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Sialic acids on B cells are crucial for their survival and provide protection against apoptosis

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Sialic acids (Sias) on the B cell membrane are involved in cell migration, in the control of the complement system and, as sialic acid–binding immunoglobulin-like lectin (Siglec) ligands, in the regulation of cellular signaling. We studied the role of sialoglycans on B cells in a mouse model with B cell–specific deletion of cytidine monophosphate sialic acid synthase (CMAS), the enzyme essential for the synthesis of sialoglycans. Surprisingly, these mice showed a severe B cell deficiency in secondary lymphoid organs. Additional depletion of the complement factor C3 rescued the phenotype only marginally, demonstrating a complement-independent mechanism. The B cell survival receptor BAFF receptor was not up-regulated, and levels of activated caspase 3 and processed caspase 8 were high in B cells of *Cmas*-deficient mice, indicating ongoing apoptosis. Overexpressed Bcl-2 could not rescue this phenotype, pointing to extrinsic apoptosis. These results show that sialoglycans on the B cell surface are crucial for B cell survival by counteracting several death-inducing pathways.

sialic acids | B cell development | extrinsic apoptosis | Siglec

Significance

Sialic acids are carbohydrates attached to membrane glycoproteins or membrane glycolipids as so-called sialoglycans. These sialoglycans on immune cells have been implicated in cell signaling and cell migration. This study shows that protection of B cells against programmed cell death is an additional function of sialoglycans. Mice without sialoglycans on B cells exhibit a severe B cell deficiency.

Like other mammalian cells, lymphocytes are covered with a dense layer of glycans, the glycocalyx. This glycocalyx is built from 10 major monosaccharides in different glycosidic linkages creating a rich array of glycan structures, which are part of proteins and lipids (1). In most cases, the glycan structures are capped with sialic acids (Sias), to form sialoglycans. Due to their exposed position, Sias have a major impact on functions of lymphocytes by influencing cell–cell communication, cellular signaling, and cell migration (2, 3). Sias are ligands for sialic acid–binding immunoglobulin-like lectins (Siglecs), which are involved in the inhibition of cell signaling in immune cells and thereby regulate immune responses (4). Sia-containing ligands are also recognized by selectins, which are crucial receptors regulating immune cell migration (3). Furthermore, sialoglycans dampen the complement system, as they can be bound by the complement regulating fluid-phase protein factor H (5). By this interaction Sia promotes discrimination between host and pathogen cellular surfaces, protecting the host from attack by its own complement system.

Lymphocytes carry Sias in either α2,3- or α2,6-glycosidic linkage to the underlying monosaccharide galactose or *N*-acetylgalactosamine and less frequently in α2,8 linkage to other Sia molecules in di- or polysialylated glycans. Linkage- and acceptor-specific sialyltransferases are required to transfer Sia to nascent glycoconjugates, a reaction that strictly depends on the activation of Sia to its cytidine-5′-monophospho-diester (CMP)-Sia that is in turn catalyzed by the nuclear enzyme CMP-sialic acid synthase (CMAS) (6). CMAS-deficient mice lack all sialoglycans on all cellular surfaces and die in utero at around embryonic day E9.5. This embryonic lethality is caused by deficits in the development of extraembryonic tissues due to a maternal complement attack against fetal extraembryonic tissues, e.g., trophoblast cells (7). Trophoblasts are extensively sialylated in the wild type, but not in $Cmas^{-/-}$ embryos and consequently the immune protection of the fetus failed. This normal fetal immune protection is most likely achieved by complement factor H binding to α2,3-linked Sias on trophoblasts and inactivating maternal complement.

Since sialoglycans are implicated in B lymphocyte (B cell) signaling and since a complete knockout (KO) of *Cmas* is lethal, we generated a B cell–specific KO of *Cmas* to examine the function of sialoglycans on B cells. B cells are generated in the bone marrow where they differentiate through distinct stages from pro-B cells to pre-B cells and to immature B cells, which leave the bone marrow and populate secondary lymphoid organs such as spleen and lymph nodes. During their differentiation in the bone marrow they rearrange their immunoglobulin genes at the heavy chain and light chain loci. During this process they enter a proliferation stage, which is dependent on the shortly expressed pre-B cell receptor (pre-BCR) and also driven by interleukin-7 (IL-7) that is

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produced locally in the bone marrow (8). After leaving the bone marrow, immature B cells first reach the spleen via the blood where they mature in so-called transitional stages until reaching the mature stage of long-lived follicular B cells with a half-life of about 5 to 6 wk (9, 10). Mature peripheral B cells depend on two central survival signals, the BCR and the survival cytokine BAFF (11, 12). Intracellular PI3-K signaling is crucial for survival of B cells in the periphery (13). The population of follicular B cells is exchanged after some weeks by newly formed immature B cells from the bone marrow. Although this process likely involves apoptotic processes, experimental detection of apoptosis is usually low in naïve resting B cells. After B cell activation a higher percentage of apoptotic B cells can be detected in the germinal centers during an ongoing immune response (14).

On B cells, sialic acids are mainly found in α2,6 and α2,3 linkages in glycoconjugates and can form *cis* bonds with Siglecs on the same cell surface. Our previous work focused on the function of the B cell inhibitory receptors CD22 (Siglec-2) and Siglec-G (15). We demonstrated that *cis* binding to Sia-containing ligands is crucial for regulation of inhibitory functions of these Siglecs. CD22 binds specifically to α2,6-linked Sia. It has been shown that CD22 clusters in homo-oligomers distinct from those of the BCR by binding to other sialylated CD22 molecules (16, 17). Upon B cell activation, these CD22 nanodomains fuse with BCR nanodomains on the surface, causing CD22 to exert its inhibitory effect through activation of the bound protein tyrosine phosphatase, SHP-1 (15). We have shown that a mutation in the ligand-binding domain of CD22 in CD22-R130E mice leads to a stronger association of CD22 with the BCR and enhanced signaling inhibition, as detected by reduced Ca^{2+} responses (18). This can be explained by the fact that the deficient binding of Sia results in the formation of smaller CD22 nanodomains, which become more motile on the B cell surface and are therefore more likely to be associated with the BCR (19). Interestingly, mice with a mutation in the gene encoding the sialyltransferase ST6Gal-I, which generates α2,6-linked sialic acids (CD22 ligands) show a similar phenotype to the CD22-R130E mice (20, 21). When two mutations were combined in CD22 × ST6Gal-I double-deficient mice, B cell signaling was similarly enhanced as in CD22-deficient mice, indicating a dominant effect of the CD22 mutation. These data revealed the crucial involvement of α2,6-linked Sia in the regulation of the inhibitory function of CD22 on B cells (16, 22).

In contrast to CD22, Siglec-G binds in a broader fashion to α2,3- or α2,6-linked Sia (23). Siglec-G is also an inhibitory receptor on B cells, but Siglec-G–deficient mice show higher BCR-induced Ca^{2+} signaling just in a subpopulation of B cells, the B-1 cells, which have special functions in the immune system (24, 25). To investigate the interaction between Siglec-G and its ligands, we also generated mice with a mutated ligand-binding domain of Siglec-G (Siglec-G R120E mice). In Siglec-G R120E mice there was less association of Siglec-G with the BCR observed, so they showed an opposite phenotype to the CD22-R130E mice (26). The molecular basis for this very different regulation of Siglec-G by ligand binding is not known. One way to study this further is by analyzing the function of the responsible sialyltransferases in genetic models. However, six different enzymes generating α2,3-linked Sia (ST3Gal) are encoded by six different genes. Single KO mice for at least five of these *St3gal* genes exist and analyzed so far, do not show any changes in B lymphocyte numbers or functions (27). However, $CD8^+$ T cell homeostasis and survival is impaired in *St3Gal1*$^{-/-}$ mice (28, 29). The role of α2,3-linked Sia on the surface of B lymphocytes has not been studied in detail so far.

In order to remove all *cis* ligands for CD22 and Siglec-G, we decided to employ an alternative approach by generating a B cell–specific deletion of the *Cmas* gene in mice by crossing *Cmas*-floxed mice (7) with mb1-cre mice (30) to remove all Sia from the B cell surface. We expected that CMAS deficiency would not strongly influence the B cell population, as observed for the individual sialyltransferase-deficient mouse lines. Surprisingly, however, B cells without sialoglycans did not survive in peripheral lymphatic organs. There was a strong reduction of B cell numbers in the lymphatic periphery of B cell–specific *Cmas*-KO mice, revealing a much more general function of Sia on B cells. The involved mechanisms and the consequences of this loss of B cells are examined in detail in this manuscript.

Results

B Cell–Specific *Cmas* KO Mice Have a Strong B Cell Deficiency in the Periphery. In order to study the function of Sia on the B cell surface we generated B cell–specific *Cmas* KO mice. To obtain a B cell–specific KO, we crossed *Cmas* floxed (*Cmas*$^{fl/fl}$) mice (7) to mb1-cre mice (30). In the resulting B cell–specific *Cmas* KO mice we first analyzed the consequence of this mutation on B cell subsets. In these mice, we found a constantly decreasing number of B cells during B cell differentiation in the bone marrow (Fig. 1 *A* and *B*). While bone marrow pro-B cells, in which mb1-cre is first expressed (30), were found in normal numbers, there was a decrease of pre-B and immature B cells and an almost complete loss of mature B cells. Surprisingly, hardly any B cells were detected in the spleen of B cell–specific *Cmas* KO mice. There was a strong reduction of transitional T1, T2, and mature B cells, affecting both follicular and marginal zone B cells (Fig. 1 *C* and *D*). This B cell deficiency was also found in all other peripheral lymphoid organs, including lymph nodes and the peritoneal cavity, as well as in the blood (*SI Appendix*, Fig. 1). This resulted in a complete block of plasma cell differentiation, affecting plasma cells in spleen and bone marrow and leading to absent serum antibodies of all classes in B cell–specific *Cmas* KO mice (*SI Appendix*, Fig. 2).

We performed lectin staining by flow cytometry to determine the degree of loss of sialylation on different B cell populations of B cell–specific *Cmas* KO mice. For this analysis, B cells of *Cmas*$^{fl/fl}$ and *Cmas*$^{fl/fl}$ × mb1-cre mice were stained either with *Sambucus nigra* agglutinin (SNA), specific for α2,6-linked Sia or with *Maackia amurensis* agglutinin (MAA II) binding α2,3-linked Sia. While pro-B cells still expressed quite normal levels of sialoglycans, cell surface sialylation was increasingly lost on later B cell differentiation stages (Fig. 2*A*). Mature B cells from the bone marrow of B cell–specific *Cmas* KO mice had lost all α2,6-linked Sia, similar to a control mouse with a ST6Gal-I deficiency, in which the responsible sialyltransferase is deleted. *Cmas* KO mature B cells additionally lost α2,3-linked Sia (Fig. 2*A*). In the spleen, both α2,6-linked Sia, as well as α2,3-linked Sia was almost completely absent on all B cell subsets of B cell–specific *Cmas* KO mice, but normally expressed on T cells (Fig. 2 *B*–*D*). We also used the lectins *Erythrina cristagalli* agglutinin (ECA) and peanut agglutinin (PNA) recognizing terminal β1,4-linked galactose or β1,3-linked galactose, respectively. In wild-type B cells, most galactose residues are masked by a terminal sialic acid and therefore cannot be bound by these lectins. Accordingly, we observed increasing ECA and PNA binding on differentiating B cells of B cell–specific *Cmas* KO mice, but not on *Cmas*$^{fl/fl}$ control B

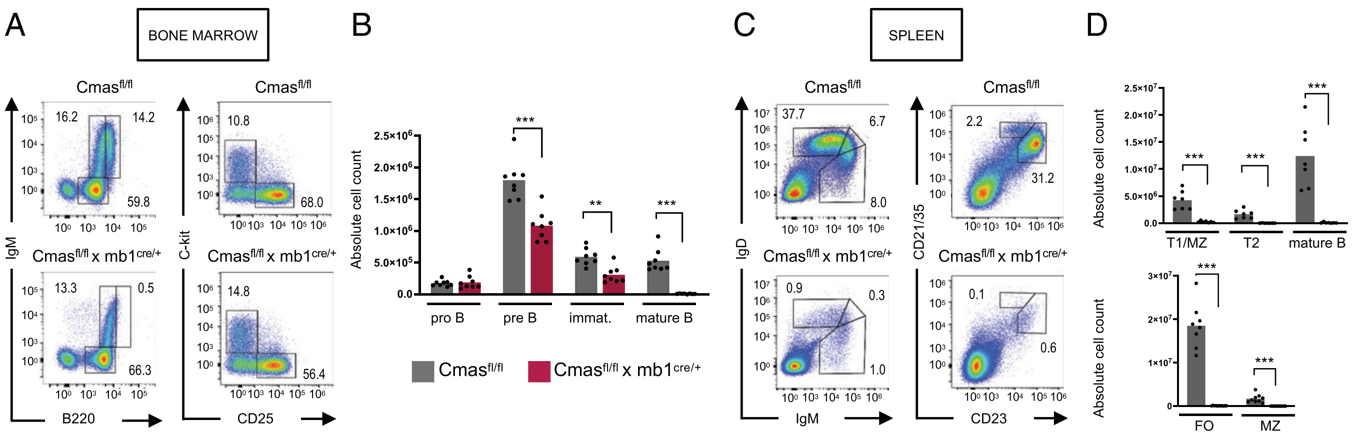

Fig. 1. Strongly reduced B cell numbers in the spleen of B cell–specific *Cmas* KO mice. B cell development of Cmas$^{fl/fl}$ × mb1$^{cre/+}$ mice and control mice (Cmas$^{fl/fl}$) were analyzed via flow cytometry. (*A*) B cell development in bone marrow, pro-B cells (B220^{med} c-kit^{pos}), pre-B cells (B220^{med} CD25^{pos}), immature B cells (B220^{pos} IgM^{pos} IgD^{neg}) and recirculating mature B cells (B220^{pos} IgM^{pos} IgD^{pos}). (*B*) Total cell numbers of the different populations in bone marrow per femur; $n = 8$. (*C*) B cell populations in spleen, gated on T1/MZ B cells (IgM^{pos} IgD^{neg}), T2 B cells (IgM^{high} IgD^{high}), mature B cells (IgM^{med} IgD^{pos}), as well as follicular B cells (FO) (CD21/35^{pos} CD23^{pos}) and marginal zone B cells (MZ) (CD21/35^{high} CD23^{low}). (*D*) Total cell numbers of the different B cell populations per spleen; $n = 6$ to 8. Symbols represent individual animals. Data are representative of eight independent experiments. Significance was calculated by using the Mann–Whitney *U* Test. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

cells (*SI Appendix*, Fig. 3). In conclusion, we found the expected downmodulation of Sia from the B cell surface of B cell–specific *Cmas* KO mice. The more sialoglycans were missing on the cell surface, the more the respective B cell populations were deleted.

***Cmas* KO Pro-B Cells Have a Defect in Cell Accumulation.** As we observed an increasing loss of hyposialylated B cells upon B cell differentiation in the bone marrow of B cell–specific *Cmas* KO mice, we wanted to address possible mechanisms. Therefore, we sorted pro-B cells from the bone marrow of *Cmas*$^{fl/fl}$ and *Cmas*$^{fl/fl}$ × mb1-cre mice and cultured them with or without IL-7, which is a crucial cytokine for survival and proliferation of progenitor B cells. We found a similar cell division of both types of pro-B cells in these IL-7 cultures until day 5, as measured by increasing cell numbers. From day 5 until day 8 *Cmas*$^{fl/fl}$ pro-B cells further increased in numbers, whereas numbers of *Cmas*$^{fl/fl}$ × mb1-cre pro-B cells did not increase further (Fig. 3*A*). Interestingly, when we followed the loss of Sia from the surface of *Cmas*$^{fl/fl}$ × mb1-cre pro-B cells in these cultures by lectin staining by flow cytometry, we observed a constantly decreasing SNA and MAA II binding between day 0 and day 8, however, not quite reaching the low level of Sia on immature bone marrow B cells ex vivo (Fig. 3*B*). We assume that once the sialic acid content on the pro-B cell surface drops below a critical threshold, the cell numbers stop increasing. We excluded differential IL-7 receptor (IL-7R) expression, as the IL7R expression was comparable on B lineage cells of *Cmas*$^{fl/fl}$ and *Cmas*$^{fl/fl}$ × mb1-cre mice (*SI Appendix*, Fig. 4*A*).

B Cells of B Cell–Specific *Cmas* KO Mice Show an Activated Phenotype and an Altered Ca^{2+} Response. Next, we characterized the remaining B cells of *Cmas*$^{fl/fl}$ × mb1-cre mice. As Siglec functions on B cells are regulated by binding to Sia *in cis* (15), we first determined the CD22 (Siglec-2) and Siglec-G expression levels. Compared with the wild type, both Siglecs were expressed at much lower levels on B cell populations in bone marrow and spleen (*SI Appendix*, Fig. 4 *B*–*G*). Since these two Siglecs are known to inhibit BCR-induced Ca^{2+} signaling, we analyzed Ca^{2+} signaling on immature B cells from the bone marrow and the spleen. Bone marrow cells were gated as CD24^{high}, B220^{low}, which includes pre-B and immature B cells and a lower Ca^{2+} response upon anti-IgM stimulation was observed in these cells from *Cmas*$^{fl/fl}$ × mb1-cre mice (Fig. 4 *A* and *C*). In contrast, a higher initial peak, but shorter lasting Ca^{2+} response was observed in splenic immature B cells of *Cmas*$^{fl/fl}$ × mb1-cre mice (Fig. 4 *B* and *D*). We furthermore observed up-regulation of the B cell activation markers MHC II and CD86 on B cells of *Cmas*$^{fl/fl}$ × mb1-cre mice (Fig. 4 *E* and *F*). Thus, the remaining peripheral B cells in *Cmas*$^{fl/fl}$ × mb1-cre mice show an activated phenotype and have decreased expression of inhibitory Siglecs on the surface.

Complement Deficiency Cannot Fully Rescue the B Cell Defect of *Cmas* KO Mice. We then investigated the mechanism of the severe reduction of B cells in peripheral lymphoid organs of B cell–specific *Cmas* KO mice. Since α2,3-linked Sia can bind to complement factor H to inhibit complement activation (5) and since maternal complement attack to the fetus is the dominant cause in lethality of CMAS-deficient mice (7), we first analyzed the role of complement for the B cell deficiency of B cell–specific *Cmas* KO mice. For this purpose, we crossed *Cmas*$^{fl/fl}$ × mb1-cre mice to complement C3-deficient mice ($C3^{-/-}$). C3 is a central complement factor that is activated by all three complement pathways (31). We reasoned that if complement was involved in the deletion of B cells, we would be able to rescue the B cell deficiency of conditional *Cmas* KO mice by an additional genetic deficiency of C3. Although we observed a significant rescue of B cell numbers of most B cell populations in both bone marrow and spleen of *Cmas*$^{fl/fl}$ × mb1-cre × $C3^{-/-}$ mice, when compared with *Cmas*$^{fl/fl}$ × mb1-cre mice, the rescue remained minor and did not restore normal B cell numbers (Fig. 5). In addition to soluble inhibitors of complement, such as factor H, there are also receptors on host cells that can inhibit complement attack to the host. One such cell bound inhibitory complement receptor is decay accelerating factor (DAF), also termed CD55 (32). To examine a possible compensatory mechanism of *Cmas* KO B cells for evasion of complement attack by up-regulation of DAF/CD55, we determined its expression and found rather a clear downmodulation of this inhibitory receptor on *Cmas* KO B cells (*SI Appendix*, Fig. 5). Thus, the B cell deficiency of B cell–specific *Cmas* KO

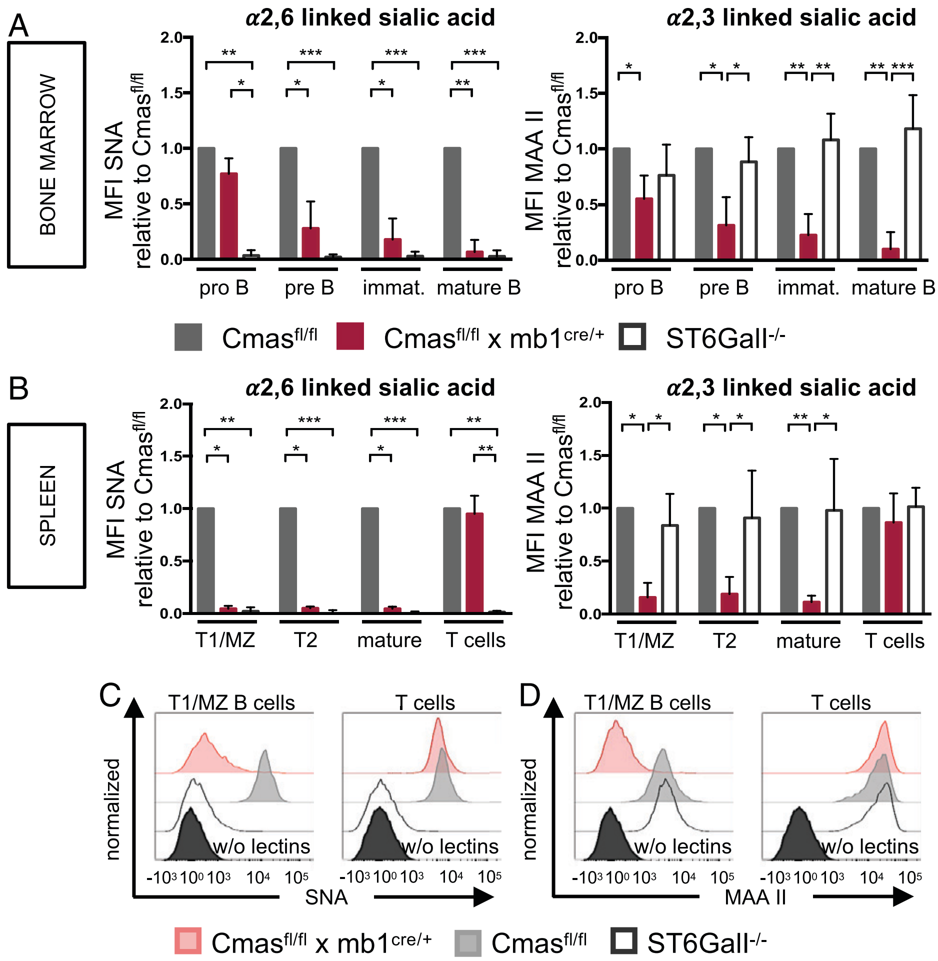

Fig. 2. *Cmas* deficiency leads to down-regulation of sialic acids on the cell surface after the pro-B cell stage. Analysis of sialylation of B cell populations of Cmas$^{fl/fl}$ x mb1$^{cre/+}$ and Cmas$^{fl/fl}$ control animals via flow-cytometry analysis with different lectins (SNA, NeuAcα2,6Gal; MAA II, NeuAcα2,3Gal). ST6GalI$^{-/-}$ mice were used as controls for SNA staining of α2,6-linked sialic acids. (*A*) Display of the mean fluorescence intensity (MFI) of bound lectins of B cell populations in bone marrow, normalized to the control. Cell populations were defined as in Fig. 1. (*B*) Display of the MFI of bound lectins of B cell populations in spleen, normalized to the control. B cell populations were defined as in Fig. 1. T cells are CD3^{pos}. Mean ± SD of four to five individual animals per genotype. Data are representative of four independent experiments. Statistical significance was calculated by using the Mann–Whitney *U* Test. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001. (*C* and *D*) Representative histograms of lectin stainings of splenic T1/MZ B cells or T cells, (*C*) SNA and (*D*) MAA II. Immat., immature.

mice cannot be explained by a complement attack due to lack of membrane-bound Sia as a dominant mechanism.

B Cells of *Cmas* KO Mice Show a Reduced BAFF Receptor Expression and Function. Next, we analyzed whether B cell survival of B cell–specific *Cmas* KO mice was affected by decreased sensitivity to B cell survival cytokines. BAFF is an important cytokine for B cell survival in the lymphatic periphery and the BAFF receptor (BAFF-R) is up-regulated during B cell maturation (11). We found that *Cmas* KO B cells do not up-regulate their BAFF-R during maturation in vivo (*SI Appendix*, Fig. 6 *A*–*C*). We therefore cultivated magnetic activated cell sorting (MACS)-sorted pro-B/pre-B/immature B cells (B220^{pos} IgD^{neg}) from the bone marrow in the presence of BAFF and determined the survival rate of immature B cells by measuring spontaneous apoptosis by quantifying the sub-G1 peak. While addition of BAFF led to a higher survival of control *Cmas*$^{fl/fl}$ immature B cells, it did not have any effect on *Cmas* KO immature B cells (*SI Appendix*, Fig. 6*D*). For this assay we had to work with immature B cells of the bone marrow that do not express high BAFF-R levels, because mature B cells of *Cmas*$^{fl/fl}$ × mb1-cre mice are too sparse. We conclude that a defective up-regulation of the BAFF-R may contribute to the lower survival of *Cmas* KO B cells.

B Cells of *Cmas* KO Mice Die by Extrinsic Apoptosis. Finally, we examined, whether apoptosis is responsible for the B cell deficiency of B cell–specific *Cmas* KO mice. For this purpose, we performed intracellular stainings of B cell populations from bone marrow and spleen with an antibody that recognizes activated caspase 3. Caspase 3 is an effector caspase that is activated both by intrinsic, as well as by extrinsic apoptosis pathways (33). We detected a massive level of apoptosis, as indicated by up to 25% activated caspase 3–positive cells in mature B cells from the bone marrow and spleen of B cell–specific *Cmas* KO mice compared with *Cmas*$^{fl/fl}$ mice (Fig. 6 *A*–*D*). Thus, this strongly up-regulated apoptosis level of mature B cells of *Cmas* KO mice seems to be the major reason for the B cell deficiency. Intrinsic apoptosis pathways are regulated by an interplay of pro- and antiapoptotic proteins, one antiapoptotic protein being Bcl-2. We examined whether we could rescue the B cell deficiency of *Cmas* KO mice by overexpressing Bcl-2. Therefore, we crossed *Cmas*$^{fl/fl}$ × mb1-cre mice to a Bcl-2 transgenic (Bcl2^{tg}) mouse line that overexpresses Bcl-2 specifically in B cells (34). As expected, we obtained higher B cell numbers in all immature, transitional and mature B cell populations of *Cmas*$^{fl/fl}$ × Bcl2^{tg} mice, when compared with *Cmas*$^{fl/fl}$ control mice (Fig. 6 *E* and *F*). However, the Bcl-2 transgene did not rescue the B cell deficiency of *Cmas*$^{fl/fl}$ × mb1-cre mice (Fig. 6 *E* and *F*).

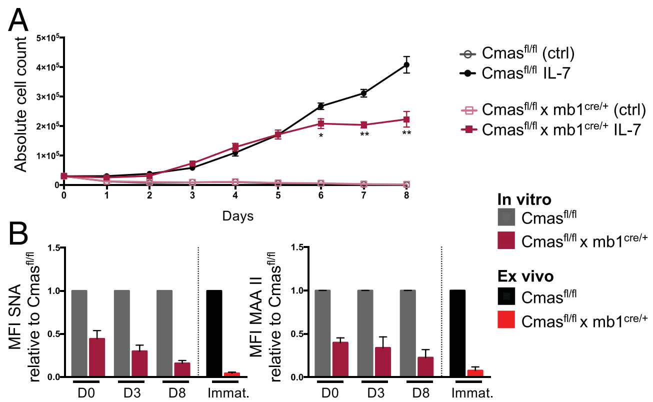

Fig. 3. *Cmas*-deficient pro-B cells show a cell division defect in IL-7 cultures. In vitro analysis of sorted pro-B cells ($B220^{med}$ $c\text{-}kit^{pos}$) from the bone marrow of $Cmas^{fl/fl}$ x $mb1^{cre/+}$ and $Cmas^{fl/fl}$ control mice. Pro-B cells were cultured for 8 d with 10 ng/mL recombinant murine IL-7 and counted daily. (*A*) Total cell numbers per well per day, representative of four experiments. Statistical significance was calculated by using the Mann–Whitney *U* Test. $*P \leq 0.05$; $**P \leq 0.01$. (*B*) Flow cytometry analysis of glycosylation using lectins SNA (NeuAcα2,6Gal) and MAA II (NeuAcα2,3Gal) of the cultured pro-B cells on days 0, 3, and 8 and of immature B cells (immat.) directly obtained from the bone marrow for comparison. Shown is the MFI in relation to the control ($Cmas^{fl/fl}$) of three independent experiments, $n = 3$.

Since Bcl-2 as an antiapoptotic protein mainly affects intrinsic apoptosis pathways, we conclude that apoptosis is involved in the loss of B cells in B cell–specific *Cmas* KO mice, but that intrinsic apoptosis pathways are not the major mechanism.

Extrinsic apoptosis pathways in B cells can be triggered by Fas, which is up-regulated on germinal center B cells. Fas stimulation by the Fas ligand (FasL) activates the initiator caspase 8–inducing apoptotic processes inside the cell (35). To study the contribution of extrinsic apoptosis processes to the B cell deficiency in B cell–specific *Cmas* KO mice, we first determined the level of Fas expression on their B cells. We found an up-regulation of Fas expression on mature *Cmas* KO B cells (Fig. 7 *A* and *B*). This finding has to be evaluated cautiously, however, since the anti-Fas antibody applied showed slightly increased binding to B cells that were desialylated by sialidase treatment (*SI Appendix*, Fig. 7 *A* and *B*). To study Fas functionally, we used an in vitro culture system, in which we stimulated B cells of *Cmas* KO and control mice by human FasL and subsequently determined cleaved caspase 8 with an intracellular staining. Total bone marrow cells were stimulated for 1 d with FasL, then stained for cleaved caspase 8 in pro-B/pre-B or in immature B cells of the bone marrow. Mature B cells of the bone marrow or of the spleen were not included because they did not survive under in vitro culture conditions. We observed a higher level of cleaved caspase 8 in pro-B/pre-B as well as in immature B cells of $Cmas^{fl/fl}$ × mb1-cre mice, compared with $Cmas^{fl/fl}$ mice, without FasL stimulation (Fig. 7*C*). The level of cleaved caspase 8 further increases in immature control B cells upon FasL stimulation, but not in immature B cells of *Cmas* KO mice. We also included $Cmas^{fl/fl}$ × mb1-cre mice and controls in the complement $C3^{-/-}$ background and obtained overall similar results (Fig. 7*C*). The remaining question then was, which cells would trigger extrinsic apoptosis by the Fas or related receptors in Cmas KO B cells. Interestingly, when we cultured bone marrow cells from $Cmas^{fl/fl}$ × mb1-cre and $Cmas^{fl/fl}$ control mice to measure spontaneous apoptosis, we noted a difference for the two culture conditions. When MACS-sorted pro-B/pre-B/immature B cells were cultured, there was no difference in the spontaneous apoptosis between the KO and control mice. However, when total bone marrow cells were cultured, a higher

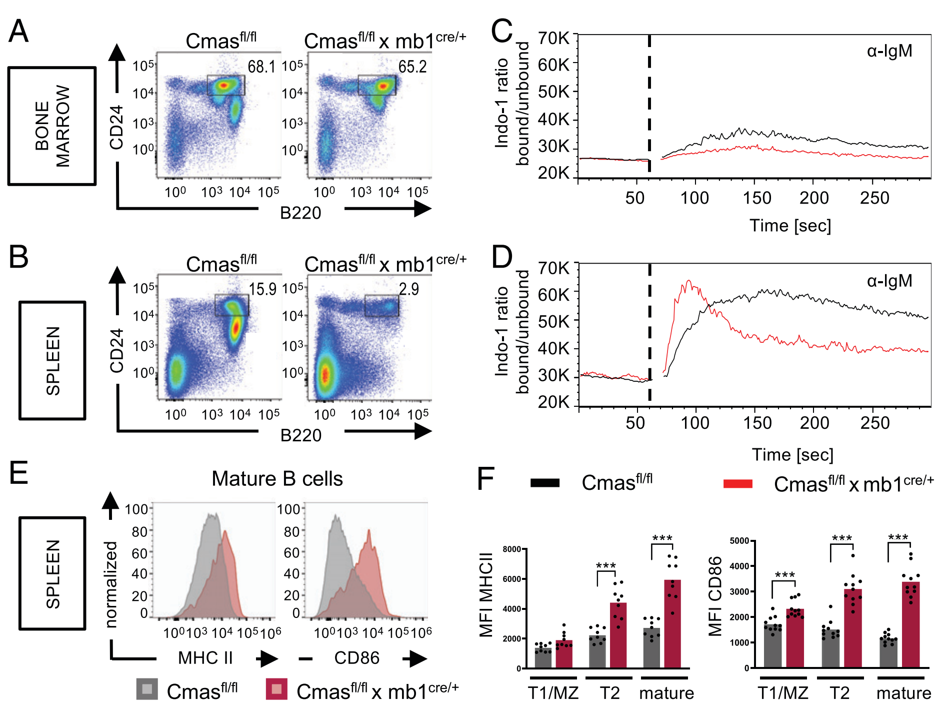

Fig. 4. *Cmas*-deficient splenic B cells show a preactivated phenotype with altered calcium mobilization after BCR stimulation. Calcium mobilization of immature B cells ($CD24^{hi}$, $B220^{low}$) from $Cmas^{fl/fl}$ x $mb1^{cre/+}$ and control ($Cmas^{fl/fl}$) mice was analyzed via flow cytometry in bone marrow (*A*) and spleen (*B*). Cells were preloaded with Indo-1. After a 50-s baseline measuring, B cells from bone marrow (*C*) and spleen (*D*) were stimulated with 26 μg/mL anti-IgM $F[ab]_2$ and the ratio of bound to unbound Indo-1 was determined. Data shown are representative of six experiments; $n = 6$. (*E* and *F*) Analysis of the activation markers MHC II and CD86 at the surface of T1/MZ B cells (IgM^{pos} IgD^{neg}), T2 B cells (IgM^{high} IgD^{high}), and mature B cells ($B220^{pos}$ IgM^{pos} IgD^{pos}) from the spleen via flow cytometry. (*E*) Representative histograms of mature B cells. (*F*) Display of the MFI of MHC II and CD86 of the different B cells populations in spleen. Data are representative of 11 independent experiments. Symbols represent individual animals. Statistical significance was calculated by using the Mann–Whitney *U* Test. $***P \leq 0.001$.

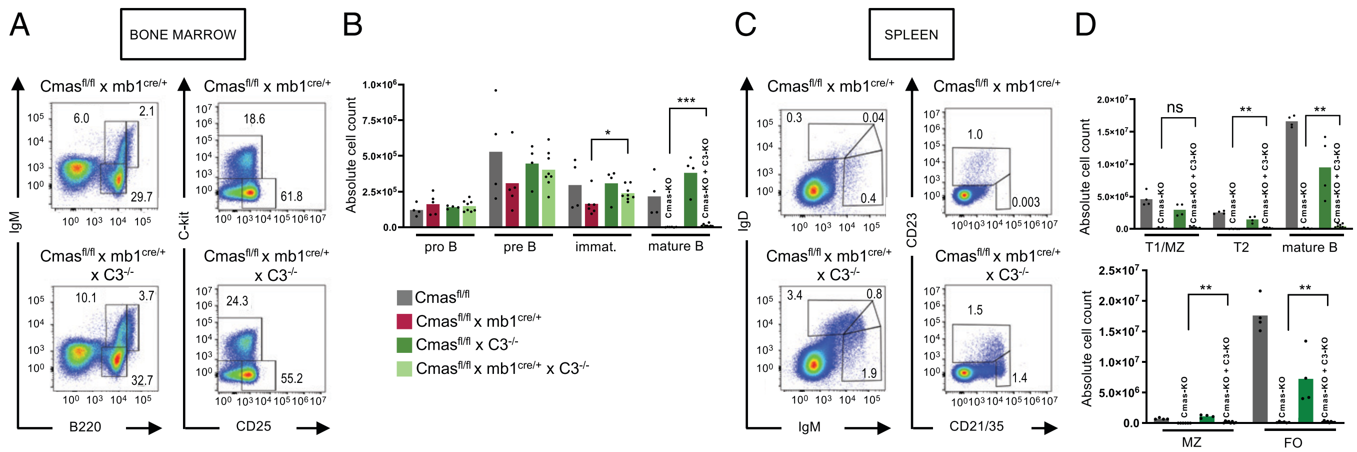

Fig. 5. Complement C3 deficiency cannot rescue the B cell deficiency of Cmas$^{fl/fl}$ x mb1$^{cre/+}$ mice. B cell development in Cmas$^{fl/fl}$ x mb1$^{cre/+}$ mice, crossed with C3$^{-/-}$ mice with a deficient complement system and controls. (*A*) B cell development in bone marrow, B cell populations defined as in Fig. 1. (*B*) Total cell numbers of different B cells populations in bone marrow per femur; *n* = 4 to 8. (*C*) B cell populations in spleen, defined as in Fig. 1. (*D*) Total cell numbers of different B cell populations per spleen; *n* = 4 to 8. Data are representative of six independent experiments. Symbols represent individual animals. Statistical significance was calculated by using the Mann–Whitney *U* Test. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$, ns: not significant.

degree of apoptosis of B cells was detected in *Cmas*$^{fl/fl}$ × mb1-cre mice, compared with *Cmas*$^{fl/fl}$ mice (*SI Appendix*, Fig. 7 *C* and *D*). This suggests that non-B lineage cells in these cultures trigger extrinsic apoptosis. In conclusion, the high rate of apoptosis induction found in *Cmas* KO B cells is to a large extent triggered by an extrinsic apoptotic pathway. This apoptosis induction is independent of the complement system. Finally, we examined whether *Cmas* KO B cells accumulate in the liver, as liver hepatocytes express an asialoglycoprotein receptor (Ashwell-Morell receptor) that depletes desialylated platelets and clears desialylated proteins (36, 37). We did not observe an accumulation of *Cmas* KO B cells in the liver, when compared with control mice (*SI Appendix*, Fig. 7*E*).

Discussion

In this study we found an unexpected severe B cell defect in mice that do not express sialoglycans on the B cell surface. This strong phenotype was surprising, as mice with genetic deficiencies in sialyltransferases did not show any major changes in B

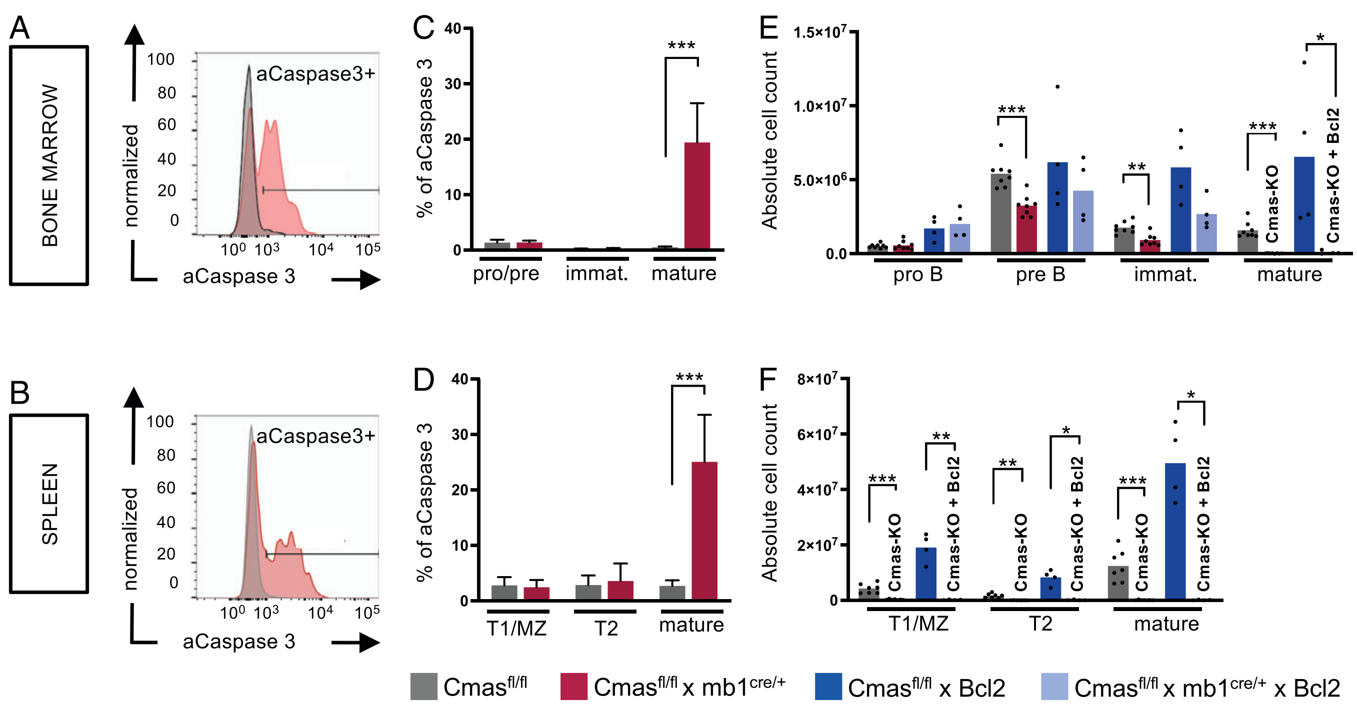

Fig. 6. *Cmas*-deficient mature B cells show strongly increased apoptosis without rescue by overexpression of anti-apoptotic Bcl-2. (*A*–*D*) Analysis of activated caspase 3 (aCaspase 3) via intracellular staining of different B cell populations in bone marrow (*A* and *C*) (pro-B/pre-B cells (B220^{pos} IgM^{neg}), immature B cells (B220^{pos} IgM^{pos} IgD^{neg}), recirculating mature B cells (B220^{pos} IgM^{pos} IgD^{pos}), and spleen (*B* and *D*), defined as in Fig. 1, of CMAS$^{fl/fl}$ x mb1$^{cre/+}$ mice compared with control mice (CMAS$^{fl/fl}$). (*A* and *B*) Representative histograms of aCaspase 3 in mature B cells. (*C* and *D*) Display of mean ± SD of aCaspase 3; *n* = 7. Total cell numbers of B cell populations in bone marrow per femur (*E*) and spleen (*F*) in CMAS$^{fl/fl}$ x mb1$^{cre/+}$ mice compared with control mice (CMAS$^{fl/fl}$), *n* = 8, as well as in CMAS$^{fl/fl}$ x mb1$^{cre/+}$ x Bcl2^{tg} mice compared with control mice (CMAS$^{fl/fl}$ x Bcl2^{tg}) with B cell–specific overexpression of anti-apoptotic Bcl-2, *n* = 4. Symbols represent individual animals. Data are representative of seven independent experiments (*A*–*D*) and four independent experiments (*E* and *F*). Statistical significance was calculated by using the Mann–Whitney *U* Test. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

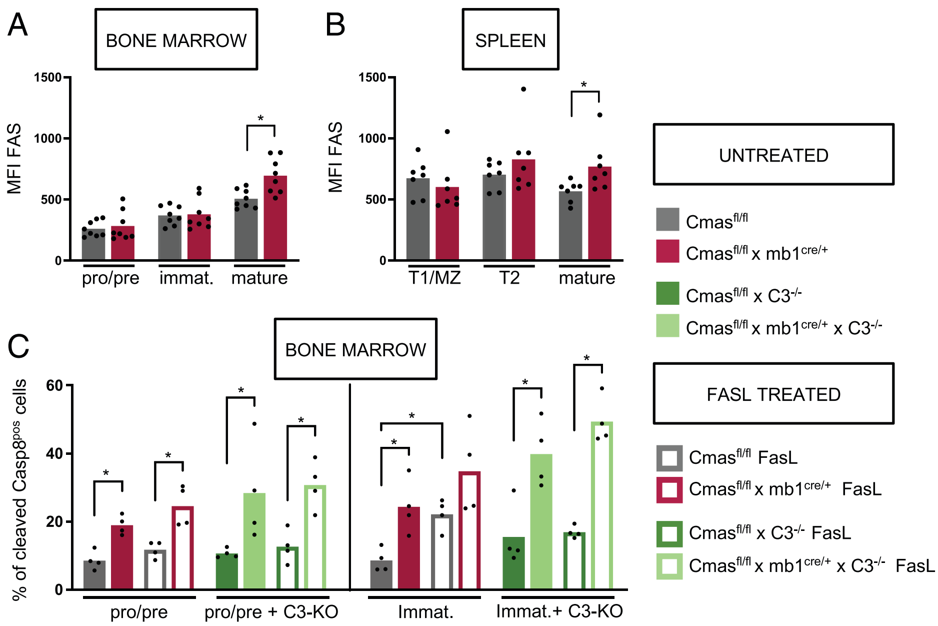

Fig. 7. Extrinsic apoptosis as detected by cleaved caspase 8 is increased in *Cmas*-deficient pro-B/pre-B and immature B cells. (*A* and *B*) Expression of the surface receptor FAS analyzed via flow cytometry in different B cell populations in bone marrow (*A*) and spleen (*B*) of CMAS$^{fl/fl}$ x mb1$^{cre/+}$ mice compared with control mice (CMAS$^{fl/fl}$); $n = 7$ to 8. (*C*) Stimulation of bone marrow cells with or without 200 ng/mL Fc-Fas ligand (FasL) for 1 d and subsequent staining for cleaved caspase 8 (Casp8). Probes were analyzed via flow cytometry, showed is the percentage of cells positive for cleaved Casp8 of the different populations; $n = 4$. Symbols represent individual animals. Solid bars are untreated probes, empty bars are treated with FasL for 1 d. Data are representative of five independent experiments. Statistical significance was calculated by using the Mann–Whitney *U* test. $*P \leq 0.05$.

cell populations. In particular, mice lacking α2,6-linked Sia (ST6Gal-I–deficient mice) have normal B cell populations (21), and in mouse lines where sialyltransferases for α2,3-linked Sia are deficient (ST3Gal-I, ST3Gal-IV, and ST3Gal-VI–deficient mice) no changes in B cell numbers were reported (27, 28). Also, mice lacking α2,8-linked Sia (ST8Sia-II and ST8Sia-IV–deficient mice) had no grossly changed B cell numbers (38). Sia expression on B cells has so far been mainly thought to influence Siglec binding and thereby affecting B cell signaling or to regulate selectin-mediated cell migration (2–4). Our findings of B cell–specific *Cmas* KO mice point to a much broader function of sialoglycans on lymphocytes.

We observed a gradually increasing loss of B cells in B cell–specific *Cmas* KO mice upon B cell differentiation in the bone marrow, with pre-B cells being mildly affected, immature B cells showing a stronger reduction, and mature B cells being almost completely absent. This reflects the cre expression by the mb1 promoter, which is active first in pro-B cells and is continuously expressed during B cell development. However, as also mature stages of B cell differentiation could develop, a severe block in B cell differentiation is unlikely. The block of cell division after a couple of days of pro-B cell cultures with presence of IL-7 rather points to a proliferation or survival defect of *Cmas*-deficient B lineage cells. The cre-mediated deletion of the *Cmas* gene in pro-B cells leads to a delay of Sia loss on the cellular surface, which is due to the protein half-life of the CMAS protein and also due to the turnover of glycoproteins on the B cell surface. We could follow this gradual loss of sialylation both in the IL-7 cultures over several days in vitro, as well as during in vivo B cell differentiation by lectin stainings, detecting either Sia or the exposed galactose due to the loss of terminal Sia. We interpret the results of these lectin stainings and the phenotype of the mice in the way that below a certain threshold of sialoglycan expression, CMAS-deficient B cells cannot survive. Whether defective proliferation or increased apoptosis of *Cmas* KO pro-B cells is involved in the IL-7 cultures was not determined. There was also no indication of a preferred differentiation of *Cmas* KO pro-B cells to pre-B cells in these cultures. At least for peripheral naïve B cells that do not proliferate without activation, a survival defect is the only likely explanation for the observed phenotype. As no accumulation of *Cmas* KO B cells in the liver was found, we conclude that removal of desialylated B cells by the asialoglycoprotein receptor on hepatocytes is no major mechanism involved here (39). However, we cannot completely exclude the involvement of this pathway. As peripheral B cells of B cell–specific *Cmas* KO mice disappear in all peripheral lymphoid organs, including the blood, spleen, and lymph nodes, the major cellular deletion probably happens after exit from the bone marrow, possibly in the spleen or in the vascular system.

The remaining peripheral B cells of B cell–specific *Cmas* KO mice show an activated phenotype, which is typical for lymphopenic mice, as observed also for T cells in a lymphopenic environment (40). Furthermore, we found an anti-IgM–induced Ca^{2+} response of immature splenic *Cmas* KO B cells that resembles the response of the B1a B cell population in mice where CD22 and Siglec-G both carry mutations that lead to loss of Sia binding (CD22-R130E × Siglec-G–R120E mice) (41). The Ca^{2+} response of splenic immature *Cmas* KO B cells was characterized by an initial higher peak, but a faster declining response. The immature B cells of *Cmas* KO mice are CD23^{neg}, CD21^{low}, IgM^{pos}, so they resemble the phenotype of B1a cells, except lacking the expression of CD5. Thus, this Ca^{2+} response of immature *Cmas* KO B cells lacking cell surface sialylation resembles the Ca^{2+} response of B cells from mice where both Siglecs cannot bind their Sia ligands (41). Since *Cmas* KO B cells also showed reduced CD22 and Siglec-G expression, their Ca^{2+} responses were not identical to the one of B cells of CD22-R130E × Siglec-G–R120E mice. It was previously also shown in ST6Gal-I–deficient mice that their B cells expressed lower levels of CD22. ST6Gal-I deficiency also increased IgM and CD22 endocytosis, which is both relevant for our findings (20).

Mice with a complete *Cmas* deficiency in all cells die at embryonic day 9.5 due to a maternal complement attack against the fetal tissue (7). This is likely due to the loss of the protective role of complement factor H. Factor H binds to polyanionic substances such as glycosaminoglycans as well as to 2,3-linked Sia on the cellular surface and protects from excessive C3 deposition/activation as it may occur in the alternative pathway of complement activation (42). The specificity of Sia binding has been shown by a structural analysis of factor H cocrystallized with an α2,3-linked Sia containing trisaccharide and C3b (5). In mice with complete *Cmas* deficiency, injection of cobra venom factor, resulting in exhaustion of the maternal complement C3, rescued the early embryonic lethality (7). Therefore, a complement attack due to defective factor-H binding to B cells without surface Sia was expected to be a likely mechanism for the peripheral B cell deficiency of B cell–specific *Cmas* KO mice. The marginal rescue of B cell numbers in $Cmas^{fl/fl}$ × mb1-cre mice when crossed to the $C3^{-/-}$ mice showed that complement attack to Sia-deficient B cells contributes to the B cell deficiency, but does not comprise the major mechanism. As the BAFF receptor was not properly up-regulated on *Cmas* KO B cells and as immature bone marrow B cells of $Cmas^{fl/fl}$ × mb1-cre mice did not show a survival advantage upon BAFF addition, we conclude that this can also contribute to the B cell deficiency. Follicular B cells of spleen and lymph nodes normally express much higher BAFF-receptor levels (11), but could not be tested from $Cmas^{fl/fl}$ × mb1-cre mice due to too low numbers.

The major mechanism causing the B cell deficiency of B cell–specific *Cmas* KO mice seems to be a strong induction of apoptotic processes. The up to 25% mature B cells with activated caspase 3 from *Cmas* KO mice is a very high proportion of apoptotic cells, which has never been detected in naïve B cells from wild-type mice. Only germinal center B cells normally show some degree of apoptosis, leading to detection of 3 to 5% of germinal center B cells of normal mice with activated caspase 3 (14). Therefore, it was quite notable that this strong apoptosis-induced B cell deficiency of *Cmas* KO mice could not be rescued by overexpressed Bcl-2. Bcl-2 is an antiapoptotic protein that inhibits the proapoptotic Bax and Bak proteins. Bax and Bak dimerize and stimulate cytochrome-c release from mitochondria, triggering the intrinsic apoptotic pathway (33, 43). The Bcl-2 transgenic mouse line has been used to rescue reduced B cell numbers due to apoptosis in several other mouse models, including one in which BAFF was inhibited (44–46). Our result of a complete failure to rescue the B cell deficiency of *Cmas* KO mice by overexpression of Bcl-2 indicates only a minor contribution of the intrinsic pathway to the observed apoptosis and argues against BAFF-receptor reduction being the sole cause of B cell loss. Instead, the high levels of cleaved caspase 8 in *Cmas* KO B cells ex vivo clearly show involvement of the extrinsic apoptotic pathway as a major mechanism in the B cell deficiency of B cell–specific *Cmas* KO mice. Interestingly, ST3Gal-I–deficient mice had decreased $CD8^+$ T cell numbers in the periphery due to spontaneous apoptosis. Also in this case, transgenic overexpressed Bcl-2 could not rescue the $CD8^+$ T cell defect, indicating a similar mechanism as observed here (28, 29).

Our in vitro culture assay did not show a clear involvement of the Fas/FasL pathway in the induction of extrinsic apoptosis in *Cmas* KO B cells. The higher Fas expression found on mature B cells of B cell–specific *Cmas* KO mice is hard to evaluate, as the used anti-Fas antibody showed a higher binding to sialidase-treated B cells. However, it is nevertheless possible that the Fas pathway contributes to the induction of extrinsic apoptosis in vivo. Otherwise, other death-inducing receptors such as TNF receptors may be involved. Interestingly, it was shown that sialylation of Fas by the ST6Gal-I provides protection against Fas-mediated apoptosis in a cancer cell line (47). α2,6-linked sialylation of Fas prevented Fas internalization, which is required for apoptotic signaling. A similar pathway was also described for the related TNF receptor 1 (48). Therefore, *Cmas* KO B cells will likely react with a stronger Fas/TNF receptor signaling response to its ligands. As the Fas ligand and TNF are expressed broadly by many cell types, receptor stimulation by ligands produced by stroma cells or immune cell types may happen in lymphatic organs such as spleen and lymph nodes or in blood vessels triggering these apoptotic responses of *Cmas* KO B cells. Interestingly, decrease of Sia from the surface of lymphocytes has been detected as an "eat-me" signal for phagocytic cells, which may be involved here as well. Neuraminidase treatment could enhance this phagocytic process (49). Furthermore, $CD8^+$ T cells of ST3Gal-I–deficient mice or wild-type $CD8^+$ T cells treated with neuraminidase were sensitive to PNA-induced apoptosis (28). As PNA binds to β1,3-linked galactose lacking terminal Sia and as PNA also binds to *Cmas* KO B cells, an endogenous lectin binding to these glycan structures could also be involved in the apoptosis induction observed here. But this needs further experimental exploration.

In conclusion, we have described a mechanism for how sialoglycans on the surface of B lymphocytes protect these cells from apoptosis. We think that this mechanism is of general importance also for other cell types. Cancer cells often up-regulate their sialic acids on the surface. This is discussed as an escape mechanism against recognition of the immune system or as a mechanism to engage inhibitory Siglec receptors on tumor-infiltrating myeloid cells (50). These mechanisms may exist, but our data presented here also point to a further role of high Sia expression on cancer cells: It may provide protection from apoptosis. Our findings highlight that interfering with sialoglycans on cancer cells may therefore be a possible therapeutic strategy in this disease. Such glycan targeting on cancer cells has already been tested experimentally (51, 52).

Materials and Methods

Mice. $Cmas^{fl/fl}$ mice were generated as described (53) and crossed with $mb1^{cre/+}$ mice (30) to generate B cell–specific *Cmas* KO mice. B cell–specific $Bcl2^{tg}$ mice (34) were kindly provided by David Vöhringer, University Hospital Erlangen, Erlangen, Germany. Complement-deficient $C3^{-/-}$ mice (54) were kindly provided by Falk Nimmerjahn, University of Erlangen, Erlangen, Germany. All mice lines are kept on a C57BL/6 background. Experiments were performed in accordance with the German law for protection of animals, after approval by the animal welfare committee.

Cell Preparation for Flow Cytometry. Single-cell suspensions of bone marrow, spleen, and lymph nodes were prepared as described in detail in *SI Appendix*, *SI Methods*.

Lectin Staining. For glycosylation analysis cells were stained with biotinylated lectins from Vector Labs (SNA, MAAII, ECA, and PNA) for 20 min at 4 °C after 2% performic acid fixation, after extracellular Abs staining as described above. Subsequently, cells were washed and incubated with streptavidin conjugates for 20 min at 4 °C.

Intracellular Caspase Staining. For intracellular active caspase 3 and cleaved caspase 8 staining, cells were incubated 10 min at room temperature to allow spontaneous apoptosis or directly taken from cell culture plates. Subsequently, extracellular staining was performed as described above. Cells were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) according to the manual, and intracellular staining was conducted using the Abs anti-active caspase 3 (clone C92-605; BD Biosciences) and anti-cleaved caspase 8 (clone D5B2; Cell Signaling Technologies).

Calcium Mobilization Assays. Calcium mobilization assays of bone marrow or splenic cells were performed as described in *SI Appendix*, *SI Methods*.

MACS-Mediated Cell Purification. To obtain pro-B/pre-B/immature B cells, bone marrow cells were stained with Fc-block, anti–B220-PE, and anti–IgD-biotin as described above. Subsequently, cells were stained with streptavidin microbeads according to Milteny Biotec protocol and negatively selected with LD columns. IgD^{neg} cells were stained with anti-PE microbeads (Milteny Biotec) and $B220^{pos}$ selected via LS columns.

Cell Culture for Sub-G1 Phase Detection. Total bone marrow cells or MACS purified pro-B/pre-B/immature B cells were cultured in RPMI 1640 media containing 5% fetal calf serum, 1.2 mM L-glutamin, 50 μM β-mercaptoethanol, 100 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, 1× nonessential amino acids (all ingredients obtained from Gibco). The 2×10^6 bone marrow cells and 5×10^5 pro-B/pre-B/immature B cells were seeded per 96-well plate and incubated for 3 d at 37 °C and 5% CO_2. Every day apoptotic cells in sub-G1 phase were determined with DAPI staining (described above).

BAFF Cell Culture. MACS-purified pro-B/pre-B/immature B cells were cultured with or without 200 ng/mL recombinant murine BAFF (BioLegend) in the same media composition as mentioned before. The 1.25×10^5 cells were seeded per 96-well plate and incubated for 4 d at 37 °C and 5% CO_2. Every day apoptotic cells in sub-G1 phase were determined with DAPI staining (described above).

Pro-B Cell Culture. Pro-B cells (c-kit^{pos} $B220^{pos}$) were sorted via AriaII (BD Biosciences) after staining with anti-CD117 (c-kit) and anti-B220 as described above. Cells were cultured with or without recombinant murine IL-7 (10 ng/mL; BioLegend) in the same media composition as mentioned before. The 3×10^4 cells were seeded per 96-well plate and incubated for 8 d at 37 °C and 5% CO_2. Cells were counted via a Neubauer counting chamber, and at days 0, 3, 8 cells were stained with lectins and antibodies as described above.

FasL Cell Culture for Cleaved Caspase 8 Detection. Bone marrow cells were cultured with or without 200 ng/mL human Fc-FasL in RPMI 1640 media (as above). The 2×10^6 cells were seeded per 48-well plate and incubated for 1 d at 37 °C and 5% CO_2. Cells were stained with anti-IgD, anti-IgM, anti-CD19, anti-B220, and intracellular staining was conducted with anti-cleaved caspase 8 as described above.

ELISA Determination of the Serum Antibody Titer. ELISA experiments were done as described in *SI Appendix*, *SI Methods*.

Sialidase Treatment. Bone marrow and splenic cells were isolated as described above. Part of the cells (1×10^6 cells per 100 μL phosphate buffered saline (PBS)) were incubated with 0.0001 U/μL sialidase (*Arthrobacter ureafaciens,* kindly provided by Martina Mühlenhoff, Hannover Medical School, Hannover, Germany) at 37 °C for 30 min. Subsequently, cells were washed with PBS containing 1% bovine serum albumin, 0.01% sodium azide and 4 mM EDTA and stained with different Abs as described above.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism software. Unpaired Mann–Whitney *U* test was used to evaluate significance, which were plotted only for the relevant data. Statistical data are presented as mean ± SD.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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