CD93 is required for maintenance of antibody secretion and persistence of plasma cells in the bone marrow niche

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Plasma cells represent the end stage of B-cell development and play a key role in providing an efficient antibody response, but they are also involved in numerous pathologies. Here we show that CD93, a receptor expressed during early B-cell development, is reinduced during plasma-cell differentiation. High CD93/CD138 expression was restricted to antibody-secreting cells both in T-dependent and T-independent responses as naive, memory, and germinal-center B cells remained CD93-negative. CD93 was expressed on (pre)plasmablasts/plasma cells, including long-lived plasma cells that showed decreased cell cycle activity, high levels of isotype-switched Ig secretion, and modification of the transcriptional network. T-independent and T-dependent stimuli led to re-expression of CD93 via 2 pathways, either before or after CD138 or Blimp-1 expression. Strikingly, while humoral immune responses initially proceeded normally, CD93-deficient mice were unable to maintain antibody secretion and bone-marrow plasma-cell numbers, demonstrating that CD93 is important for the maintenance of plasma cells in bone marrow niches.

Aiolos | Blimp-1 | differentiation | humoral immunity | immunoglobulin

Serum Ig is crucial for life-long protection against previously encountered pathogens (1). Given the relatively short half-life of Ig in vivo, antibodies have to be continuously secreted by plasma cells (PC) (2). This production is tightly regulated to guarantee efficient long-lasting responses and to avoid autoimmunity.

Durable Ig responses require T-cell help and proceed in 2 phases. The rapid initial B-cell activation peaks around day 6 and produces short-lived plasmablasts that secrete relatively low-affinity IgM and IgG antibodies independent from germinal centers (GC). The second, slower response is initiated in parallel in the B-cell follicles and involves GC formation, in which B cells undergo affinity maturation, class-switch recombination yielding high-affinity PC and memory cells after 10 to 14 days. A proportion of these PC migrate to the bone marrow (BM) or to sites of inflammation, where they secrete antibodies for extended periods of time (3, 4). The mechanisms regulating the generation and the survival of BM long-lived PC (LLPC) are only partly understood. Nevertheless, it has been shown that transcription factors, such as Blimp-1, Irf-4, and Xbp-1 are required for these differentiation steps. Blimp-1 is known to be both necessary (5) and sufficient (6) for PC differentiation and has been shown to be involved in the maintenance of LLPC in the BM (7). Levels of Blimp-1 correlate tightly with PC maturation (8). Although Blimp-1 expression is essential to generate fully functional PC, it is not required for the earliest steps in antibody secreting cell (ASC) differentiation (9, 10).

The survival of BM PC is thought to be dependent from signals provided by survival niches (11) to a limited number of cells, implying that there is competition between newly generated and resident PC in the BM (12, 13). Further understanding of BM PC homeostasis is particularly crucial for the treatment of autoimmune diseases, but has proven difficult to tackle, as PC do not divide and have lost most of the surface markers that allow efficient targeting (14). Thus, discovery of new PC biomarkers could provide new potential therapeutic targets.

CD93 is expressed early during B-cell differentiation in the BM, before being down-regulated upon maturation in the spleen (15, 16). The function of CD93, however, remains elusive. Here we show that CD93 expression is reinduced during PC differentiation. LLPC express high levels of CD93 in the BM, whereas CD93 expression was found neither on GC B cells nor on memory B cells. Strikingly, while B-cell responses initially proceeded normally, CD93-deficient mice were unable to maintain antigen-specific Ig levels and BM PC numbers in T-dependent (TD) immunizations, demonstrating that CD93 is crucial in the maintenance of PC in the BM.

Results

CD93 and CD138 Expression in ASC and Preplasmablasts in TD and T-Independent Immune Responses. We have previously shown that Lat^{Y136F}-mutant mice have a pronounced increase in PC numbers expressing CD93 (17). Four populations of ASC and preplasmablasts could be distinguished based on CD93 and CD138 expression: double negatives (DN), CD93 single positives (SP), CD138 SP and double positives (DP) [supporting information (SI) Fig. S1 *A* and *B*]. The CD93 SP B cells were weakly positive for intracellular IgG_1, whereas the two CD138-expressing cell subsets were strongly positive and represented ASC. DN cells were negative for intracellular IgG_1 and contained preplasmablasts (Fig. S1*C*).

To address, whether in WT mice CD93 expression was induced during PC differentiation, we immunized BALB/c mice with mouse mammary tumor virus (MMTV) or with the hapten nitrophenol coupled to chicken γ-globulin (NP-CGG). It is well established that MMTV infection induces a strong extrafollicular plasmablast population in the draining lymph node, which can readily be identified by flow cytometry as large MHCII intermediate ($MHCII^{int}$) $B220^{low}$ (refs. 18 and 19 and Fig. 1*A*). This population, peaking 6 days after infection, also contained a minor percentage of $CD11c^+$ and $GR1^+$ plasmacytoid dendritic cells (<2%, data not shown). At this time, the cells were heterogeneous for CD93 and CD138 expression and the 4 populations could be distinguished (see Fig. 1*A*). To further analyze the composition of these subpopulations,

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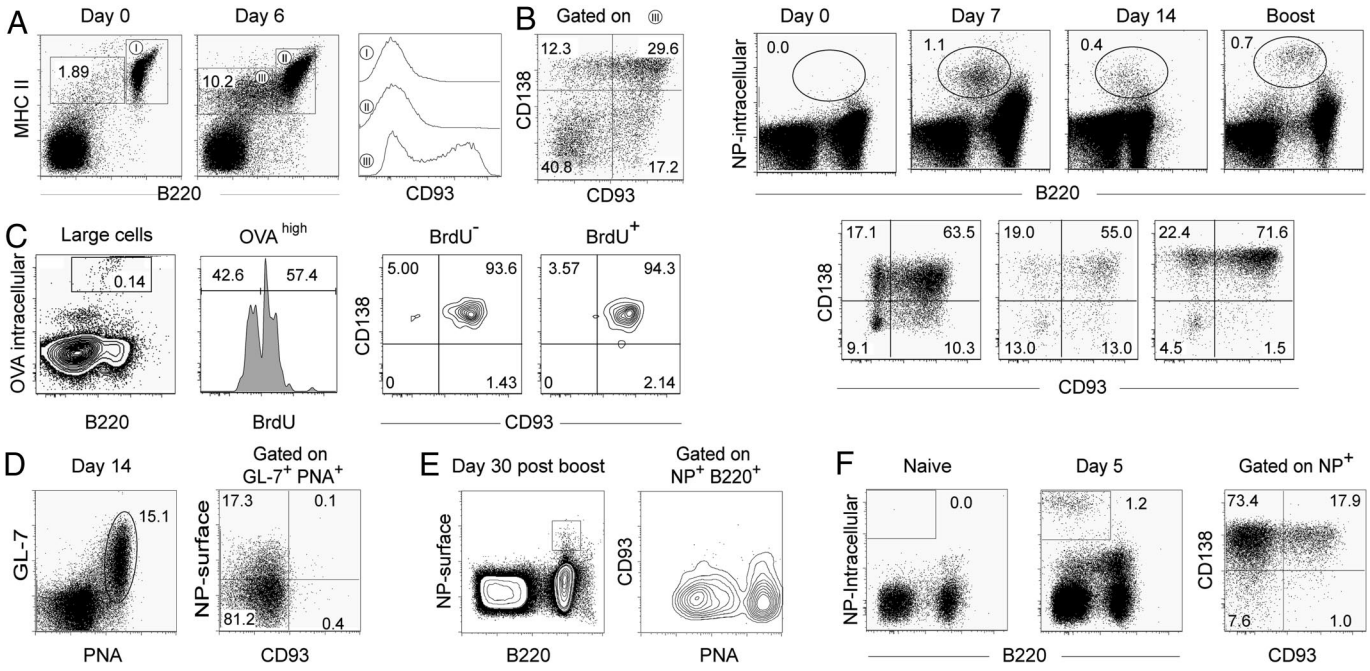

Fig. 1. CD93 is expressed on ASC after MMTV and NP-CGG immunizations. (*A*) Plasmablasts and B cells in the draining lymph nodes of MMTV-immunized and control BALB/c mice. Numbers indicate the percentages of cells. (*B*) FACS analyses of lymph node cells of C57BL/6 mice at different time points after footpad immunization with 50 μg of NP-CGG in alum. NP-intracellular$^+$, B220^{int} cells (ASC) were analyzed for CD93 and CD138 expression. (*C*) FACS analysis 40 days after boost immunization of BALB/c mice with ovalbumin (OVA) protein. Mice were fed with BrdU containing water for 20 days before analysis. LLPC in the BM were detected by intracellular staining for OVA-specific antibodies and low BrdU incorporation. (*D*) Expression of CD93 and surface NP-specific Ab was assessed on GL7$^+$PNA$^+$ B cells 14 days after NP-CGG immunization. (*E*) Memory B cells, defined as B220$^+$ NP-surface$^+$, present 30 days after boost were analyzed by FACS for the expression of CD93. (*F*) FACS analyses of splenic cells before and 5 days after i.v. immunization with 30 μg of NP-Ficoll. B220$^-$NP-intracellular$^+$ ASC were analyzed for the expression of CD93 and CD138.

we used MMTV-injected *Blimp-1$^{gfp/+}$* reporter mice and measured Blimp-1 expression. As shown in Fig. S2, the large majority of cells had up-regulated Blimp-1.

Kinetic analyses suggested that DN differentiated into DP through 2 pathways through the induction of CD93 SP or CD138 SP intermediates (Fig. S3). Immunohistology performed on lymph node sections confirmed that the CD93$^+$ cells localized in the medullary cords and colocalized with IgG_{2a} staining (Fig. S4 and ref. 18).

To follow antigen-specific B-cell responses in BM and spleen, we used the well-established TD NP-CGG response. The NP-specific ASC population was mostly DP, indicating that CD93 was up-regulated in the follicular as well as in the extrafollicular response (Fig. 1*B*). To assess CD93 expression on LLPC present in the BM, BrdU incorporation was used. As LLPC do not proliferate, this procedure allows distinguishing these cells from newly formed ASC (4). As shown in Fig. 1*C*, the large majority of antigen-specific ASC in the BM were CD93$^+$, regardless of their proliferative capacity. In contrast, GC and memory B cells remained negative for CD93 during the course of the experiment (Fig. 1 *D* and *E*, and data not shown). To induce a T independent (TI) II response, mice were injected with NP-Ficoll. Under these conditions, only a fraction of the NP-specific ASC generated expressed CD93 (Fig. 1*F*). Altogether, these results showed that CD93 is induced in both TD and TI immune responses, and that it is a reliable marker of (pre)plasmablasts and PC, including LLPC in the BM.

Functional Features of CD93-Expressing ASC Subsets Induced After MMTV Infection. We next assessed the differentiation capacity of the ASC and preplasmablast subsets that are discriminated on the basis of CD138 and CD93 expression. FACS-purified subpopulations from draining lymph nodes of BALB/c mice 6 days after MMTV infection were recultured for 24 h in vitro and subsequently analyzed by FACS (Fig. 2*A*). This experiment clearly showed that the DN B cells could differentiate into CD138 SP and DP cells. Moreover, after in vitro activation and reculture of DN cells, the CD93 SP subpopulation was also induced (Fig. S5*A*). In both experimental settings, the CD93 SP and the CD138 SP subpopulations had the ability to differentiate into DP ASC, whereas the DP population retained its phenotype (see Fig. 2*A* and Fig. S5*A*). DP- and SP-sorted cells with reduced CD93 and CD138 expression after reculture represented early apoptotic Annexin V$^+$ cells, which were excluded from Fig. 2*A*. Yields of surviving cells after reculture were between 10% (DP) and 35% (DN), excluding preferential survival of rare contaminating cells instead of differentiation. As PC differentiation is ultimately associated with exit from the cell cycle, we measured the percentage of proliferating cells in each subpopulation present in the draining lymph node 4.5 or 6 days after MMTV immunization. Mice were injected i.p. with BrdU 12 or 2 h before analysis. Both at day 4.5 and day 6, the DP population contained less BrdU$^+$ cells, but the difference was significant only at day 6 (Fig. 2*B*).

Class-switch recombination, was examined by subjecting purified cells to immunohistological staining with anti-IgG_{2a}, which is the major isotype induced by MMTV. Both CD138 SP and DP were strongly $IgG_{2a}{}^+$, while the CD93 SP were weakly positive and the DN remained negative (Fig. 2*C*). ELISPOT analysis performed on purified subpopulations confirmed that the DN and CD93 SP subsets contained few IgM- or IgG2a-producing ASC. Interestingly, whereas both DP and CD138 SP contained equivalent numbers of IgM ASC, the amount of IgG_{2a} ASC was strongly increased in DP, as an additional evidence of increased maturation (Fig. 2*D*).

PC terminal differentiation is controlled by a small group of transcription factors, namely Blimp-1, Irf-4, and Xbp-1 (20). Bcl6 and Pax5 inhibit terminal differentiation, which are essential for the GC activity and the maintenance of the naive B-cell phenotype

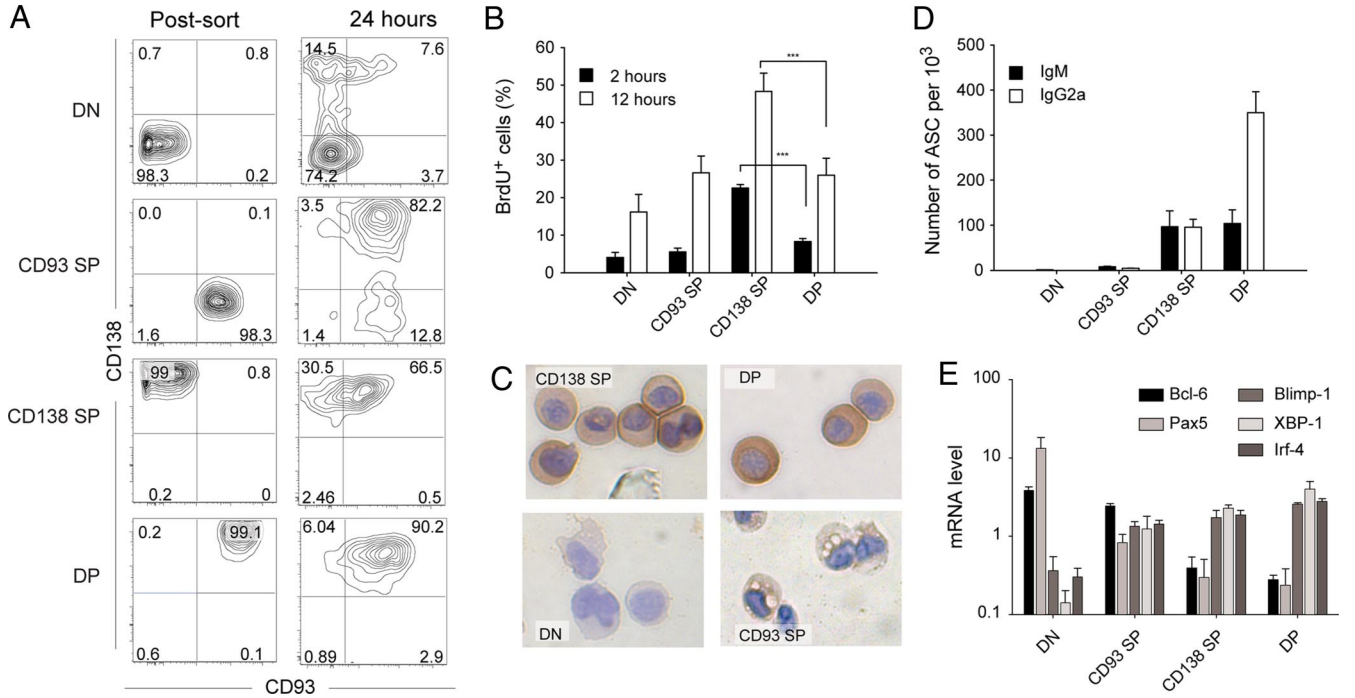

Fig. 2. Characteristics of the 4 B-cell and ASC subpopulations defined by the expression of CD138 and CD93. (*A*) Six days after MMTV immunization, plasmablasts from draining lymph nodes were sorted by FACS based on expression of B220, CD138, and CD93 expression and recultured for 24 h. Only live cells defined as DAPI$^-$ Annexin V$^-$ were included in the FACS analysis. (*B*) MMTV-immunized mice (6 days old) were pulsed i.p. with 300 μg of BrdU and incorporation was assessed in the different populations present in the draining lymph nodes after 2 or 12 h; mean $\pm$ SD of 3 independent experiments. **, $P < 0.01$, CD138 SP compared with DP subset by Student's *t* test. (*C*) The 4 subsets of FACS-purified plasmablasts were fixed and stained with anti-IgG$_{2a}$ (*brown*). Nuclei were counterstained with Mayer's Hematoxylin (*blue*). Data are representative of 3 independent experiments. (*D*) FACS-purified populations were analyzed by ELISPOT for IgM- and IgG$_{2a}$-secreting cells. (*E*) mRNA levels of *Bcl6, Pax5, Blimp-1, Irf-4,* and *Xbp-1* in the 4 FACS-purified subpopulations were investigated by real-time RT-PCR. Expression was normalized to Pol2A and Pol2G. Data are represented as mean $\pm$ SD of triplicate samples. The experiment was performed twice with similar results.

(21–23). To confirm the stepwise differentiation, the mRNA expression of these factors was assessed by quantitative RT-PCR. The negative regulators of differentiation, Bcl6 and Pax5, were progressively decreased in differentiation from the CD93 SP, to the CD138 SP and in the DP (Fig. 2*E*). In contrast, the positive regulators of ASC regulation, namely Blimp-1, Xbp-1, and Irf-4 were progressively induced in the different subpopulations. The molecular data combined with the reculture assay, the cell cycle, and the class-switch recombination analyses demonstrated that the coexpression of CD93 and CD138 could be used as a marker of increased maturation stages of ASC.

The Role of Blimp-1 and Aiolos in CD93 Expression. Mice deficient for *Blimp-1* and *Aiolos* have defects at different stages of PC differentiation (8, 9, 24). To determine whether CD93 is a direct target of Blimp-1 regulation, we analyzed mice heterozygous or homozygous for the *Blimp-1^{gfp}* allele (*Blimp-1$^{gfp/+}$* and *Blimp-1$^{gfp/gfp}$*).

Analysis of *Blimp-1$^{gfp/+}$* mice revealed that CD93 was predominantly observed on GFP^{high} cells, which correspond to LLPC (8). Similarly, after in vitro B-cell activation, CD93 was largely restricted to Blimp-1/GFP positive cells (Fig. S6 *A* and *B*). Sort and reculture experiments using *Blimp-1$^{gfp/+}$*-reporter B cells supported our differentiation scheme (Fig. S5*B*). Stimulation of *Blimp-1$^{gfp/gfp}$* Blimp-1-deficient B cells lead to an early block in PC differentiation at the stage of preplasmablasts, which express GFP, secrete very low amounts of Ig, and lack CD138 expression (9) (see Fig. S6*B*). After LPS stimulation, CD93 induction was severely reduced in GFP$^+$ *Blimp-1$^{gfp/gfp}$* cells but was normal in GFP-negative B cells (Fig. S6 *C*, *lane 1*). After stimulation of *Blimp-1$^{gfp/gfp}$* B cells with anti-CD40 + IL-4/IL-5, the induction of CD93 was higher in GFP$^+$ cells than after LPS activation, but severely reduced compared to the heterozygous controls (see Fig. S6*C*, *Top*). Taken together, these results indicate that CD93 induction is not directly dependent on Blimp-1 but rather correlates with the formation of mature ASC, which are highly dependent on Blimp-1 expression.

Aiolos$^{-/-}$ B cells show alterations in later stages of PC development and *Aiolos$^{-/-}$* mice fail to retain LLPC in their BM (24). Analysis of *Aiolos$^{-/-}$ Blimp-1$^{+/gfp}$* mice, revealed that CD93 expression on both spleen and BM ASC was normal or even elevated (Fig. S7). In line with this, induction of CD93 on *Blimp-1$^{gfp/+}$Aiolos$^{-/-}$* B cells after LPS or anti-CD40 + IL-4/IL-5 stimulation was comparable in GFP$^+$ cells and even increased in undifferentiated GFP$^-$ B cells (see Fig. S8 and data not shown). These results indicate that CD93 is not a direct target of Aiolos and that the absence of LLPC is not because of diminished CD93 expression.

CD93 Is Dispensable for ASC Differentiation but Is Crucial for the Maintenance of LLPC. Despite the expression of CD93 during the early stages of B-cell development, CD93 deficiency has no major consequence on the early B-cell development in the BM or the percentage of the different B-cell subsets in the periphery (Fig. S9 and ref. 25).

To determine if CD93 per se is involved in the differentiation of PC, *CD93$^{-/-}$ Blimp-1$^{gfp/+}$* B cells were activated with LPS or anti-CD40 + IL-4/IL-5 in vitro. This led to the induction of GFP$^+$ plasmablasts, similar to CD93$^{+/+}$*Blimp-1$^{gfp/+}$* control B cells (Fig. 3*A*). In the absence of CD93, a similar expression pattern for CD138 was observed when compared to WT cells, indicating that CD93 deficiency does not lead to altered plasmablast differentiation in vitro.

To determine the function of CD93 on ASC in vivo, we next examined the B-cell response in mice deficient for CD93. Immunization with MMTV showed that the extrafollicular response was unaffected in absence of CD93 (Fig. 3*B*). As expected, all of the

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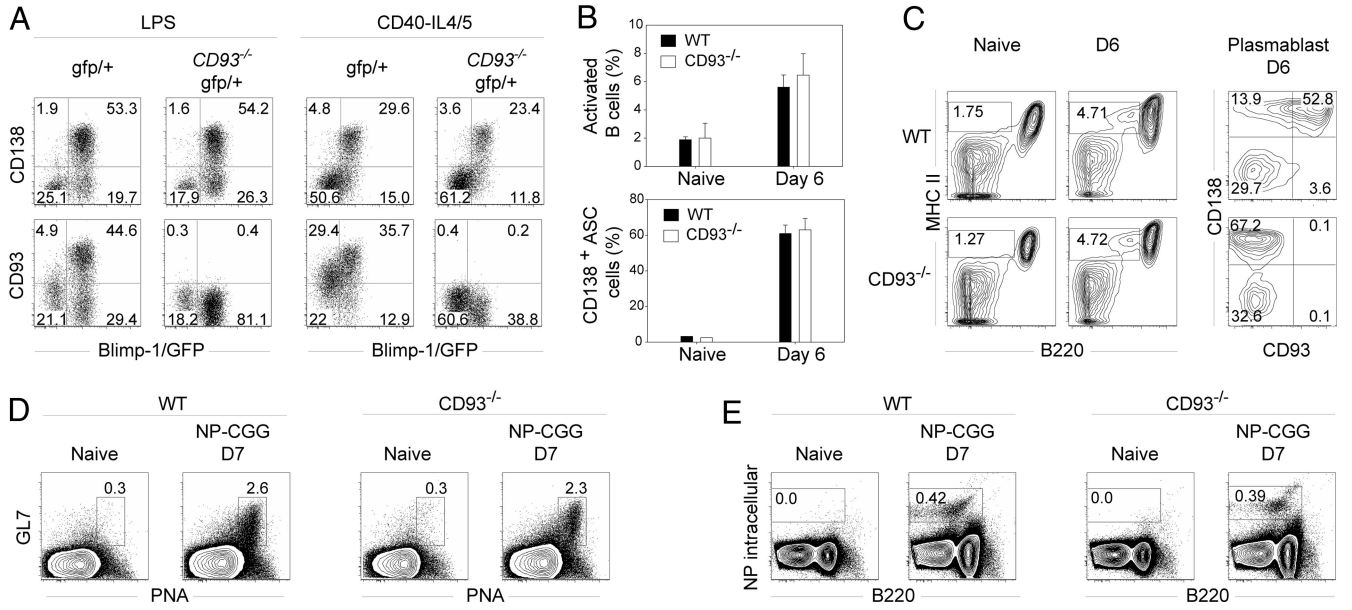

Fig. 3. CD93 is dispensable for ASC differentiation in vitro and for early stages of B-cell responses after TD immunization in vivo. (*A*) MACS-purified splenic B cells from *Blimp-1*$^{gfp/+}$ and *CD93*$^{-/-}$ *Blimp-1*$^{gfp/+}$ mice were activated in vitro with LPS or anti-CD40 + IL-4/IL-5 for 5 days and analyzed by flow cytometry. (*B*) CD93-deficient and control mice were immunized with MMTV and the frequency of B220^{-}MHCII^{int}CD138^{+} ASC was determined in lymph nodes by FACS. Bars are the means ± SD of 3 independent samples. (*C*) FACS analysis of one representative experiment from (*B*). (*D*) CD93$^{-/-}$ and WT mice were immunized with NP-CGG. The percentages of B220^{+}PNA^{+}GL-7^{+} germinal center cells in the draining lymph node were investigated by flow cytometry before and 7 days after immunization. (*E*) The frequency of NP-intracellular^{+}B220^{int} ASC was analyzed in the same organs. Data are representative of 3 independent experiments.

CD138^{+} cells remained CD93^{-} (Fig. 3*C*). In addition, immunization with NP-CGG showed that the GC reaction and the formation of NP-specific ASC occurred normally in these mice (Fig. 3 *D* and *E*). Determination of the serum Ig titres confirmed these observations and showed equivalent NP-specific IgG_1 Ig production during the initial phase of the response (Fig. 4*A*).

In contrast, analyses performed at later time points showed a significant decrease in high-affinity serum IgG_1 after day 20 and in total NP-specific IgG_1 serum after day 30 for total NP-specific antibodies (see Fig. 4*A*). After the boost, the CD93-deficient mice were able to respond normally but again showed a statistically significant decrease in Ig levels at later time points (see Fig. 4*A*). Similarly, analyses performed after NP-Ficoll immunization (TI type II response) showed no difference between CD93-deficient and the WT mice at the peak of the response but significantly decreased NP-specific Ig levels later during the response (Fig. 4*B*). Moreover, the absolute number of NP-specific PC in the BM of CD93-deficient mice measured 60 days after a boost with NP-CGG of CD93-deficient mice was lower than that observed in the controls (Fig. 4*C*). The reduction in Ig titers (see Fig. 4*A*) and BM PC (see Fig. 4*C*) were in the comparable range of 30 to 60%.

To determine whether migration or survival of PC in BM is affected by the absence of CD93, we immunized CD93-deficient and control mice and transferred splenocytes from the immunized mice 6 days after boost into WT recipients. Interestingly, and in contrast to WT splenocytes, transfer of CD93-deficient splenocytes failed to maintain high NP-specific Ig titers in recipient mice (Fig. 4*D*). To follow the migration of ASC after transfer, ELISPOT assays were performed on spleen and BM 1 or 36 days after transfer. One day after transfer, equal numbers of NP-specific cells were present in the spleen and the BM of the recipient mice receiving either CD93-deficient or WT cells (Fig. 4*E*). However, 36 days after transfer, low and comparable numbers of NP-specific PC of either genotype were observed in the recipient spleens. Strikingly, however, at this time point the number of NP-specific cells present in the BM of recipient mice receiving CD93-deficient splenocytes was markedly reduced compared to mice transferred with WT cells.

These results were confirmed in mixed BM chimeras. Irradiated WT mice were reconstituted with CD45.2 CD93-deficient and CD45.1 WT BM cells. The frequency of cells from both origins was analyzed in total BM and in NP-specific PC 30 days after boost immunization. This showed that the ratio of WT to CD93-deficient cells was significantly higher in NP-specific cells than in total BM cells, indicating that WT PC survive preferentially in BM (Fig. 4*F*). Altogether, these data indicate that CD93 is required for the maintenance of LLPC in the BM and, thus, for long-term protective Ig levels in the serum.

Discussion

Improved tools for tracking PC in normal and pathological conditions are required for better understanding and potential improvement of therapy (14). The long-standing assumption that PC are short-lived and produced continuously from cycling precursors has been challenged by recent experiments, demonstrating that a large part of Ig in autoimmune disease are produced by LLPC (26). Given the incomplete response to current available treatments targeting CD20^{+} or dividing cells that leave LLPC unaffected, a direct targeting could offer a treatment with faster clinical response and be possibly more efficient.

CD93 is a transmembrane protein with unknown function (27, 28). This article shows that CD93 is expressed during differentiation on plasmablasts and PC. ASC differentiation and PC maturation correlate well with changes in surface markers, Ig secretion, isotype switch, cessation of cell cycle (20), as well as with pronounced modifications at the transcriptional level, including the induction of the transcription factor Blimp-1, which in turn leads to expression changes of more than 250 genes (29). Analyses of these parameters in the 4 subsets defined by the expression of CD93 and CD138 demonstrated a progressive maturation from the DN B cells to the DP PC. Reculture assays further indicated that this process can follow 2 different pathways, the CD138 SP being favored in vivo and both pathways being observed after in vitro activation. The signals leading to the one or the other remain unclear.

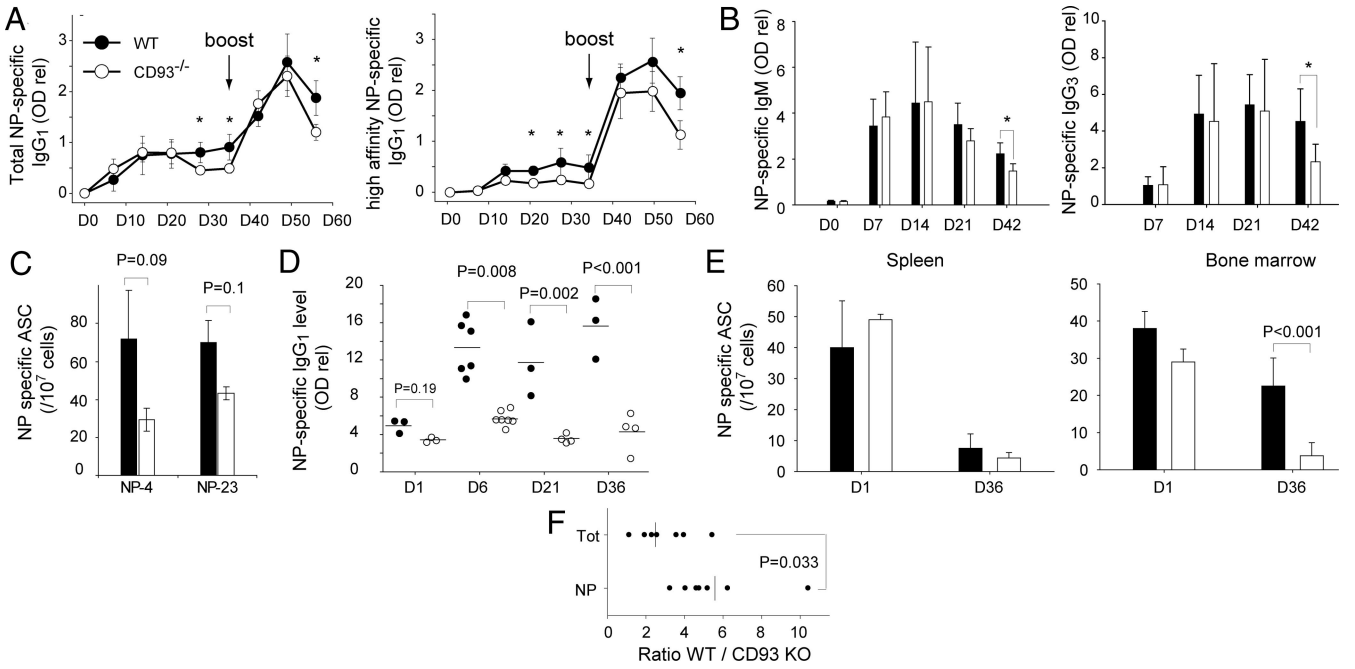

Fig. 4. CD93 expression is required for the maintenance of LLPC in the BM. (*A*) ELISA of total and high affinity NP-specific IgG_1 in the serum of CD93-deficient and WT mice after NP-CGG immunization and boost. Data are the mean ± SD of 12 mice. Three additional experiments ($n = 9$) gave similar results. (*B*) Mice were immunized with NP-Ficoll and the concentration of IgM and IgG_3 NP-specific Ig level was monitored by ELISA. (*C*) CD93-deficient and control mice were immunized and boosted 30 days later with NP-CGG. The frequency of high affinity and total NP-specific ASC present in the BM was monitored by ELISPOT at day 30. Data are the mean of 3 mice. (*D*) Transfer of WT or CD93-deficient splenocytes into WT naive recipient 6 days after boost immunization. Level of NP-specific IgG_1 monitored by ELISA in the sera at different time points after transfer of WT cells (*black circles*) or $CD93^{-/-}$ cells (*open circles*) into WT recipient. (*E*) Number of NP-specific ASC present in spleen and BM day 1 and 36 after transfer of WT cells (*black bar*) or CD93-deficient cells (*open bar*) as detected by ELISPOT. Results were pooled from 3 mice for each condition. (*F*) Lethally irradiated WT (Ly5.1) mice were reconstituted with a 1:1 mixture of CD45.1 WT and CD45.2 $CD93^{-/-}$ bone marrow. These chimeric mice were immunized and boosted with NP-CGG as described above. Thirty days later the ratio of cells from WT and $CD93^{-/-}$ origin in total BM or in NP-specific cells was investigated. *, $P < 0.05$, CD93 compared with WT mice by Student's *t* test. When the normality test failed, a Mann-Whitney Rank Sum Test was performed.

To determine the molecular mechanisms regulating CD93 expression, we analyzed its expression in the absence of Blimp-1 or Aiolos, 2 transcription factors known to be required during PC differentiation. Although CD93 expression was strongly impaired in the absence of Blimp-1, the initial induction of low levels of CD93 on activated Blimp-1-deficient B cells suggests that CD93 does not directly depend on Blimp-1, but rather correlates with differentiation stages of ASC defined by Blimp-1 expression. It has been demonstrated that PC generated in the absence of Aiolos cannot be retained in the BM. Analysis of Aiolos-deficient PC differentiation, however, revealed that CD93 is not a direct target of Aiolos.

Despite extensive studies both in humans and rodents, the function of CD93 in adhesion or phagocytic processes remains elusive and its function in PC differentiation had not been previously analyzed (28, 30, 31). While CD93 appears dispensable for the early phase of the humoral immune response, our results demonstrated that its absence resulted in an inability to maintain high antigen-specific Ig levels in the sera. This correlated directly with a decrease in the absolute number of LLPC observed in the BM of CD93-deficient mice after secondary immunization. To distinguish between the effect of PC migration to the BM or maintenance in this compartment, adoptive transfer and mixed BM chimeras were used. This showed a pronounced defect in the maintenance of BM PC and high levels of serum Ig for CD93-deficient cells.

While the function of CD93 in adhesion has not been demonstrated, its protein structure suggests an important role in this process. The cytoplasmic tail of human CD93 contains a highly charged juxtamembrane domain of 15 aa shared with other adhesion molecules, such as CD43, CD44, and intercellular adhesion molecules. It has been shown to interact with the moesin protein, a member of the Ezrin/Radixin/Moesin family (32). Moesin is known to be important in linking transmembrane proteins to the cytoskeleton. This contributes to a redistribution of the actin cytoskeleton that has been shown to be essential for phagocytosis, migration, and adhesion (33, 34). As this molecule seems to be implicated in survival in BM niches, it makes sense that GC and memory B cells do not express this marker. While it is unclear whether CD93 is directly involved in adhesion, our results indicate that CD93 is critical for the maintenance of LLPC in their BM survival niches.

Materials and Methods

Mice and Immunization. Lat^{Y136F} (35), $CD93^{-/-}$ (25), $Blimp\text{-}1^{gfp/+}$ (8), and $Aiolos^{-/-}$ (36) mice were previously described. C57BL/6, BALB/c, and CBA/Ca mice were purchased from Harlan Olac. $Blimp\text{-}1^{gfp/gfp}$ mice were generated by fetal liver reconstitution as described earlier (8). Animals were bred in the facility at the Swiss Institute for Experimental Cancer Research. All experiments were done in agreement with Institutional and Swiss regulations.

Six- to 8-week-old mice were injected s.c. into the hind footpad with MMTV(SW) (37). Alternatively, mice were immunized i.p. with 50 μg of alum precipitated NP-CGG (Biosearch Technologies), 100 μg of alum precipitated OVA or i.v. with 30 μg of NP-Ficoll (Biosearch Technologies). To follow the response in popliteal lymph nodes, 20 μg of NP-CGG was injected s.c. in the footpad. Boosts were performed i.p. 30 days later with 50 μg NP-CGG or 100 μg soluble OVA. BrdU (Sigma-Aldrich) was administrated as a 0.8 mg/ml solution in the drinking water (light protected and changed every second day), or 1 mg of BrdU was injected in PBS i.p.

Antibodies, Flow Cytometry, and Cell Sorting. Single-cell suspensions were stained with the following mAb from Becton Dickinson (BD) PharMingen: MHCII (2G9), Nk1.1 (PK136), CD138 (281–2), GL-7 (Ly77), IgD (11–26c.2a) IgM (R6–60.2), CD4 (L3T3), CD11b (M1/70), CD21/CD35 (7G6), CD43 (S7), CD80 (16–6A1), CD86 (GL-1); from eBioscience: CD23 (B3B4), CD93 (AA4.1), B220 (RA3–6B2), PNA (Sigma-Aldrich); from Biolegend: CD5 (53–7.3), CD62L (Mel-14), CD24 (M1/69),

CD8 (53–6.7), BP-1 (6C3). Biotinylated mAbs were visualized with streptavidin-PE-Cy5.5 (eBioscience). NP_{40}-PE was from Biosearch technologies and OVA from Molecular Probes. BrdU staining was performed using the BrdU-FITC flow kit from BD according to the manufacturer's instructions. DAPI (Molecular Probes) and Annexin V (BD PharMingen) were used to exclude dead cells. FACS data were collected with a BD FACSCalibur, FACSCanto, or FACS LSRII cell analyzer, and analyzed on FlowJo (Tree Star). Cells were sorted on a FACSAria (BD) with a purity of 95 to 99%.

In Vitro Culture. $CD19^+$ splenic B cells were purified by MACS (Miltenyi Biotec) using anti-CD19 beads. The purity was >90%. Stimulation cultures were performed in complete DMEM (Invitrogen Corporation) complemented with 10% FCS (Brunschwig), 10 mM Hepes (Invitrogen Corporation), 20-μg/ml gentamycin (Invitrogen Corporation), and 50 μM β-mercaptoethanol (Invitrogen Corporation) with anti-CD40 (FGK.45; 10 μg/ml) and IL-4/IL-5 or LPS (Sigma-Aldrich; 5 μg/ml). ASC populations were FACS sorted and recultured in complete RPMI for 24 or 48 h in the absence of additional signals.

ELISA and ELISPOT. Serum or supernatant levels of total IgM, IgG_1, IgG_{2a}, IgE, and NP-specific Ig were quantified by ELISA using polyclonal goat Abs specific for mouse Ig isotypes for detection (Caltag Laboratories) and *o*-phenylenediamine developing reagents (Sigma-Aldrich). To detect anti-NP Ig, plates were precoated with NP_{23} or NP_4-BSA (Biosearch Technologies). Total amount or NP-OVA-specific ASC were assessed by ELISPOT using standard techniques.

Immunohistological Analysis. Purified ASC were centrifuged on polylysine-coated slides (Menzel-Gläzer) using cytospin (Thermo Electron Corporation). Slides were stained with biotinylated anti-IgG_1 or anti-IgG_{2a} and quantified using streptavidin-HRP (Jackson Immunosearch Laboratories). Counterstaining was done using Mayer's hematoxylin. Immunofluorescent staining on acetone-fixed frozen lymph node sections were performed using standard techniques. The following reagents were used: anti-B220-biotin (RA3–6B2, Caltag Laboratories), anti-CD4 (RM4–5; eBioscience). Anti-CD93 was provided by P. Gasque, University of Wales College of Medicine, Cardiff, UK (38). For detection, Alexa 488-conjugated streptavidin (Molecular Probes), APC conjugated anti-rat Ig (Jackson Immunosearch Laboratories), and Cy3 conjugated anti-rabbit Ig (Jackson Immunosearch Laboratories) were used.

Quantitative mRNA Expression. RNA isolation and quantitative RT-PCR using SYBR Green mix on Light Cycler (Roche) were performed as described elsewhere (39). Primers used are listed in Table S1. Amplification plots were analyzed using the second derivative method with LC data analysis 3.5 Software (Roche). Relative expression of mRNA was determined with qBase (40), using *Pol2A* and *Pol2G* as reference genes.

Adoptive Transfer and BM Chimeras. Spleens were isolated from WT or $CD93^{-/-}$ mice 6 days after NP-CGG boost immunization. Total splenocytes were injected i.v. into WT recipient mice, which were killed 1 and 36 days after transfer. Blood was obtained on days 6, 15, and 21. Spleen and BM were analyzed by ELISPOT assay for the frequency of NP-specific ASC. Ratios were corrected relative to the number of NP-specific donor cells for each genotype. Mixed BM chimeras were generated by reconstituting 2×450 Rad irradiated C57BL/6 mice with 10^7 BM cells from $CD93^{-/-}$ and CD45.1 donor mice at a 1:1 ratio. Eight weeks later, mice were immunized with NP-CGG as described above.

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