRL/LPR mice and 2-mo-old C57BL/6 mice served as positive (+) and negative (−) controls. One dot represents Ab concentration in serum of one mouse. (E) Cells of spleen and PerC were stained for different T and B cell markers by FACS. Left panels show B220$^+$ T (CD19$^+$ CD3$^+$) and B (CD19$^+$ CD3$^+$) cells. Right panels show analysis of B1 cells in the different lymphatic sites. One dot represents cell numbers of one mouse. Summary data of three independent experiments are presented. Groups were compared using Student $t$ test. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. ns, not significant.
B1 cell numbers were not altered in younger Dkk3<sup>−/−</sup> mice, but were significantly increased in adult mice lacking DKK3. The B1 cell population is self-renewing (44), and the spleen has been shown to be an important organ for B1 cell maintenance (47). Because we found increased proliferation in the spleen and decreased apoptosis of B1 cells of Dkk3<sup>−/−</sup> mice, we conclude that DKK3 limits the self-maintenance capacity of B1 cells. SiglecG has been shown to control the expansion of the B1 cell population (31). Therefore, the decreased expression of SiglecG in Dkk3<sup>−/−</sup> mice might contribute to the increase in B1 cell numbers. Another explanation may represent the increased secretion of the cytokine IL-10 in Dkk3<sup>−/−</sup> mice, as IL-10 has been demonstrated to act as a growth factor for the B1 cell population and contribute to their self-maintenance (48–51).

Because we did not observe differences in B1 cell numbers in neonatal mice, 2 and 4 wk old, nor in the BM of Dkk3<sup>−/−</sup> mice, we suggest that B1 cell development may not be directly impaired by DKK3. However, it has been proposed that the selection process of MZ and B1 cells during development is similar, as these cells also exhibit similar functions (17, 34, 52). Because we found that MZ B cell development was affected by DKK3 in early phases of B cell development (in neonatal, 2- and 4-wk-old mice, but not in adult mice), we do not like to exclude that B1 cell development may also be changed in the absence of DKK3.

The observation that B cell numbers were altered in Dkk3<sup>−/−</sup> mice only after recombination of the BCR genes raised the possibility that DKK3 may affect BCR signaling. This view is supported by three observations. First, BCR signaling and functional modulation of molecules affecting BCR signaling could impact on FO and B1 cell numbers (31, 53). We found increased BCR signaling in B cells of Dkk3<sup>−/−</sup> mice by assessing calcium influx. Second, SiglecG is inhibiting BCR-induced calcium signaling (31). Therefore, the observed lower expression of this molecule in B cells of Dkk3<sup>−/−</sup> mice may contribute to the increased calcium signaling. Third, increased BCR signaling leads to less receptor editing in B cells (33). Accordingly, we found lower numbers of λ-expressing B cells in Dkk3<sup>−/−</sup> mice. Taken together, these results strongly suggest that DKK3 might be a protein influencing BCR signaling.

In addition to the λ-chain and SiglecG, EBF1 was found to be downregulated in absence of DKK3 by microarray gene analysis and determined by IPA to be responsible for the observed B cell phenotype of Dkk3<sup>−/−</sup> mice (Supplemental Table I). EBF1 has been shown to be crucial for B cell development (54). Furthermore, it was demonstrated to be required for B cell commitment, pro-B cell development, and subsequent transition to the pre-B cell stage (55). Lack of this molecule leads to loss of B cells at the pre-B cell step, which reflects our observations in Dkk3<sup>−/−</sup> mice (54). Whether or not DKK3 affects B cell development by regulating EBF1 expression requires further investigations.

DKK3 is highly expressed in the fetus, including fetal liver (56, 57). This early DKK3 expression might regulate B1 cell development, as the fetal liver is one of the most important sites for B1 cell development (8). Furthermore, we found DKK3 expression in developing B cells themselves in the BM, but not in mature B cells, which implies the importance of DKK3 during B cell development.

Our BM chimera experiments demonstrated that both intrinsic and environmental non-B cell–derived DKK3 could contribute to B cell development. DKK3 expression in mature B cells was not detectable. However, adoptive B1 cell transfer experiments showed that the observed phenotype was dependent on the origin of the transferred B cells, isolated either from DKK3–sufficient or -deficient mice, as well as on environmental DKK3. This raises two options. Either the DKK3 expression observed during development may change the genetic program of the B cell long-lasting, or DKK3 in the environment is taken up by B cells and alters their behavior over a longer period. Both possibilities might reflect an imprinting mechanism. Also, mature B cells not expressing DKK3 possess an altered B cell function, with increased Ab responses as well as increased secretion of the cytokine IL-10. Furthermore, treatment of MRL/LPR mice with neutralizing DKK3 Abs lead to aggravated disease, demonstrating that environmental DKK3 is able to act on B cells.

It has been reported that DKK3a binds to integrin α6β in zebrafish (58). However, to date, no surface receptor for DKK3 could be identified in mice or humans. Kataoka et al. (59) demonstrated that recombinant human DKK3 is internalized by induced pluripotent stem cell–derived embryoid bodies via endocytosis, which could represent a mechanism also used by B cells and their progenitors. Because we demonstrated that DKK3 expression by B cell progenitors alone is sufficient to support their development, DKK3 might also act in an autocrine manner on B cells. Secreted DKK3 may be taken up and interact with intracellular signaling molecules. For example, DKK3 was shown to interact with β-TrCP in the cytoplasm, thereby acting as a negative regulator of Wnt signaling (60). Wnt signaling, in turn, has been connected to B cell development in different studies (61–63). Further investigations are necessary to reveal the exact molecular mechanisms and possible binding partners of DKK3 function.

IgM levels in serum were increased in Dkk3<sup>−/−</sup> mice in the steady state. On one hand, these higher levels of B1-secreted natural IgM may protect Dkk3<sup>−/−</sup> mice against different bacterial and viral infections (64, 65). In contrast, Dkk3<sup>−/−</sup> mice might be more prone to autoimmunity. In the C57BL/6 background, no signs of spontaneous autoimmune disease were observed. However, in the MRL/LPR background, a higher level of autoimmunity was detected in mice treated with the neutralizing DKK3 Ab. These mice showed an increase in B1 cell numbers similar to C57BL/6 mice deficient for DKK3, correlating with increased disease parameters. Also, in other studies, increased B1 cell numbers were associated with increased autoimmunity (37, 66–69). However, whether this correlation is causal or rather compensatory for the autoimmune disease must be resolved, as B1 cells were also described to have regulatory potential as part of the described regulatory B cells (70–72). The accelerated disease in anti-DKK3–treated mice might be due to a stronger BCR signaling and a therewith connected higher potential to react pathogenically.

In summary, we identified DKK3 as a novel regulator in determining B cell fate and function. Other DKK family members are necessary during embryonic development (19). Hence, the involvement of DKK3 in B cell development fits well into the capacity of the DKK family to regulate developmental processes.

Because B cell function can be influenced by environmental DKK3, further exploration of the molecular mechanisms will help to progress to therapeutically relevant strategies.

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**Disclosures**

The authors have no financial conflicts of interest.


Supplemental Figure 1. B cell numbers and frequencies are altered in Dkk3−/− mice analyzed at different sites and ages of mice.

FACS analysis of B cells from spleen and peritoneal cavity (PerC), peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) of 8-12 weeks, 4 and 2 weeks old C57BL/6 (wt) and Dkk3−/− mice. B1 cells are gated as CD19+B220intCD43+, B2 cells as CD19+B220high, follicular B cells (FO) as CD19+B220highCD23+ CD21/35int, marginal zone B cells (MZ) as CD19+B220highCD23intCD21/35high, B1a cells as CD43+CD5+, B1b cells as CD43+CD5− and a third B1 population being CD43intCD5+ was also analyzed. Summary data of at least three independent experiments are shown. One dot represents cell numbers of one mouse. Mean is indicated for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).
Supplemental Figure 2. Analysis of B cell development in absence of DKK3.

(A) B cell development is impaired in Dkk3\textsuperscript{−/−} neonatal mice. FACS analysis of B cells isolated from PerC of neonatal C57BL/6 (wt) and Dkk3\textsuperscript{−/−} mice. All analyzed B cell subsets were B220\textsuperscript{+}CD19\textsuperscript{+}. Progenitors were CD93\textsuperscript{+}IgM\textsuperscript{−}, T1 were CD93\textsuperscript{+}IgM\textsuperscript{+}CD23\textsuperscript{−}; T2+T3 were CD93\textsuperscript{+}IgM\textsuperscript{++}CD23\textsuperscript{−}; B2 were CD93\textsuperscript{+}IgM\textsuperscript{+}CD23\textsuperscript{+} and B1 cells were CD93\textsuperscript{−}IgM\textsuperscript{−}CD23\textsuperscript{−}CD21\textsuperscript{−}CD5\textsuperscript{−}.

(B) FACS gating strategy of Prepro+Pro−, Pre−, Immature, mature B2 and B1 B cells from leukocytes of adult bone marrow.

(C) B cell progenitor numbers are not altered in BM of Rag2\textsuperscript{−/−} mice deficient for DKK3. FACS analysis of B cells (CD19\textsuperscript{+}B220\textsuperscript{+}) in BM of wt.Rag2\textsuperscript{−/−} and Dkk3\textsuperscript{−/−}Rag2\textsuperscript{−/−} mice.

(D) A Dkk3\textsuperscript{−/−} B cell origin does not lead to reduced B1 and T cell numbers in mixed BM chimeras. FACS analysis of mixed BM chimeras generated by injection of T and B cell depleted BM cells from wt mice (CD45.1\textsuperscript{+}) and Dkk3\textsuperscript{−/−} RA/EG\textsuperscript{+/−} mice (eGFP\textsuperscript{+}) which were mixed at a ratio of 1:1 and transferred to either wt.Rag2\textsuperscript{−/−} or Dkk3\textsuperscript{−/−}Rag2\textsuperscript{−/−} mice. 5 weeks after transfer, mature T cells (CD5\textsuperscript{+}CD43\textsuperscript{−}CD3\textsuperscript{+}) in spleen and mature B1 cells (CD19\textsuperscript{+}B220\textsuperscript{low}CD43\textsuperscript{+}CD5\textsuperscript{+}) in PerC were analyzed as control population for B2 cells.

A, B and C show summary data of three independent experiments. One dot represents cell numbers of one mouse. Mean is indicated for each group. Groups were compared using student t-test (ns= not significant; *=p<0.05; **=p<0.005; ***=p<0.0005).
Supplemental Table I: Prediction of the humoral immune response in \textit{Dkk3}^{-/-} versus wt B cells by IPA software including genes with a fold change of at least 1.7 times.

<table>
<thead>
<tr>
<th>Humoral immune response</th>
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<th>Associated molecules</th>
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<tr>
<td>quantity of B lymphocytes</td>
<td>1.37E-04</td>
<td>EBF1$^1 \downarrow$, Igl$^3 \downarrow$</td>
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<tr>
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<tr>
<td>lack of B-2 lymphocytes</td>
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<tr>
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<tr>
<td>quantity of immunoglobulin</td>
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<tr>
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<td>EBF1$\downarrow$, Igl$\downarrow$, S1PR2$^4 \uparrow$, SIGLECG$\downarrow$, SLA$\downarrow$</td>
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<td>EBF1$\downarrow$, FCER2$\downarrow$, Igl$\downarrow$, SIGLECG$\downarrow$</td>
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</tbody>
</table>

$^1$ EBF = Early B cell factor  
$^2$ $\downarrow$ = downregulation in Dkk3^{-/-} mice  
$^3$ Igl = immunoglobulin lambda chain  
$^4$ $\uparrow$ = upregulation in Dkk3^{-/-} mice
Supplemental Figure 3. DKK3 deficient mice do not have an altered antibody response to TNP-Ficoll but have increased levels of LPS-specific IgM upon LPS treatment.

(A) C57BL/6 (wt) and Dkk3^{-/-} mice were immunized with TNP-Ficoll in dPBS i.p. on day 0. Serum was analyzed by ELISA for TNP-Ficoll specific antibody levels of IgM and IgG3. Data are pooled from two independent experiments and one dot represents the antibody levels of one mouse. Horizontal bars represent the mean for each group.

(B) C57BL/6 (wt) and Dkk3^{-/-} mice were treated with LPS in dPBS i.p. on day 0. Serum was analyzed by ELISA for total and LPS-specific antibody levels of IgM. Data are pooled from two independent experiments and one dot represents the antibody levels of one mouse.

Groups were compared using student t-test (ns= not significant; *=p<0.05; **=p<0.005; ***=p<0.0005).