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Regulation of B-lineage cells by caspase 6

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Abstract

The caspase (Casp) family of proteases regulate both lymphocyte apoptosis and activation. Here, we show that Casp6 regulates early B-cell development. One-week-old Casp6 knockout (Casp6 KO) mice have significantly more splenic B-cell subsets than wild-type (WT) mice. Adult Casp6 KO mice have normal levels of total splenic B cells but have increased numbers of B1a B cells and $CD43^+$ "transitional" or splenic red pulp (RP) B cells. These results suggested that Casp6 may function to control B-cell numbers under nonhomeostatic conditions and during B-cell development. Consistent with this model, reconstitution of B cells was dysregulated in Casp6 KO mice after sublethal irradiation. Furthermore, bone marrow pro-B, pre-B and immature B-cell numbers were significantly higher in 1-week-old Casp6 KO mice than in 1-week-old WT mice. Casp6 KO pro-B cells proliferated more in response to IL-7 than WT pro-B cells, suggesting that Casp6 regulates early B-cell responses to IL-7. Indeed, adult and aged Casp6 KO mice had elevated numbers of $IL\text{-}7\alpha R^+Sca1^+$ precursors of common lymphoid progenitors, suggesting Casp6 may help regulate progenitors of B cells and early B-lineage cells. Casp6 regulates B-cell programs both during early development and after antigen stimulation in the periphery.

Keywords

B cells; bone marrow; caspase; lymphopoiesis; spleen

INTRODUCTION

The mammalian caspase (Casp) family of cysteine aspartate-specific proteases are involved not only in cell death pathways including apoptosis and autophagy, but also in the maturation of proinflammatory cytokines and lymphocyte activation.[1–4] Casps generally are divided into three groups: activators of cytokines, initiator Casps and effector Casps. Casps not only mediate apoptosis but also regulate nonapoptotic functions including cell

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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development, proliferation and differentiation.[4–6] For example, Casp3 cleavage of the transcription factor GATA-1 impairs erythroblast development.[7] In the innate immune system, Casp3 can inhibit maturation of dendritic cells, while macrophage differentiation requires Casp3 and Casp8.[8,9] Furthermore, Casp6 and Casp8 are essential for myeloid cell differentiation.[5,10–12]

Casp8 and Casp3 regulate lymphocyte cell fate through a number of mechanisms. Beyond regulating apoptosis, Casp8 has a number of other functions.[13,14] Depending on the cell type and levels of adaptors like FADD and $cFLIP_L$, Casp8 can regulate the production of IL-1β, prosurvival pathways or necroptosis mediated by the receptor-interacting protein kinases-1 (RIPK1) and RIPK3 pathway.[14,15] Casp8 is also essential for hematopoietic progenitor cell proliferation and the differentiation of macrophages.[16] In B cells, Casp8 controls activation by TLR agonists and germinal center responses,[17,18] while Casp3 regulates B-cell activation in part by cleaving its substrate, the cell cycle inhibitor p21Cip1.[19]

The effector Casp, Casp6, like Casp8 and Casp3, has been implicated in a number of cellular processes ranging from apoptosis and neurodegeneration to cell differentiation, cell proliferation and oogenesis.[1,4,20–22] However, Casp6 has functions distinct from those other caspases. Substrates of Casp6, such as Casp8, SATB1, $p27^{Kip1}$, Notch1 and lamin A, and telomerase control transit through the cell cycle, cell survival and development.[4,20,23,24,25] In particular, a number of studies have implicated Casp6 in axon degeneration and neurodegenerative diseases including Alzheimer's disease and Huntington's disease.[26] However, relatively little is known about the role of Casp6 in the immune system and lymphocyte development.

Casp6 is cleaved and activated early after B cells are stimulated to enter the cell cycle.[27,28] Using Casp6 knockout (Casp6 KO) mice, we found that Casp6 regulates B-cell differentiation into plasma cells by modifying cell cycle entry.[28] Rather than proliferating, activated Casp6 KO B cells differentiate into plasma cells and produce antibodies. To further analyze the role of Casp6 in B cells, we examined early B-cell development in young Casp6 KO mice. Neonatal Casp6 knockout (Casp6 KO) mice have significantly more splenic and bone marrow (BM) B-cell subsets than wild-type (WT) mice. Casp6 KO early B-cell precursors express higher levels of cyclin D2 than WT counterparts and proliferate more in response to IL-7, suggesting that Casp6 functions to regulate early B-cell responses to IL-7.

RESULTS

Neonatal Casp6 KO mice have more splenic B cells than WT mice

To investigate the *in vivo* effects of Casp6 on early B-cell development, we first compared the number of splenic B-cell subsets at different times during development in neonatal and adult Casp6 and WT mice (Figure 1) as we described.[29] The gating strategy used for identifying splenic B-cell subsets is shown in Supplementary figure 1). In 1-week-old mice, the absolute numbers of splenic $IgM^{hi}IgD^{lo}CD21^{lo}$ transitional 1 (T1), $IgM^{hi}IgD^{hi}CD21^{lo}$ transitional 2-follicular precursor (T2-FOP), $IgM^{hi}IgD^{hi}CD21^{hi}$ transitional 2-marginal zone precursor (T2-MZP), $IgM^{lo}IgD^{hi}CD21^{lo}$ follicular (FO) and $IgM^{hi}IgD^{lo}CD21^{hi}$ marginal

zone (MZ) B-cell subsets were all significantly higher in Casp6 KO mice than in WT mice (Figure 1a).

In contrast, in 3-week-old mice, the numbers of T1, T2-FOP and MZ B-cell subsets were lower in Casp6 KO mice than in WT mice while levels of FO B cells were the same. By the time Casp6 KO mice became adults, they had normal numbers of all B-cell subsets (Figure 1a). Both 3-week-old and adult Casp6 KO mice had normal levels of splenic $CD4^+$ and $CD8^+$ T cells (data not shown). In 7-week-old WT mice, transitional B-cell numbers were lower than at 3 weeks and mature B-cell levels had increased, as previously described.[30] However, the absolute cell number of transitional B cells in Casp6 KO mice did not change significantly between 3 and 7 weeks. This suggests that the transitional B-cell pool in Casp6 KO mice, unlike in WT mice, had already developed by 3 weeks of age.

B-1 B cells develop early in life in the fetal liver and in adults are found principally in the peritoneal cavity and gut-associated lymphoid tissues.[31]

Therefore, we also compared peritoneal B-1 B-cell numbers in 3-week-old WT and Casp6 KO mice based on their expression of CD5, CD11b and CD43, which are not expressed by conventional B-2 cells. $B220^+CD11b^+$ B-1 cells can be subdivided into two subpopulations, $CD5^+$ B-1a B cells and $CD5^-$ B-1b B cells.[31] Peritoneal B-1a B cells were significantly increased in 3-week-old Casp6 KO mice, compared to WT mice, but peritoneal B-1b B-cell and $B220^+CD11b^-$ B-2 B-cell numbers were normal (Figure 1b). Again, by 6–9 weeks of age, levels of peritoneal B-1a B-cell numbers in Casp6 KO mice were normal (data not shown). The increased numbers of peritoneal B-1a B cells and of splenic B cells in young Casp6 KO mice together suggest that early B-cell development in peripheral lymphoid tissues is accelerated in the absence of Casp6.

Activated transitional B cells are increased in Casp6 KO mice

CD43 is an activation marker that is expressed on BM B-cell precursors, immature B cells and B-1 B cells but not on mature splenic B cells. After rapid cell proliferation, large pro-B cells downregulate CD43 expression as they become small pre-B cells.[31,32] $CD43^+$ immature cells found in the spleen have an activated phenotype, are increased in cell size and have enhanced survival *in vitro*.[33]

Because B-cell development was accelerated in 1- to 3-week-old Casp6 KO mice, we next compared CD43 expression and the size on splenic B-cell subsets from young (3-week-old) WT and Casp6 KO mice. The percentage of $CD43^+$ cells was increased in both the T1 and T2-FOP subsets of 3-week-old Casp6 KO mice compared with WT mice (Figure 1c). In addition, the cell size of $CD43^+$ T1 and T2-FOP B cells from Casp6 KO mice was relatively larger than that of WT counterparts (data not shown). These results suggest that the accelerated B-cell development in Casp6 KO mice may be due in part to an increase in activated $CD43^+$ transitional B cells.

B-cell reconstitution in sublethally irradiated WT and Casp6 KO mice

The increased numbers of transitional B cells in neonatal Casp6 KO mice suggested that Casp6 may function to regulate B cells under nonhomeostatic conditions. To assess this

possibility, WT and Casp6 KO mice were sublethally irradiated (500R), and then the reconstitution over time of splenic B-cell subsets was monitored by flow cytometry (Figure 2). The trend was that B cells in Casp6 KO mice reconstituted somewhat faster than in WT mice. However, the results were only significant at a few time points because of variability within the groups seen in three independent experiments.

In line with the data in young mice (Figure 1c), we found increased frequencies of $CD43^+$ cells in Casp6 KO mice within the T1 and FO B-cell subsets early postreconstitution (Supplementary figure 2), again suggesting an acceleration of B-cell development in the BM and activation of TR B cells in the absence of Casp6.

B-cell development is also dysregulated in the bone marrow of Casp6 KO mice

To assess further a possible role for Casp6 in regulating numbers of developing lymphocytes, we compared numbers of B- and T-cell precursors in the BM and the thymus in neonatal WT and Casp6 KO mice (gating strategy, Supplementary figure 3). At 1 week of age, $B220^{+/lo}$ cell frequencies were increased in Casp6 KO mice (Figure 3a). $B220^{+/lo}IgM^-CD43^+$, IgM^-CD43^- and IgM^+CD43^- cell populations, corresponding to pro-B, pre-B and immature B cells were all significantly elevated in neonatal BM of Casp6 KO mice (Figure 3a, b), and, as in the case of splenic B-cell subsets, the differences between WT and Casp6 KO mice subsided as mice grew older. Casp6 KO mice also had somewhat more total thymocytes and in particular more $CD4^+CD8^+$ double-positive cells than WT mice. This trend toward increased thymocytes continued through to 8 weeks of age (Supplementary figure 4); however, the differences were not as striking as for BM B-lineage cells and did not reach statistical significance.

Casp6-deficient early B-lineage cells are hyper-responsive to IL-7

Since B-cell precursors were elevated in the BM of young Casp6 KO mice and since early B-cell differentiation is regulated by IL-7,[34] we decided to examine the response of Casp6 KO BM cells to IL-7. We isolated $B220^-$ or $B220^+$ BM cell fractions and cultured them with IL-7 *in vitro*. After 4 or 7 days, cells were counted and analyzed by flow cytometry for frequencies of small pro-B ($CD43^+B220^{lo}IgM^-$, FSC<400), large pro-B ($CD43^+B220^{lo}IgM^-$, FSC>400), pre-B ($CD43^-B220^{lo}IgM^-$), immature B ($CD43^-B220^{lo}IgM^+$) and mature B cells ($B220^{hi}IgM^+$). Both $B220^-$ Casp6 KO cell cultures (Figure 4a, b) and $B220^+$ Casp6 KO cell cultures (Figure 4c, d) produced more small and large pro-B cells than WT cell cultures and also produced more immature or mature B cells. This effect was not spontaneous but IL-7-dependent since we found no differences between WT and Casp6 KO cell cultures without IL-7 stimulation (Figure 4b, d). Differences in cell differentiation, particularly, an increase in the number of small pro-B, large pro-B and immature Casp6 KO B-lineage cells was observed in response to graded doses of IL-7 (data not shown). In contrast to IL-7 treatment, the responses to graded doses of SCF (1–100 ng mL^{-1}) were not different between Casp6 KO and WT cells (data not shown).

Since Casp6 KO $B220^-$ cells treated with IL-7 gave rise to more early B-lineage cells, we examined them in more detail. *Ex situ* Casp6 KO $B220^-$ BM cells expressed more cyclin D2 than WT $B220^-$ counterparts (Supplementary figure 5a), suggesting that more Casp6 KO

B220$^-$ BM may be in cell cycle or more readily enter the cell cycle. In support of this, the increased expression of Cyclin D2 seemed to be restricted to the large buoyant cell fraction (i.e. cells isolated from a 50/60% Percoll gradient) from the B220$^-$ BM cells (Supplementary figure 5a). To examine this further, we layered BM cells obtained from adult WT and Casp6 KO mice over Percoll density gradients and separated them into fractions that contained large buoyant cells (enriched for cells in cell cycle) or small dense cells (primarily cells in G0/G1). Similar numbers of buoyant and dense cells were obtained from WT and Casp6 KO adult BM (data not shown). We then cultured the cell fractions with graded doses of IL-7. After 10 days in culture, the buoyant B220$^-$ Casp6 KO cells, unlike dense cells, expanded in response to IL-7 more than buoyant WT B220$^-$ cells, producing as many as fivefold more cells (Figure 4e). The increased response of Casp6 KO BM B-lineage cells to IL-7 was not simply due to a difference in IL-7α receptor (IL-7αR) expression as the levels of both IL-7αR/CD127 and γ chain were similar on WT and Casp6 KO pro-B, pre-B and immature B cells (Supplementary figure 5B). We conclude that buoyant B220$^-$ Casp6 KO cells expand more in response to IL-7 than their WT counterparts.

Casp6 regulates early precommon lymphoid progenitors (pre-CLPs)

The absolute numbers of B progenitor cells decline in aged B6 mice and are dramatically lower in 10- to 24- month-old mice, in part because of suboptimal responses to IL-7.[35] Thus, we decided to test if Casp6 might play a role in age-associated changes of IL-7αR$^+$ progenitors of B cells. Hematopoietic progenitor cells do not express markers expressed on mature hematopoietic cells, such as B220, CD3, CD11b/Mac-1, Ly6C/G (Gr1) or Ter119, that is they are lineage-negative (Lin$^-$); however, they do express Ly6A/E (Sca1) and c-kit, and thus, sometimes are designated as LSK$^-$ (Lin$^-$Sca1$^+$Kit$^+$) cells.[36] LSK$^-$ cells mature into non-self-renewing cells, some of which give rise to common lymphoid progenitors (CLPs) that express little or no Sca1 but are IL-7αR$^+$.[35,37] An IL-7αR$^+$ population of Lin$^-$Sca1$^+$ cells has been described that appears to contain precursors of the Sca1$^-$ CLPs,[38] or what we will term “pre-CLPs.” The CLP compartment, while quite heterogeneous, can be defined as a population of Lin$^-$IL-7αR$^+$Sca1$^-$CD93$^+$ cells.[35,37] When cultured in the presence of IL-7, this population of cells differentiates into B220$^+$CD19$^-$ and B220$^+$CD19$^+$ B-cell lineage-committed progenitors.[39]

We obtained BM from 3- or 18-month-old WT or Casp6 KO mice, and using flow cytometry, we quantified the number of Lin$^-$IL-7aR$^+$Sca-1$^+$CD93$^-$CD19$^-$ pre-CLPs,[38] as well as the IL-7αR$^+$ CLPs/early B-cell progenitors (EBPs) cells[35,39] that are Lin$^-$IL-7αR$^+$Sca-1$^-$CD93$^+$CD19$^-$ (Figure 5a, b). Casp6 KO mice at either 3 months of age or at 18 months of age had significantly more IL-7αR$^+$Lin$^-$Sca-1$^+$ pre-CLPs than WT mice, but had normal numbers of EBP/CLPs (Figure 5b). At this age, the numbers of the CD4$^-$Ly6C$^-$IL-7αR$^+$CD19$^-$CD93$^+$CD43$^+$B220^{lo}CD24$^-$ pre–pro-B cells also were not different between WT and Casp6 KO mice (data not shown). Thus, Casp6 appears to regulate the levels of pre-CLPs in the BM both in adult and aged mice.

We next tested whether Casp6 deficiency also affects the responses of pre-CLPs to IL-7 stimulation *in vitro*. We sorted pre-CLPs from 6-month-old WT or Casp6 KO mice, cultured them in the presence of IL-7 for 10 days and then analyzed the frequencies of EBPs and B-

lineage cells (pre–pro-B and pro-B), based on the expression of B220, CD93, CD19 and CD43. Results from two independent experiments showed that the overall frequencies of $CD93^+$ cells did not differ between WT and Casp6 KO cell cultures. However, we found four-to fivefold increases in the frequencies of $B220^{lo/+}CD43^+$ cell in Casp6 KO cell cultures, compared to WT cell cultures (Figure 5c, d). The majority of these cells were $CD19^+$ and large in size (FSC > 400) (data not shown), resembling the phenotype of large pro-B cells. Based on these data, we conclude that, in the absence of Casp-6, IL-7-driven differentiation of pre-CLPs/EBP into pro-B cells is accelerated.

DISCUSSION

Casp6 plays a role in programmed cell death and regulating entry into the cell cycle.[2,4,21,28] In this study, we now show that Casp6 plays a role in shaping the course of B-cell development. Compared to WT counterparts, 1-week-old Casp6 KO mice have significantly more splenic immature and mature B-cell subsets and more BM pro-B cells and pre-B cells (Figure 1a, Figure 3). Yet, adult Casp6 KO mice have normal numbers of BM and splenic B-lineage cells. Early B-cell development in both the BM and peripheral lymphoid tissues is accelerated in the absence of Casp6, but a role for Casp6 is not evident once B-cell homeostasis is achieved in older mice.

The B-cell lineage consists of renewing cell populations in a dynamic equilibrium or steady state.[40] An equilibrium is established in adults because of competition with other cells for limited resources and other factors. The increase in B-lineage cell numbers in Casp6 KO mice theoretically could be due to dysregulation of the rate of proliferation, death or survival of Casp6 KO B cells. However, our *in vitro* analyses did not uncover any differences between WT and Casp6 KO B cell proliferation or spontaneous cell death.[28] Furthermore, mature B and T cells in Casp6 KO mice inoculated with BrdU do not take up BrdU any faster than B and T cells in WT mice (data not shown). A more likely possibility was that Casp6 regulates how fast B-cell homeostasis is established under nonhomeostatic conditions. To explore this possibility, we irradiated groups of WT and Casp6 KO mice sublethally and then monitored the kinetics of B-cell reconstitution over time (Figure 2). Initially, Casp6 KO mice had more transitional B cells than WT mice, but by day 21, no differences were evident between the two groups. These results are consistent with a model where Casp6 can function to limit the rate of early B-cell expansion.

Irradiated Rag2 KO mice reconstituted with graded amounts of normal BM cells with B-cell-deficient (μMT) BM cells develop pre-B cells in proportion to the fraction of normal BM cells introduced.[41] When the mice have relatively few pre-B cells, then they also had reduced numbers of mature B cells. Thus, under some conditions, perhaps early in development, the numbers of pre-B cells being generated may be rate limiting. Conversely, the increase in BM pro-B and pre-B cells in neonatal Casp6 KO mice may lead to faster development of splenic B cell populations.

Not much is known about how the pool size of B-lineage cells is maintained; indeed, the rate of B-cell development in various KO mice implicated in B-cell differentiation and activation clearly is worthy of more careful analysis. FoxO proteins, like Casp6, have been implicated

in the regulation of lymphocyte homeostasis.[42] While adult Foxo3 KO mice, like Casp6 KO mice, have normal numbers of splenic B-cell subsets,[43] in direct contrast to Casp6 KO mice, they have reduced numbers of BM pro-B, pre-B and immature B cells. Unlike Foxo3 KO early B cells,[43] Casp6 KO BM cells have abnormal responses to IL-7 as measured by enhanced expansion after IL-7 treatment of pro-B, pre-B and immature B cells (Figure 4). Casp6 KO $B220^-$ BM cells examined *ex situ* expressed elevated levels of cyclin D2. Similarly, Casp6 KO splenic B cells have significantly more cells in the G1 phase of the cell cycle compared to WT B cells, implying that Casp6 might function as a brake for B cell entry into the cell cycle.[28] In the BM, Casp6 may also function as a brake, but in this case for IL-7 signaling of early B cells and their progenitors.

At 3 weeks of age, Casp6 KO mice showed an increase in B1a B cells (Figure 1b); thus, we cannot exclude the possibility that Casp6 might also regulate the early B1 B-cell precursors. It is also worth noting that the $B220^+CD43^-sIgM^-$ cell population, which was increased in neonatal Casp6 KO mice, could also contain B1-cell progenitors or other progenitor cell types.

Nevertheless, the fact that all B-lineage stages of Casp6 KO BM cells are expanded by IL-7 more than WT cells suggests that IL-7αR$^+$B220$^-$ B-cell progenitors are under Casp6 control. Our findings support this possibility: first, Casp6 KO BM $B220^-$ cells are especially responsive to IL-7, and IL-7 treatment of $B220^-$ Casp6 KO cells mainly expands $B220^+IgM^-CD43^+$ (pro-B) cells (Figure 4). Second, this difference in responsiveness was not due simply to differences in the expression of IL-7 receptor chains, which were similar in WT and Casp6 KO cells. Since IL-7 normally plays a key role in regulating the course of early B-cell development,[34,44,45] particularly at the CLP and pre–pro-B stage, dysregulated IL-7 responses in early B-lineage cells or their precursors may account for the acceleration in B-cell development present in Casp6 KO mice. In the absence of Casp6, IL-7 expands early B-cell numbers significantly, suggesting Casp6 may help regulate IL-7-mediated survival and growth via a cell trophic program as opposed to an instructive pathway.[34]

It is noteworthy that actual numbers of CLPs/EBPs and pre–pro-B cells were not elevated in adult Casp6 KO mice (Figure 5 and data not shown). Rather, an IL-7αR$^+$ population of Lin^-Sca1^+ pre-CLPs cells was expanded. A dysregulation of these cells could account for the increased numbers of BM pro-B cells and perhaps, the somewhat elevated numbers of thymocytes in Casp6 KO mice. However, in spite of this increase, actual numbers of B-lineage cells are normal in adult and aged Casp6 KO mice. This suggests that a feedback mechanism of some kind activated perhaps once the BM niche fully operates to hold B-cell expansion in check in spite of the increased numbers of pre-CLPs. We were not able to detect obvious differences in how Casp6 KO early B cells *versus* WT counterparts respond directly to IL-7, as measured, for example by STAT5a phosphorylation (data not shown). Further studies are needed to define just how Casp6 regulates IL-7 signaling. While our data suggest that Casp 6 deficiency affects early lymphocyte development at the pre-CLP stage, it is possible that other BM cell types might be affected as well.

It is interesting to compare our findings implicating Casp6 in early lymphoid development to studies demonstrating a role for Casp6 in neural development. Nikolaev *et al.*[46] showed that

Casp6 is required for axonal degeneration, which occurs during neural development. Importantly, an apoptotic pathway did not mediate this degeneration. Just as with B-lymphoid development, the nervous system needs to be sculpted during development by culling neurons and 'pruning' axons.[47] Casp6 may contribute to sculpting and pruning in both systems. Could Casp6 regulate related or analogous signaling pathways in early B cells and neural progenitors? Park *et al.*[48] found that signaling through the p75 neurotrophin receptor is defective in the absence of Casp6. Further studies are needed to determine how Casp6 affects IL-7 signaling and whether Casp6 regulates elements common to the IL-7 and neurotrophin signaling pathways.

METHODS

Mice

Casp6 KO mice were generated as described[49] with a male breeder initially kindly provided by Dr Richard Flavell (Yale University). Mice were backcrossed for more than 15 generations and housed under specific pathogen-free conditions at the University of Washington. For the B-cell reconstitution experiments, 7-week-old mice were given a sublethal dose of 500-rad whole body irradiation and given antibiotics in their drinking water. All experiments were performed in compliance with the University of Washington Institutional Animal Care and Use Committee.

Flow cytometry

Single-cell suspensions were prepared from mouse spleens or thymuses. Peritoneal cavity cells were collected by flushing the peritoneal cavity with phosphate buffered saline containing 0.5% fetal calf serum. BM cells were obtained from both tibias and femurs. One million cells of the various tissues tested were stained with mAbs as listed in Supplementary table 1. MAbs were conjugated to FITC, PE, PE-Cy7, PerCP, PerCPCy5.5, APC, APC-Cy7, ef450 or biotin. Streptavidin-FITC, -PE, and -APC were used as second-step reagents for biotinylated Abs. All antibodies used in this study were obtained from BD Bioscience or eBiosciences. Stained cells were analyzed using LSR II (BD Bioscience, San Jose, CA, USA) and the data analysis was performed using FlowJo software (Treestar, San Carlos, CA).

Proliferation assays

After red blood cell depletion by Grey's solution, BM B cells were purified by magnetic positive selection using anti-B220 MACS beads (BD Bioscience). The purity of the $B220^+$ B-cell preparations was >95%. Purified BM B cells (2.5×10^5 cells/well) were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (Life Technologies, Carlsbad, CA, USA), 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 2 mM L-glutamine and 2-ME (5×10^{-5} M) in the presence or in the absence of recombinant mouse IL-7 (R&D Systems Inc., Minneapolis, MN, USA) in 24-well plates. After indicated days of culture, BM B cells were harvested, counted and stained with anti-IgM, anti-B220 and anti-CD43, then analyzed by LSR1 using CellQuest software.

Quantitative PCR (qPCR) analyses

Total RNA was isolated using a QIAShredder and RNeasy kit (QIAgen, Valencia, CA, USA). cDNA was transcribed from purified RNA using an oligo$(dT)_{16}$ primer and Superscript III Reverse Transcriptase (Invitrogen, Waltham, MA, USA). Real-time qPCR was performed according to the manufacturer's instructions for Power SYBR® Green PCR Master Mix (Applied Biosystems Inc.) using specific primers. Samples were run in duplicate on Applied Biosystems 7900HT Sequence Detection Systems Version 2.3. Amplification was performed using one cycle at 50°C for 2 min then 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. One cycle each at 95°C for 15 s, 60°C for 15 s and 95°C for 15 s concluded the run. Test primers were compared to the 18S housekeeping gene and values were given as relative expression. The mouse primers for cyclin D2[50] and 18S used in this study are listed in Supplementary table 2.

Statistical analyses

Statistical significance between the two groups was determined by a two-tailed, unpaired Student's *t*-test or by two-way repeated measures (RM) ANOVA with Bonferroni post-test. Graphs and statistical analyses were performed using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Values are reported as mean ± s.d. $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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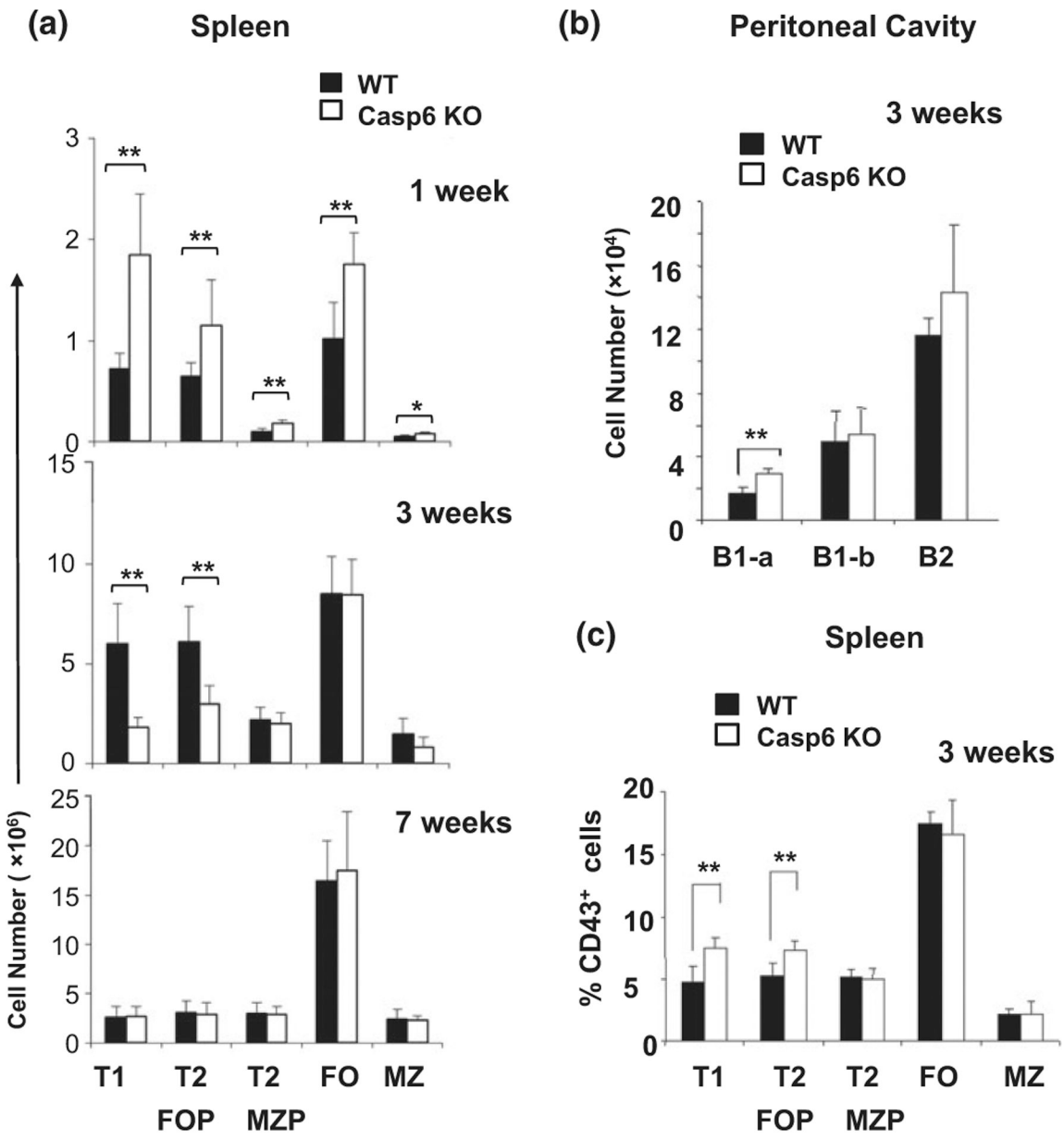

Figure 1.

Early B-cell development is accelerated in young Casp6 KO mice. **(a)** Splenic B-cell subsets of 1-, 3- or 7-week-old mice were defined as follows; T1 ($IgM^{hi}IgD^{lo}CD21^{lo}$), T2-FOP ($IgM^{hi}IgD^{hi}CD21^{lo}$), T2-MZP ($IgM^{hi}IgD^{hi}CD21^{hi}$), FO ($IgM^{lo}IgD^{hi}CD21^{lo}$) and MZ ($IgM^{hi}IgD^{lo}CD21^{hi}$). **(b)** Casp6 KO mice have increased levels of peritoneal B-1a B cells. B-cell subsets from peritoneal cavities of 3-week-old mice were determined as follows: B-1a B cells ($CD11b^{+}B220^{+}CD5^{+}$), B-1b B cells ($CD11b^{+}B220^{+}CD5^{-}$), B-2 B cells ($CD11b^{-}B220^{+}$). **(c)** Casp6 KO mice have increased levels of activated transitional B cells. Splenic cells of 3-week-old mice were stained with mAb specific for CD43/S7 with other B cell subset markers. Mean (± s.d.) percentage of $CD43^{+}$ cells in each B cell subset is shown. Black bars show the cells of WT mice and open bars show those of Casp6 KO mice. The

data are pooled from three independent experiments, using five to seven mice per genotype per experiment. Differences between the two genotypes for each cell type were assessed for statistical significance using an unpaired *t*-test. $*P < 0.05$, $**P < 0.01$.

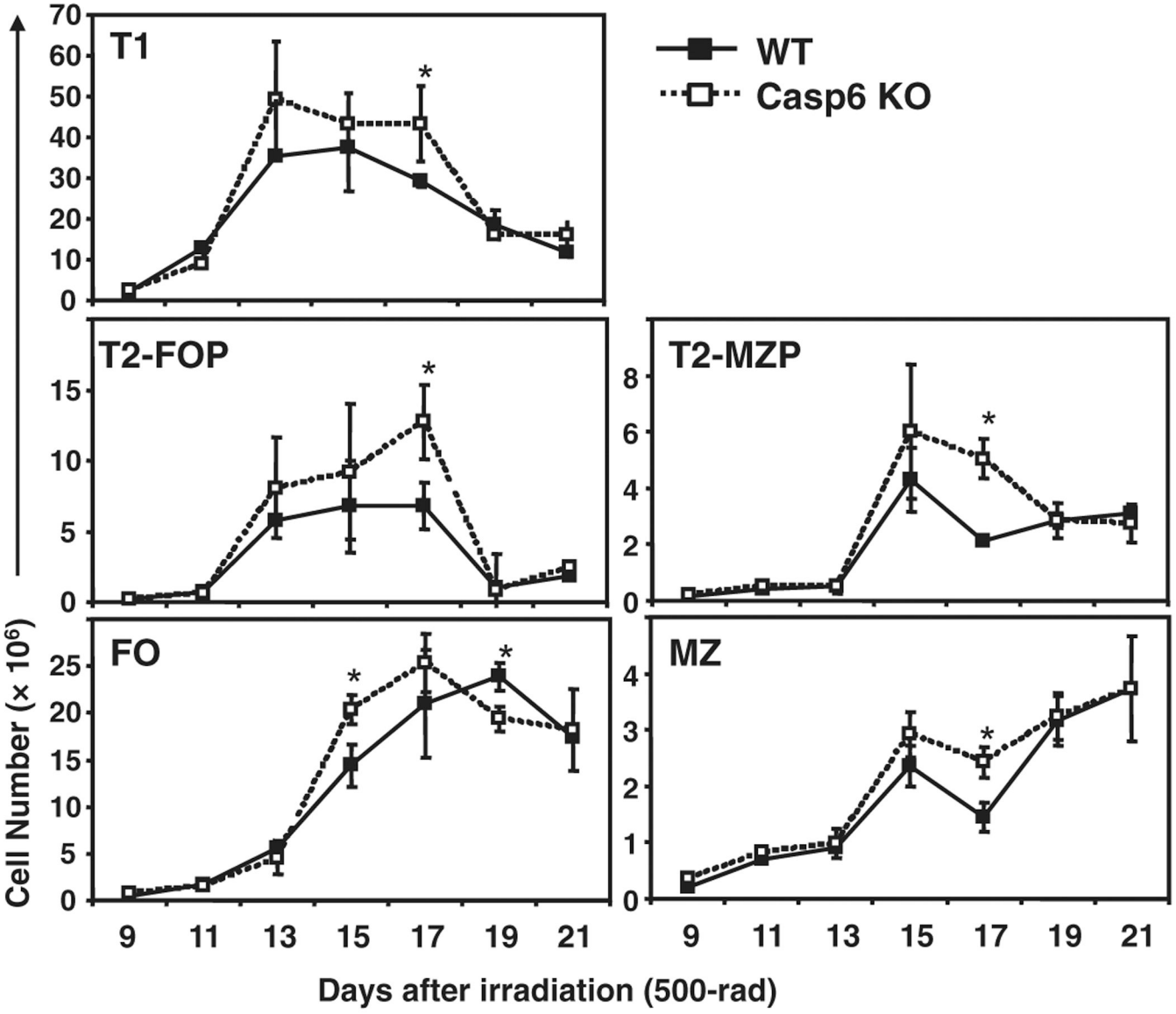

Figure 2.

B-cell reconstitution after sublethal irradiation is faster in Casp6 KO mice than in WT mice. Seven-week-old mice received 500-rad of sublethal irradiation. After the irradiation, sets of mice were sacrificed at the indicated time points and examined for splenic B-cell subset levels as in Figure 1a: black squares = WT mice and open squares = Casp6 KO mice. The mean (± s.d.) absolute cell number of each B-cell subset was calculated. The data are pooled from three independent experiments, using five mice per genotype per experiment. Statistical significance was assessed using an unpaired *t*-test. $*P < 0.05$.

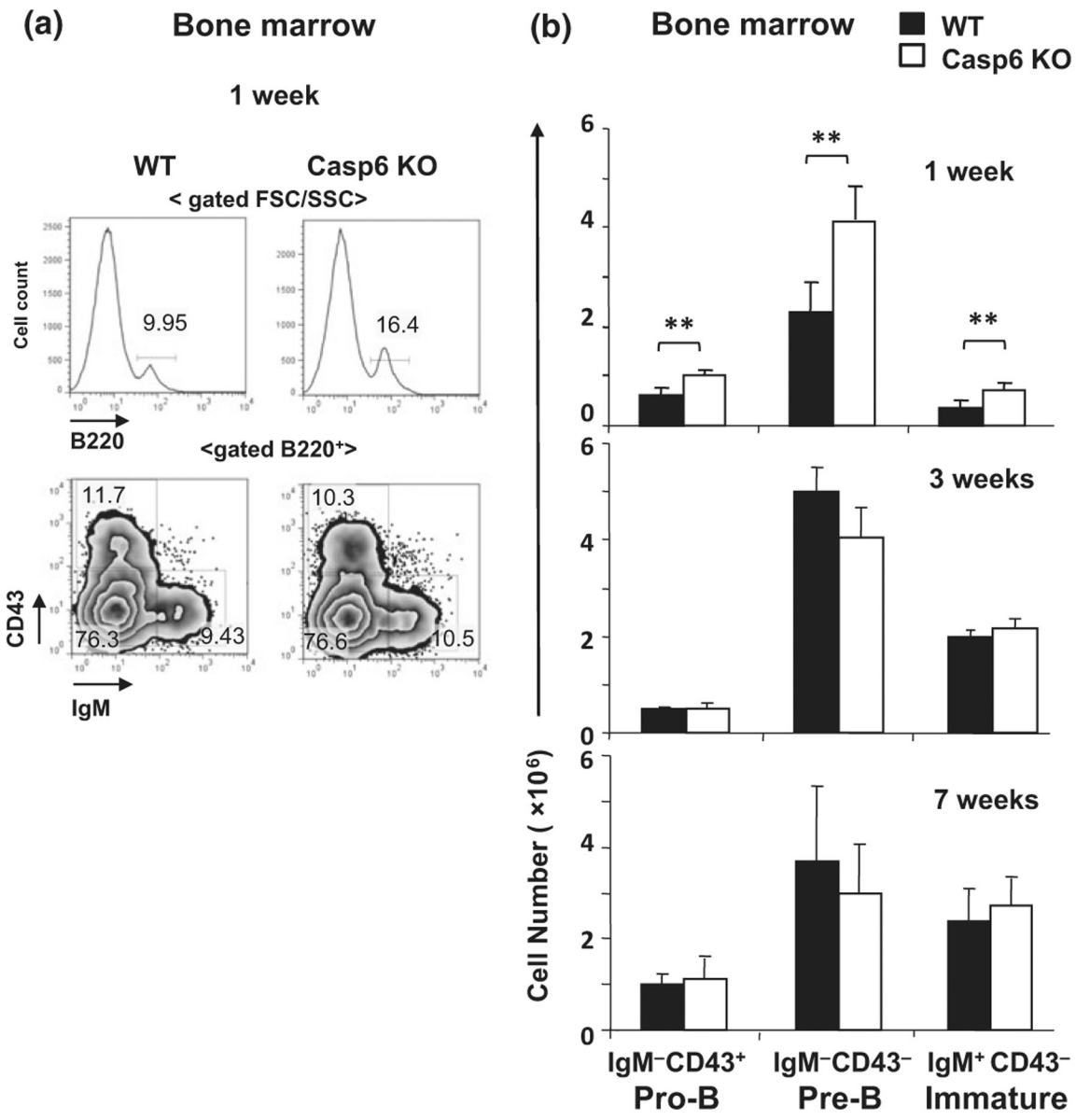

Figure 3.

Bone marrow B-lineage cells are increased in neonatal Casp6 KO mice. BM cell subsets of 1-, 3- or 7-week-old mice were analyzed by flow cytometry using a combination of B220, IgM and CD43. **(a)** Representative flow cytometric plots from 1-week-old mice showing gating strategy. **(b)** Summary results from the analysis of three independent experiments; BM B-cell subsets were defined as follows: pro-B cells ($CD43^{+}B220^{lo}IgM^{-}$), pre-B cells ($CD43^{-}B220^{lo}IgM^{-}$) and immature B cells ($CD43^{-}B220^{lo}IgM^{+}$).[30] Closed bars show the cell numbers of WT mice and open bars show those of Casp6 KO mice. Each bar represents the mean (± s.d.) of absolute cell numbers of BM cells from both tibias and femurs. The data are pooled from three independent experiments, using five to seven mice per genotype per experiment. Statistical significance was assessed using an unpaired *t*-test. $*P < 0.05$ $**P < 0.01$.

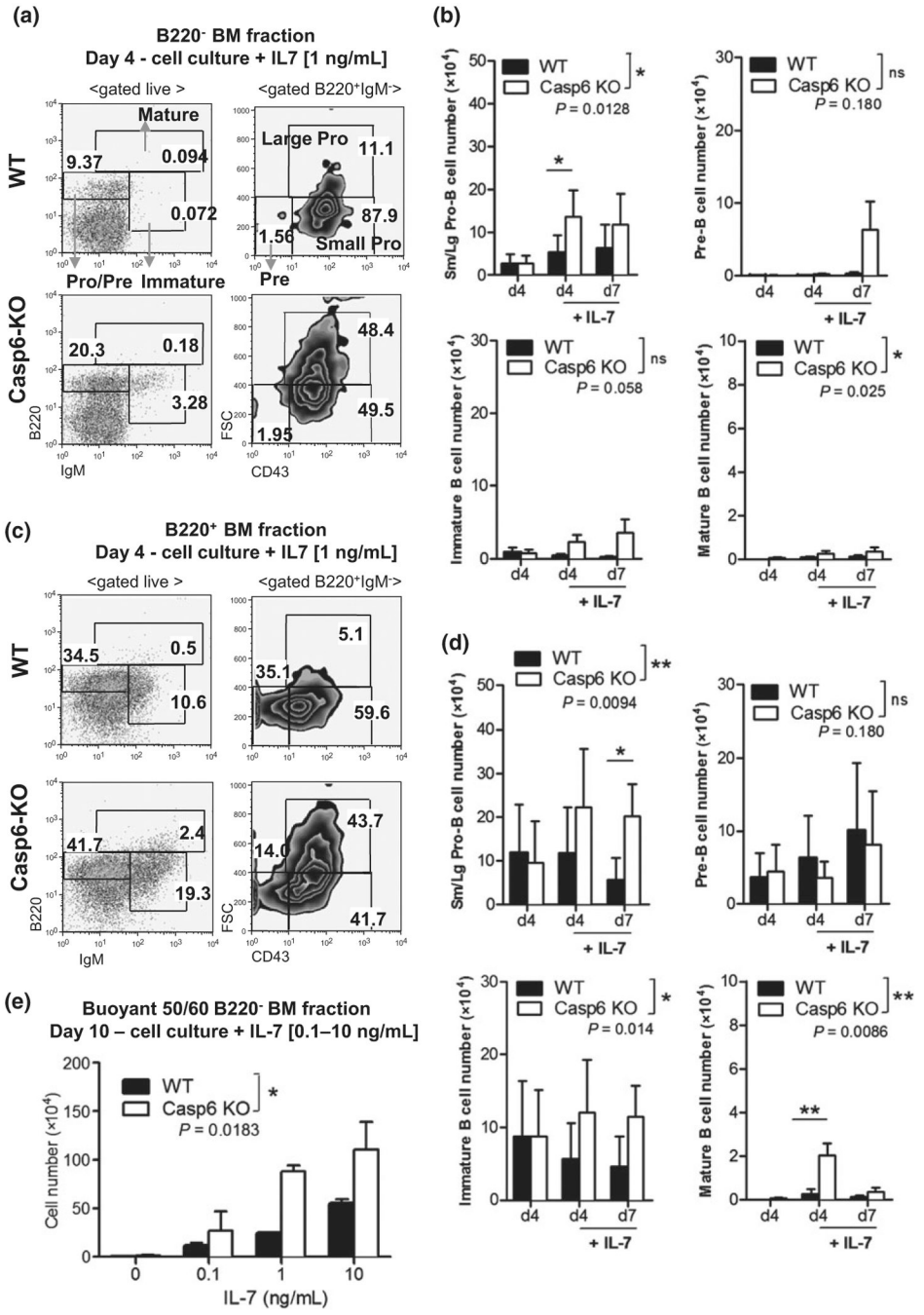

Figure 4.

Expansion of BM B-lineage cells is increased in Casp6 KO mice in response to IL-7. $B220^+$ and $B220^-$ cells were purified from the BM of 7-week-old mice. 2.5×10^5 $B220^-$ or $B220^+$ cells were cultured with IL-7 and after 4 or 7 days, cells were harvested and the absolute cell numbers of B-lineage subsets were determined. Cells were stained with B220, CD43 and IgM; BM B-cell subsets were defined as follows: small pro-B cells ($B220^{lo}IgM^-CD43^+$, FSC < 400), large pro-B cells ($B220^{lo}IgM^-CD43^+$, FSC ≥ 400), pre-B cells ($B220^{lo}IgM^-CD43^-$) and immature B cells ($B220^{lo}IgM^+CD43^-$). **(a)** Representative flow plots showing

in vitro differentiation of $B220^-$ cell fraction after 4 days in culture in the presence of 1.0 ng mL^{-1} IL-7, **(b)** Cell numbers in culture ($B220^-$ cell fraction) at day 4 with or without IL-7 stimulation and at day 7 with IL-7 stimulation; summary data of two independent experiments. **(c)** Representative flow plots showing *in vitro* differentiation of $B220^+$ after 4 days in culture in the presence of 1.0 ng mL^{-1} IL-7. **(d)** Cell numbers in culture ($B220^+$ cell fraction) at day 4 with or without IL-7 stimulation and at day 7 with IL-7 stimulation; summary data of two independent experiments. **(b, d)** Statistical analysis between the two genotypes was performed using two-way repeated measures (RM) ANOVA with Bonferroni post-test, $*P < 0.05$. **(e)** $B220^-$ BM cells from buoyant cell fractions (50/60 Percoll interface) were cultured with graded doses (0.1, 1 and 1 ng mL^{-1}) of IL-7 for 10 days, after which total cell numbers were determined. Summary data of two independent experiments. Statistical analysis between the two genotypes was performed using two-way RM ANOVA with Bonferroni post-test, $*P < 0.05$.

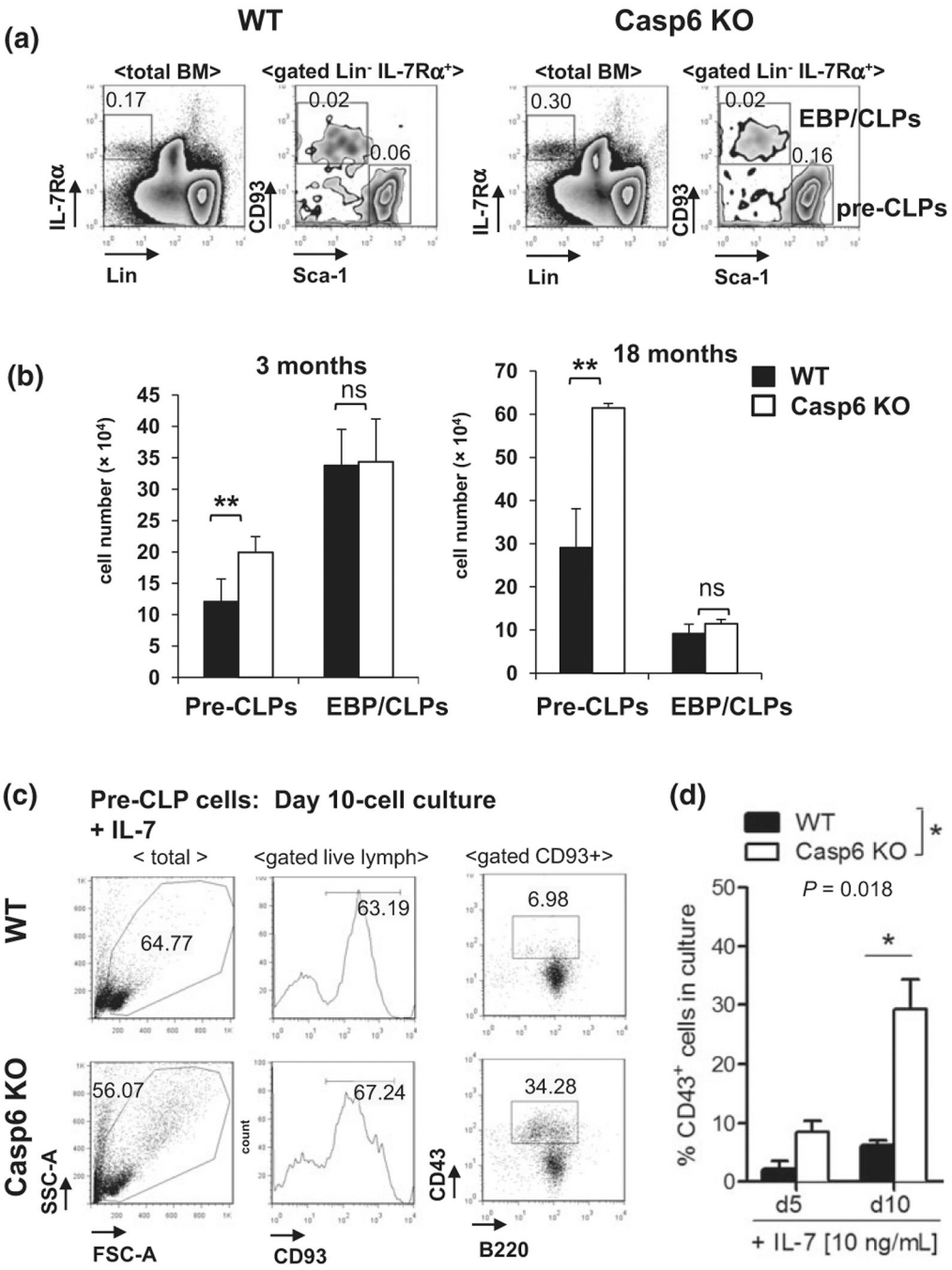

Figure 5.

Casp6 KO mice have increased numbers of pre-CLPs in BM. **(a)** Representative FACS analyses of pre-CLP and CLP/EBP cell population in BM of 18-month-old WT or Casp6 KO mice. BM cells were stained with anti-B220, anti-CD3, anti-CD11b/Mac-1, anti-Ly6C/G (Gr1) and anti-Ter119 lineage markers (Lin), anti-IL-7αR, anti-CD19, anti-CD93 and anti-Ly6A/E (Sca1) antibodies. **(b)** Numbers of pre-CLP ($Lin^{-}IL\text{-}7\alpha R^{+}Sca1^{+}CD19^{[C0]}$) and CLP/EBP ($Lin^{-}IL\text{-}7\alpha R^{+}Sca1^{-}CD93^{+}CD19^{-}$) cells from 3- or 18-month-old WT or Casp6 KO mice. Data represent the mean (± s.d.) absolute cell numbers of total BM cells from five

to six mice per group. Statistical significance was assessed using an unpaired *t*-test. $*P < 0.05$, $**P < 0.01$. **(c, d)** Frequency of large pro-B cells after culture with IL-7 is higher in Casp6 KO than in WT BM cells. FACS-sorted pre-CLP ($Lin^{-}IL\text{-}7\alpha R^{+}Sca1^{+}CD19^{-}$) cells were cultured for 5 or 10 days in IL-7 and then analyzed for the frequency of $CD93^{+}B220^{+}CD43^{+}$ cells. **(c)** Representative flow cytometric plots showing *in vitro* differentiation of pre-CLP cells after 10 days in culture with 10.0 ng mL^{-1} IL-7. Data are representative of two independent experiments using pooled BM cells isolated from four mice per group. **(d)**. Summary data of two experiments. Statistical analysis between the two genotypes was performed using two-way RM ANOVA with Bonferroni post-test, $*P < 0.05$.