Sustained antibody responses depend on CD28 function in bone marrow–resident plasma cells

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Sustained long-term antibody levels are the cornerstone of protective immunity, yet it remains unclear how they are durably maintained. A predominant theory implicates antigen-independent antibody production by a subset of long-lived plasma cells (LLPCs) that survive within bone marrow (BM). Central tenets of this model—that BM LLPCs constitute a subset defined by intrinsic biology distinct from PCs in other tissues and contribute to long-term antibody titers—have not been definitively demonstrated. We now report that long-term humoral immunity depends on the PC-intrinsic function of CD28, which selectively supports the survival of BM LLPC but not splenic short-lived PC (SLPC). LLPC and SLPC both express CD28, but CD28-driven enhanced survival occurred only in the LLPC. In vivo, even in the presence of sufficient T cell help, loss of CD28 or its ligands CD80 and CD86 caused significant loss of the LLPC population, reduction of LLPC half-life from 426 to 63 d, and inability to maintain long-term antibody titers, but there was no effect on SLPC populations. These findings establish the existence of the distinct BM LLPC subset necessary to sustain antibody titers and uncover a central role for CD28 function in the longevity of PCs and humoral immunity.

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Abbreviations used: ASC, antibody-secreting cell; BMDC, BM-derived DC; BMSC, BM stromal cell; EMSA, electromobility shift assay; LLPC, long-lived PC; PC, plasma cell; SLPC, short-lived PC.

Sustained levels of antibodies are the cornerstone of long-term immunity against infection by many pathogens, and induction of durable antibody titers is an essential characteristic of effective vaccines. As the half-life of immunoglobulin is on the order of days to weeks but protective levels of antibody may be sustained for a lifetime, continued antibody production by plasma cells (PCs) is required. How these PC populations are maintained over a lifetime remains unclear; however, two models have been proposed. The first involves continuous differentiation of antigen-specific memory B cells into short-lived PCs (SLPCs; which survive for weeks), driven by endemic/persistent antigen or by polyclonal antigen-independent B cell activators (Amanna and Slifka, 2010). However, this mechanism as the exclusive means to sustain antibody levels long term has been called into question because antibody titers can persist despite decades elapsing before antigen reexposure or with no reexposure at all (Amanna et al., 2007). Additionally, sustained antibody titers after immunization in humans does not appear to require memory B cell activation (Amanna et al., 2007), and vaccine-induced antibodies in mice are maintained over prolonged periods even in the absence of a replenishing B cell compartment (Slifka et al., 1998; Ahuja et al., 2008). To account for these observations, a second model has been proposed in which long-term antigen-specific antibody levels are maintained in an antigen-independent manner by a subset of PCs that are long lived and, in some instances, would be predicted to survive the lifetime of the host (Slifka et al., 1998; Ahuja et al., 2008; DiLillo et al., 2008). BM-resident nonproliferating PCs have been implicated as the long-lived PCs (LLPCs; Slifka et al., 1998; Manz and Radbruch, 2002), and in this model, BM LLPCs and SLPCs (in the spleen and other secondary lymphoid organs) are intrinsically distinct subsets

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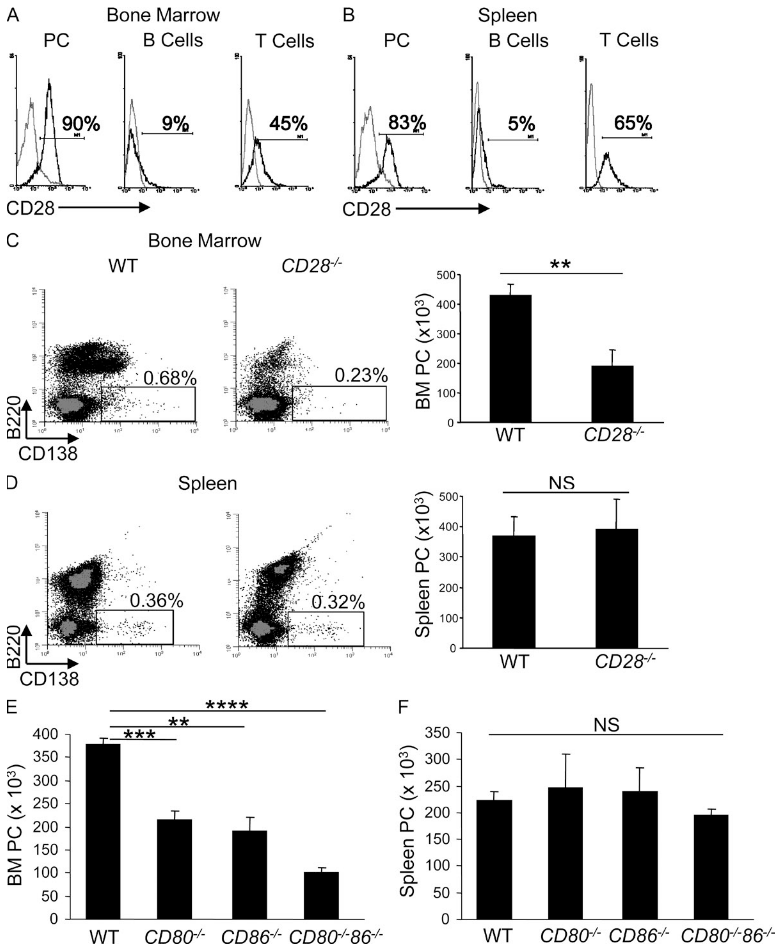

Figure 1. CD28 is expressed on splenic and BM PCs. (A and B) CD28 expression was determined in BM (A) or splenic (B) CD138$^+$ PCs, CD3$^+$ T cells, and CD19$^+$ B cells purified from WT mice. Gray lines represent isotype controls; black lines represent anti-CD28 staining. Results shown are one representative experiment of four. (C and D) Total PC numbers in BM (C) or spleen (D) of WT and CD28$^{-/-}$ mice were determined from total mononuclear cells by multiparametric flow cytometry using CD138$^+$B220$^-$ to identify PC (representative plots are shown). (E and F) PCs in BM or spleen of CD80$^{-/-}$, CD86$^{-/-}$, and CD80/86$^{-/-}$ mice were determined as in C and D. Mean ± SD of 10 mice (C and D) or of three mice (E and F) is shown. ns, not significant. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

that do not interconvert into one another (Radbruch et al., 2006) and differ in their generation, biology, longevity, and anatomical localization. It has been hypothesized that one distinction between these subsets is the ability of LLPC to use a limited number of specific BM stromal niches that are essential for their survival (Manz et al., 1997; Radbruch et al., 2006) and thus access to, competition for, and maintenance within these niches are predicted to be major determinants of the long-lived protective antibody repertoire (Moser et al., 2006).

However, although long-lived PCs have been identified in the BM, careful review of the literature reveals there is no direct evidence that BM PCs actually contribute to long-term antibody responses, as it has not been possible to selectively eliminate them while retaining other PC populations. This is closely tied to the fact that it is far from clear that BM PCs are actually a distinct PC subset as predicted by the model. No intrinsic molecular or cellular characteristics have been identified that clearly define the putative LLPC or SLPC subset, and certainly none that account for the differences in longevity. Factors involved in PC differentiation (e.g., Blimp-1 [Shapiro-Shelef and Calame, 2005; Martins and Calame, 2008], Aiolos [Cortés and Georgopoulos, 2004], and Ets-1 [John et al., 2008]), adhesion (e.g., LFA-1/VLA-4 [DiLillo et al., 2008]), and survival (e.g., FcγRIIb [Xiang et al., 2007], IL-6 [Minges Wols et al., 2002], and APRIL/BAFF [Benson et al., 2008]) appear to be important for all PCs, and none selectively affects the generation or survival of the putative PC subsets in the spleen or BM. There are specific characteristics associated with BM homing and residency by PCs, such as the expression of the chemokine receptor CXCR4 (Tokoyoda et al., 2004), reliance on the adhesion molecule CD93 (Chevrier et al., 2009), and association with reticular CXCL12$^+$ stromal cells (Tokoyoda et al., 2004), eosinophils (Chu et al., 2011), basophils (Rodriguez Gomez et al., 2010), and megakaryocytes (Rodriguez Gomez et al., 2010; Winter et al., 2010). However, it is not known whether all newly differentiated PCs can home to the BM and become long-lived by stochastically finding a BM niche, or whether the LLPC subset a priori has unique intrinsic competency to access/use the BM niche for long-term survival (Radbruch et al., 2006). And, in the latter case, it is also unknown what the molecular basis is for this competency to interact with the BM niche and how it is different from SLPC interactions in the spleen/secondary lymphoid organs.

Although CD28 has been almost entirely characterized as the prototypic T lymphocyte receptor that provides the essential costimulatory signal that, in conjunction T cell receptor/CD3 signaling, results in T cell activation (Sharpe and Freeman, 2002; Friend et al., 2006), enhanced function (Shapiro et al., 1997; Friend et al., 2006), and survival (Boise et al., 1995; Frauwirth et al., 2002), it is also expressed on the surface of PCs (Kozbor et al., 1987). Interestingly, CD28 expression in the B cells is specifically repressed by the B cell master regulator Pax-5 and de-repressed during differentiation to PC (Delogu et al., 2006). Little, however, is known about what function CD28 has in the normal B cell lineage, as its role in humoral immune responses has been predominantly attributed to helper T cell co-stimulation and germinal center formation (Shahinian et al., 1993; Ferguson et al., 1996) even though the absence of CD28 diminishes short-term primary antibody responses even with adequate T cell help (Delogu et al., 2006). For the

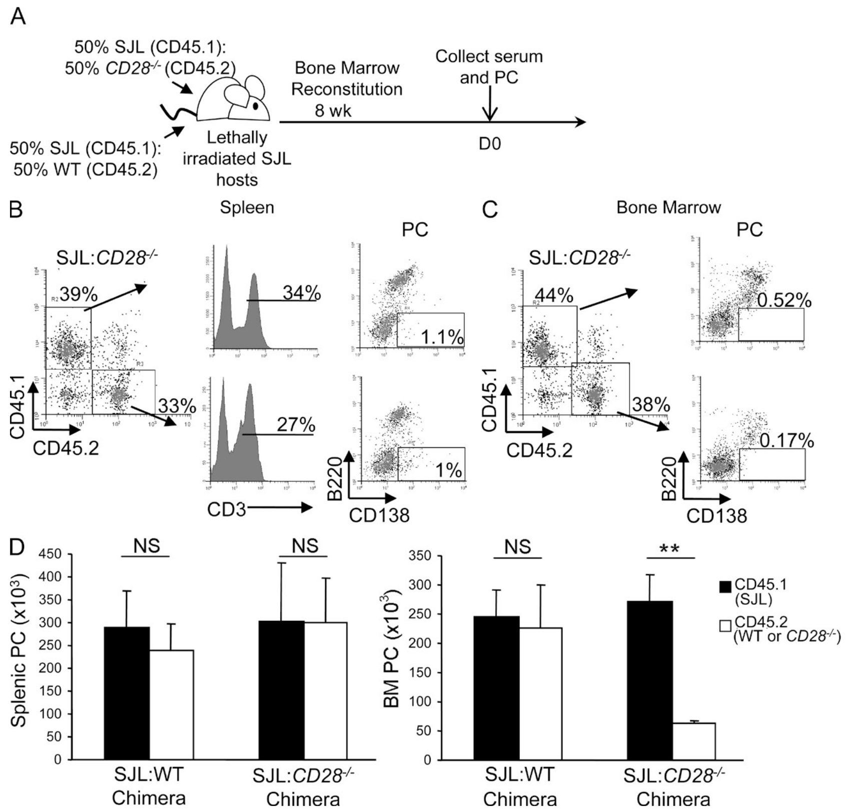

Figure 2. BM PCs from CD28$^{-/-}$ mice have a competitive repopulation disadvantage. (A) Experimental design (D, day). (B) Total splenocytes from chimeras were analyzed by multiparametric flow cytometry for percentages of CD3$^+$ T cells and CD138$^+$B220$^-$ PC. CD45.1 (SJL) splenocytes are plotted on top and CD45.2 (CD28$^{-/-}$) splenocytes on the bottom. Dot plots and histograms are representative of three mice. (C) BMs from chimeras were analyzed by multiparametric flow cytometry for percentages of CD138$^+$B220$^-$ PC. Dot plots are representative of three mice. (D) Total PC numbers in spleen (left) and BM (right) was determined from total mononuclear cells by multiparametric flow cytometry using CD138$^+$B220$^-$ to identify PC. Histograms are representative of three mice per group. Error bars represent the mean ± SD. ns, not significant. **, $P < 0.01$.

malignant BM-resident PCs in multiple myeloma, CD28 expression clinically correlates with significantly poorer prognosis (Almeida et al., 1999) and disease progression (Robillard et al., 1998), suggesting that CD28 provides the myeloma cells with a survival advantage. Consistent with this, we and others have found that CD28 activation in myeloma cells induces PI3K and NF-κB signaling (Tu et al., 2000; Bahlis et al., 2007), IL-8 production (Shapiro et al., 2001), and a prosurvival signal that protects in vitro against chemotherapy-induced death (Bahlis et al., 2007). These observations led us to examine whether intrinsic CD28 function in normal PCs plays a general or subset-specific role in regulating their survival and, thus, the longevity of antibody responses.

RESULTS

Mice lacking CD28 or CD80/CD86 have selective loss of BM PC

Throughout our studies, we examined splenic and BM PCs as the putative SLPC and LLPC subsets, respectively. In WT C57BL/6J mice, purified BM PC (Fig. 1 A) and splenic PC (Fig. 1 B) both expressed CD28 at similar levels to T cells. However, naive mice genetically deficient for CD28 (CD28$^{-/-}$) had significantly fewer PCs in the BM (Fig. 1 C, right) but equivalent numbers of splenic PC (Fig. 1 D, right) compared with WT mice as analyzed by multiparametric flow cytometry of the total mononuclear cell population (Fig. 1, C and D, left, representative plots for PCs based on their CD138$^+$B220$^-$ phenotype; Shapiro-Shelef et al., 2003, 2005). More stringent phenotypic gating for PCs (CD138$^+$B220$^-$IL-6R$^+$MHCII$^-$; Moser et al., 2006) in multiparametric flow analysis or direct enumeration of PC numbers after immunomagnetic purification from spleen or BM yielded the same significant differences (unpublished data). Similarly, unvaccinated mice lacking the CD28 ligands (CD80$^{-/-}$, CD86$^{-/-}$, and CD80/CD86$^{-/-}$) had a selective loss of BM PC (Fig. 1 E) but a comparable number of splenic PC (Fig. 1 F) versus WT. Interestingly, the loss of BM PC in the CD80$^{-/-}$ or CD86$^{-/-}$ single knockouts indicates that even though either ligand can bind CD28, they are not redundant in the context of maintaining the BM PC population. And although PCs express low levels of CD86, they do not express CD80 (unpublished data), suggesting that BM PC interaction with CD80-expressing stromal cell in the BM niche is required to sustain this PC subset (see Fig. 4).

CD28-dependent maintenance of the BM PC population in vivo is PC intrinsic

Potential reasons for the decrease in BM PC in the CD28$^{-/-}$, CD80$^{-/-}$, CD86$^{-/-}$, and CD80/CD86$^{-/-}$ mice include an intrinsic PC defect or extrinsic causes as a result of lack of T cell help or other alterations in the host microenvironment. To more definitively determine if CD28 was affecting BM PC directly in a cell-intrinsic manner, or if the selective loss of BM PC was a result of extrinsic factors, competitive repopulation studies were performed. Congenic BM chimeras were generated by transplanting 10^6 CD28$^{+/+}$ SJL (CD45.1) + C57BL/6J WT (CD45.2) or SJL + CD28$^{-/-}$ (CD45.2) BM cells at a 1:1 ratio into lethally irradiated SJL hosts (Fig. 2 A). After reconstitution, equal chimerization of CD3$^+$ T cells and CD138$^+$B220$^-$ PC was seen in the spleens of both chimeras (Fig. 2, B [representative plots from SJL:CD28$^{-/-}$ chimeras] and D [left]). However, in the BM of the SJL:CD28$^{-/-}$ chimeras both the percentage of PC contributed by the CD45.2 CD28$^{-/-}$ BM (Fig. 2 C) and the total number (Fig. 2 D, right) was substantially

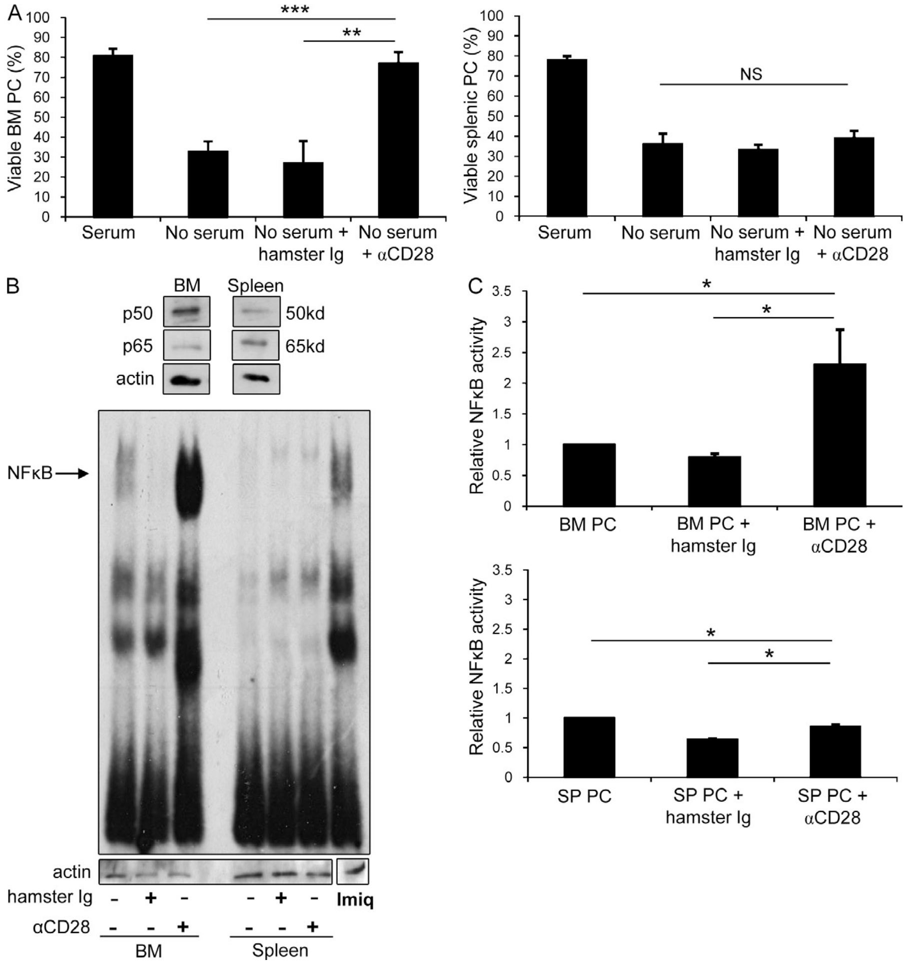

Figure 3. CD28 activation protects BM but not splenic PCs from serum starvation–induced death. (A) 2 × 10^4 purified BM or splenic PCs were cultured with or without fetal calf serum for 24 h with or without polyclonal control hamster Ig-coated beads or anti-CD28–coated beads, and viability was assessed by trypan blue exclusion. Mean ± SD of three independent experiments is shown. (B) Purified BM and splenic PCs were cultured with or without polyclonal control hamster Ig or anti-CD28 for 30 min (splenic PCs were cultured with or without 3 µM imiquimod as a positive control), and whole cell lysates analyzed by EMSA (bottom) for binding to probes containing consensus NF-κB binding sites (Bahlis et al., 2007). Immunoblot analysis (top) was performed for p50 and p65. Data are representative of four independent experiments. (C) Purified BM and splenic PCs were cultured alone, with polyclonal control hamster Ig or anti-CD28 for 60 min, and lysates were analyzed for NF-κB luciferase reporter activity. Relative NF-κB activity was determined as described in Materials and methods, and BM PC or SP PC alone were set as 1 and all other samples normalized to BM PC or SP PC alone. Mean ± SD of three independent experiments is shown. ns, not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

less than that contributed by the SJL CD45.1 marrow, which is in contrast to the equal contribution by the C57BL/6J WT marrow to the BM PC population in the SJL:WT chimeras. These data demonstrate that in the context of the same host environment where both $CD28^{+/+}$ and $CD28^{-/-}$ PCs have access to the same T cell help and the same BM microenvironment, the BM (but not splenic) PCs of $CD28^{-/-}$ origin are at a competitive disadvantage, which is consistent with a direct cell-intrinsic role for CD28 specifically in the BM PC subset.

CD28 activation enhances the survival of BM but not splenic PCs

The potential intrinsic functions of CD28 in the BM PC include regulating LLPC generation during B→PC differentiation, LLPC plasmablast proliferation, selective homing to/adhesion within the BM, and/or survival within the BM niche. Given the previous findings in T cells and myeloma cells, we first examined whether CD28 activation had a prosurvival effect in normal PCs. In vitro, anti-CD28 mAb–induced direct CD28 activation by itself (without an exogenous signal 1) protected purified WT BM PC from serum starvation–induced death (Fig. 3 A, left) but had no effect on splenic PC survival (Fig. 3 A, right). Assessment of the purified BM and splenic PC populations demonstrated that they were phenotypically and functionally similar (Fig. S1) and that there was not an excess of $CD19^{+}CD138^{+}$ IgM-secreting plasmablasts in the splenic PC population that might account for the differential responses (Kallies et al., 2004). Given that splenic PCs express CD28, the basis for this differential response was likely a result of differences in downstream signaling. We confirmed that the components of the NF-κB pathway were present, as both splenic and BM PCs express the p50 and p65 NF-κB subunits (Fig. 3 B, top). However, anti-CD28 mAb induced NF-κB signaling in BM PC (Fig. 3 B, bottom left) but not in splenic PC (Fig. 3 B, bottom right), as measured by electromobility gel shift assays. NF-κB signaling could be induced in splenic PC by the TLR 7 agonist imiquimod (Tangye and Tarlinton, 2009), demonstrating that there was not a global defect in NF-κB signaling in these cells. To further validate CD28-mediated NF-κB signaling (or lack thereof), we examined activation of NF-κB responsive gene elements using splenic and BM PC isolated from the NF-κB reporter mouse strain, which is transgenic for the IκBα promoter linked to the firefly luciferase reporter gene (Zhang et al., 2005). After 1 h of stimulation in vitro, BM PC cultured with anti-CD28 mAb had a 2.3-fold increase of relative NF-κB activity compared with BM PC cultured alone or with control hamster Ig (Fig. 3 C, top), whereas there was no effect of anti-CD28 stimulation on the NF-κB activity in the splenic PC (Fig. 3 C, bottom). Altogether, these

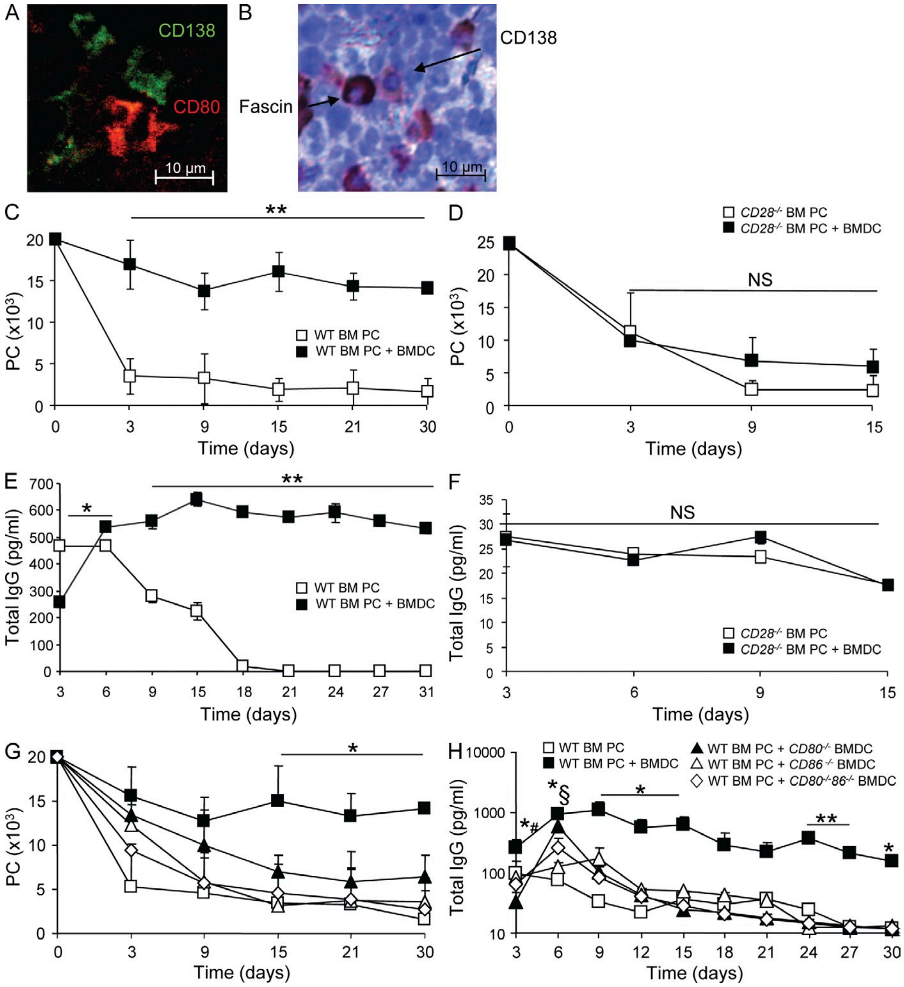

Figure 4. BMDCs interact with and support PC survival and function through CD28–CD80/CD86 interactions. (A) BM sections from WT mice were stained with antibodies against CD80 (red) and CD138 (green; image representative of four independent experiments). (B) Immunohistochemical staining from sternum sections of WT mice. Brown is fascin, identifying DCs, and pink is CD138⁺ PCs identified by arrows (image representative of two independent experiments). (C–H) Purified BM PCs were cocultured with BMDC of indicated genotypes for the indicated time periods. Total viable PC numbers were determined by 7AAD incorporation analyzed by flow cytometry (mean ± SD of three independent experiments is shown). Culture supernatants were analyzed for total IgG production by ELISA (mean ± SD is shown of one representative experiment of three). ns, not significant. *§, $P < 0.05$ PC + BMDC compared with PC + $CD80^{-/-}$ BMDC and $CD80/86^{-/-}$ BMDC; *#, $P < 0.05$ PC + BMDC compared with PC + $CD86^{-/-}$ BMDC; *, $P < 0.05$; **, $P < 0.01$.

findings suggest that compared with BM PC, CD28 on splenic PC has a higher activation threshold more characteristic of that seen in T cells (Thompson et al., 1989; Stein et al., 1994).

BM-derived DCs (BMDCs) support BM PC survival and Ig production

The selective loss of BM PC also seen in the CD80/CD86 knockouts suggests that the essential stromal cells within the BM PC survival niche (Shapiro-Shelef and Calame, 2005) express these CD28 ligands. Other work has suggested that DCs (which can have high expression of CD80 and CD86) are supportive stromal cells for the B lineage, as direct DC contact provides critical differentiation and survival signals to normal B cells (Sapoznikov et al., 2008), plasmablasts (Mohr et al., 2009), and myeloma cells (Said et al., 1997; Bogen, 2002; Kukreja et al., 2006). Additionally, BM DCs in myeloma patients are induced to produce the B lineage survival factor IL-6 (Said et al., 1997). Consistent with these studies, we have found in situ within the BM of WT mice that CD138⁺ PCs are in direct contact with CD80⁺ (Fig. 4 A) and fascin⁺ (a DC marker; Bahlis et al., 2007; Fig. 4 B) BM stromal cells (BMSCs) phenotypically resembling DC. Enumeration of PCs across entire BM sections demonstrated that 73.6% were in contact with CD80⁺ BMSC and 68.5% were in contact with fascin⁺ BMSC.

The ability of DC to support PC survival was examined in vitro in co-cultures of purified WT BM PC + WT BMDC and demonstrated seven-fold more viable PC compared with medium alone conditions out to 30 d of culture (Fig. 4 C). However, BMDC could not support $CD28^{-/-}$ BM PC survival (Fig. 4 D), indicating a central role for CD28 even within the complexity of the PC–DC cellular interaction. Co-culture with BMDC also did not support long term survival of splenic PC (Fig. S2), which is consistent with their lack of CD28 signaling, although there was enhancement of short-term survival, possibly as a result of CD28 induction of IL-6 from the DC (see Fig. 5). WT BM PC function, as measured by total IgG production, was also maintained in the BMDC co-cultures (Fig. 4 E), whereas $CD28^{-/-}$ BM PC cultured alone or with BMDC produced only low levels of IgG, IgM, and IgA (Fig. 4 F and not depicted). This was not because $CD28^{-/-}$ BM PCs were unable to make immunoglobulin, as exogenously added IL-6 induced significant IgG production (Fig. S3). Co-culture of WT BM PC with $CD80^{-/-}$, $CD86^{-/-}$, and $CD80/CD86^{-/-}$ BMDCs similarly yielded significantly less long-term PC survival and production of IgG compared with co-culture with WT BMDC (Fig. 4, G and H). Interestingly, the observation that the individual absence of CD80 or CD86 affects PC survival/function in vitro is consistent with the preceding in vivo findings and suggests that they are not simply interchangeable ligands for CD28 but have functions separate from activating CD28.

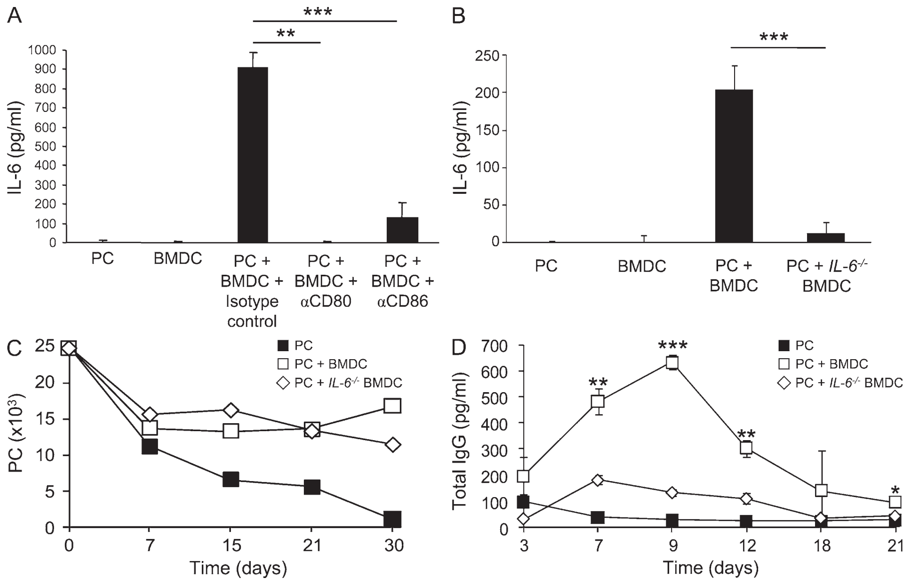

Figure 5. BM PCs induce DC IL-6 production through a CD80- and CD86-dependent mechanism. (A). 10^4 purified BM PCs were cultured with or without 10^5 WT BMDC with or without 20 µg/ml of hamster Ig (isotype control), or 20 µg/ml anti-CD80 or anti-CD86 for 24 h. IL-6 production analyzed by ELISA. (B) Purified BM PCs were cultured with or without WT or IL-6$^{-/-}$ BMDC for 24 h and IL-6 production analyzed by ELISA. (C and D) Purified BM PCs were cultured with or without WT or IL-6$^{-/-}$ BMDC. Total PC numbers and IgG production were determined as previously described. Data are presented as the mean ± SD and are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

BM PCs induce BMDC production of IL-6 that is necessary for Ig production in a CD28–CD80/CD86–dependent manner

Given that the other receptor for CD80 and CD86, CTLA4, has not been detected on normal or malignant PC (Shaffer et al., 2002; Zhan et al., 2007; Driscoll et al., 2010), one possibility is that this separate CD80/CD86 function is via their signaling directly to the DC. It has been shown in DC-mediated T cell activation that CD28 cross-linking of CD80/CD86 induces DC production of IL-6 (Orabona et al., 2004), a proinflammatory cytokine necessary for T cell activation but also a well characterized differentiation/survival factor for the B cell lineage (Kawano et al., 1988; Minges Wols et al., 2002). PCs also induce IL-6 production from the stromal microenvironment, although the specific interactions involved are unclear (Minges Wols et al., 2002). This raised the possibility that CD28 on the surface of PC also induces DC production of microenvironmental IL-6 to support PC survival/function. In vitro, although WT PC and WT BMDC did not make IL-6 by themselves, co-culture induced significant IL-6 production that is dependent on CD80 (completely) or CD86 (partially; Fig. 5 A) and was not seen when the co-cultured DC could not make IL-6 (IL-6$^{-/-}$ BMDC; Fig. 5 B). Surprisingly however, even though exogenous IL-6 has a significant prosurvival effect on BM PC cultured in medium alone (Fig. S4; Minges Wols et al., 2002), BM PC survival was unaffected when co-cultured with IL-6$^{-/-}$ BMDC compared with WT (Fig. 5 C). However, there was significantly less IgG production in IL-6$^{-/-}$ BMDC co-cultures (Fig. 5 D), suggesting that CD28 separately regulates BM PC survival directly and immunoglobulin production indirectly via CD80/CD86-mediated induction of IL-6 from the stromal DC.

Loss of CD28, CD80, or CD86 compromises BM PC survival and durable antibody responses in vivo

If CD28 function is selectively important for the maintenance of the LLPC subset, loss of CD28 in PCs may not affect total antibody levels (because SLPC would be unaffected, consistent with Delogu et al., 2006) but would compromise the survival of antigen-specific LLPC and the ability to sustain antigen-specific antibody titers long term after vaccination. To examine this, BM chimeric mice lacking CD28 only in B cell compartment were generated by tandem transplantation of WT hosts with BM from µMT mice that lack B cells but have normally functioning CD28$^+$ T cells (Tuaillon, 2000; Delogu et al., 2006) plus BM from either CD28$^{-/-}$ or WT control mice (Fig. 6 A). Analysis of chimerization showed comparable percentages of CD3$^+$CD28$^+$ T cells, whereas CD138$^+$ PCs were CD28$^+$ in WT and CD28$^-$ in CD28$^{-/-}$ chimeras (Fig. 6, B and C). The chimeras were primed and boosted with the T cell–dependent antigen NIP-ovalbumin, and serum immunoglobulin and PC numbers were assessed over 180 d. WT:µMT and CD28$^{-/-}$:µMT chimeras had equivalent total serum IgG1 levels over the 6 mo (Fig. 6 D, left). However, although the NIP-specific IgG1 titers were similar at day 7, they were significantly lower in the CD28$^{-/-}$:µMT chimeras by day 21 and back to prevaccination levels by day 180 (Fig. 6 D, right). NIP-specific IgA and IgM titers were unaffected (Fig. S5, A and B), suggesting that plasmablasts and mucosal PC are less dependent on CD28. To determine if the loss of anti-NIP antibody titers was a result of down-regulation of immunoglobulin production or loss of the LLPC population, the number of total and antigen-specific PCs was assessed. The total number of PCs in the spleen was comparable between chimeras (Fig. 6 E, left) but significantly lower in the BM of the CD28$^{-/-}$:µMT mice (Fig. 6 E, right). The frequency of NIP-specific antibody-secreting cells (ASCs) was also similar in the spleens of the chimeras (Fig. 6 F), but twofold (day 42) to sevenfold (day 180) lower in the BM of the CD28$^{-/-}$:µMT mice (Fig. 6 G). The smaller number of BM PCs in the CD28$^{-/-}$:µMT could also be a result of defective B→LLPC differentiation or LLPC BM homing versus decreased in situ survival, so the rate of decline in PC numbers in the BM over time was determined. This would be unchanged by a generation/homing defect

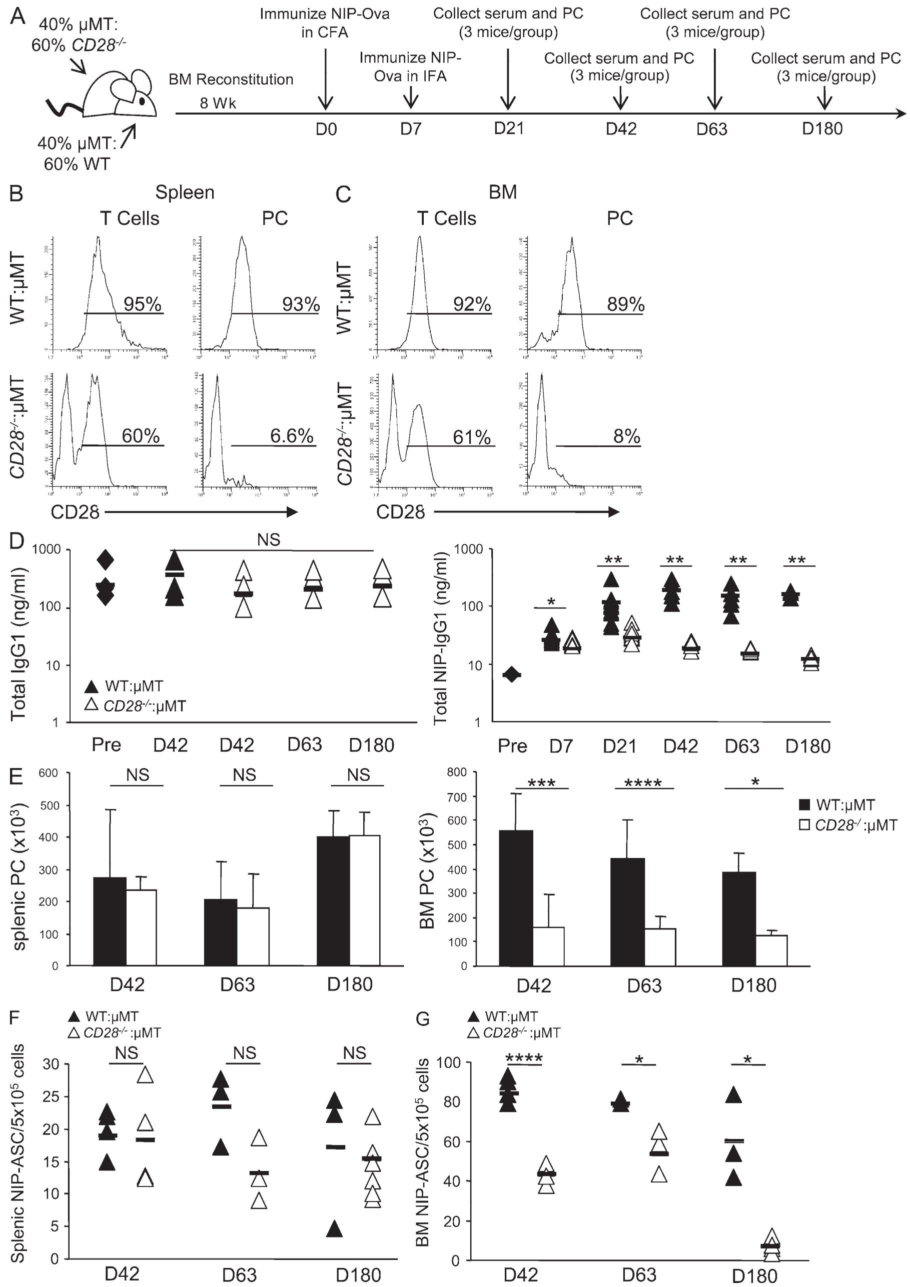

Figure 6. Loss of CD28 in the B lineage reduces long-lived antibody responses and long-lived PC numbers. (A) Experimental design. (B and C) Total splenic (B) or BM (C) mononuclear cells were analyzed in chimeras at time 0 before immunization for CD28$^+$CD3$^+$ T cells (left) and CD28$^+$CD138$^+$B220$^-$ PC (right) by multiparametric flow cytometry. (D) Total serum IgG1 and NIP-specific IgG1 was analyzed by ELISA at the time points indicated. Each point represents one mouse, with the mean indicated by black bars. (E) Total PC numbers in spleen (left) and BM (right) was determined from total mononuclear cells by multiparametric flow cytometry using CD138$^+$B220$^-$ to identify PC. Histograms are representative of six mice (day 180 WT, $n = 3$) per group. Error bars represent the mean ± SD. (F and G) Splenic (F) and BM (G) NIP-IgG ASC numbers were determined by ELISPOT. Each point represents the triplicate mean of one mouse. Mean is indicated by black bars. ns, not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

(fewer LLPC would get to the BM but, once there, would have normal survival) but accelerated by a survival defect. Consistent with the latter, the decline in the NIP-specific BM PC numbers was significantly faster in the CD28$^{-/-}$:μMT (slope = −0.29) with a half-life of 63 d versus WT:μMT chimeras (slope = −0.12; $P < 0.023$) with a half-life of 426 d, with no difference seen in rates of splenic PC decline.

These findings predict that if CD28-expressing LLPCs are interacting with CD80/CD86-expressing stromal niche DC, that loss of CD80 and/or CD86 expression will recapitulate the effect of losing PC CD28. Additionally, involvement of other CD80/CD86 binding receptors in addition to CD28 would be unmasked if the loss of the CD80 or CD86 was not equivalent to the loss of CD28. Because generating chimeric mice lacking CD80 or CD86 only in the myeloid compartment was not feasible, global CD80$^{-/-}$ or CD86$^{-/-}$ mice (and WT controls) were vaccinated and analyzed in the same fashion as for the CD28$^{-/-}$:μMT chimeras. Total serum IgG1 levels in the CD80$^{-/-}$ and CD86$^{-/-}$ mice were lower on average than in the WT mice, but this difference was not statistically significant (Fig. 7 A, left). However, NIP-specific antibody titers were significantly lower in CD80$^{-/-}$ and CD86$^{-/-}$ mice compared with WT at all time points, persisting out past 2 mo (Fig. 7 A, right). The total numbers of CD138$^+$B220$^-$ PC were comparable in the spleens of CD80$^{-/-}$, CD86$^{-/-}$, and WT mice (Fig. 7 B, left), but BM PCs were significantly decreased in CD80$^{-/-}$ and CD86$^{-/-}$ mice compared with WT (Fig. 7 B, right). Similarly, the frequency of NIP-specific ASC in the spleens of CD80$^{-/-}$, CD86$^{-/-}$, and WT mice were equivalent (Fig. 7 C) but significantly decreased over time in the BM of CD80$^{-/-}$ and CD86$^{-/-}$ mice compared with WT (Fig. 7 D). Altogether, the loss of CD80 or CD86 recapitulates the selective effect of CD28 loss on the BM PC population, supporting the model of an essential prosurvival interaction involving CD28 expressed on LLPC with CD80 and CD86 in the stromal niche.

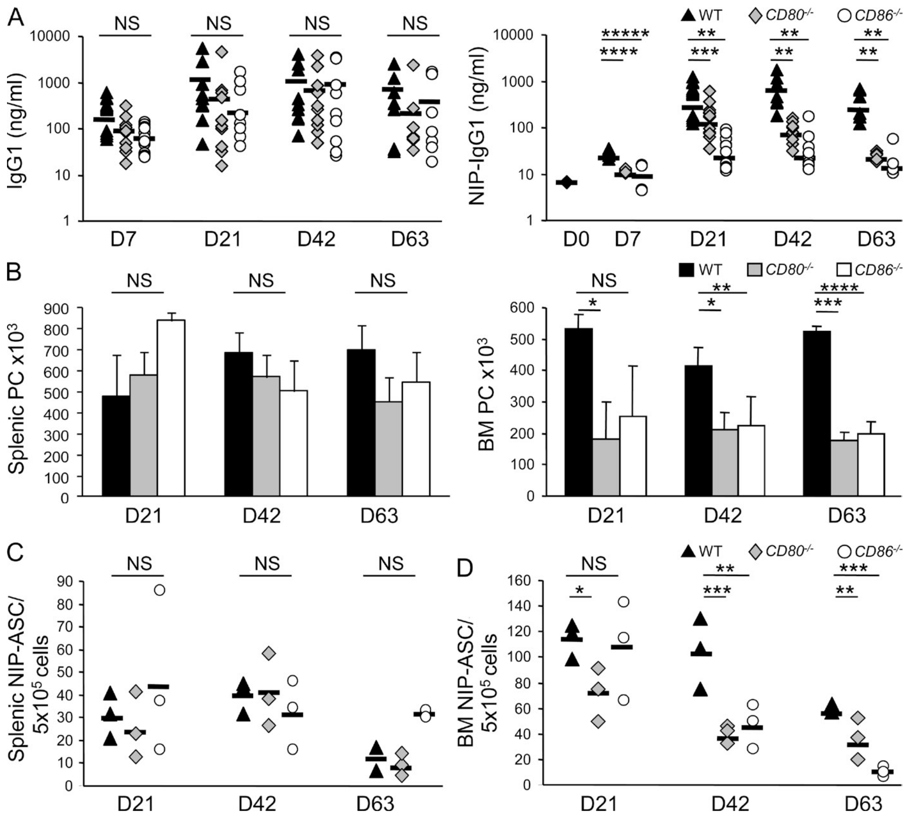

Figure 7. Loss of CD28 ligands CD80 or CD86 diminishes long-lived antibody responses and long-lived PC numbers. (A) Mice were vaccinated and total serum IgG1 and NIP-IgG1 was analyzed by ELISA at the time points indicated. Each point represents one mouse, with the mean indicated by black bars. (B) Total PC numbers in spleen (left) and BM (right) was determined from total mononuclear cells by multiparametric flow cytometry using $CD138^{+}B220^{-}$ to identify PC. Histograms are representative of three mice per group. Error bars represent the mean ± SD. (C and D) Splenic (C) and BM (D) NIP-IgG ASC numbers were determined by ELISPOT. Each point represents the triplicate mean of one mouse. Mean is indicated by black bars. ns, not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; *****, $P < 0.00001$.

DISCUSSION

Although long-lived antibody responses are a fundamental component of protective humoral immunity and are essential for effective vaccination, the molecular and cellular basis for such sustained immunoglobulin production (in particular in the absence of ongoing antigen exposure) remain poorly understood. Prolonged survival of a subset of PCs in the BM has been implicated as a key component of long-term humoral immunity; however, the intrinsic characteristics of these PCs (and if they even are a distinct subset), the basis of their longevity, and their actual contribution to durable antibody titers are not known. We have found that intrinsic CD28 function in PCs plays a previously unrecognized but essential role in maintaining long-lived antibody responses by selectively supporting the survival of BM PC. Furthermore, the intrinsic difference in CD28 signaling/function between short-lived splenic PC and long-lived BM PC is the first clear evidence (to our knowledge) that LLPC and SLPC are distinct subsets of PC. More importantly, the loss of long-term antibody titers with the selective loss of BM PC in the $CD28^{-/-}$, $CD80^{-/-}$, and $CD86^{-/-}$ mice is the first direct demonstration that BM LLPCs are necessary to sustain antigen-specific antibody levels. Altogether, our findings provide clear evidence that a distinct subset of BM-resident LLPC is necessary and sufficient to maintain long-term antibody levels, and they identify CD28 function in PCs as a central determinant of LLPC function and survival.

Although there has been extensive evidence that CD28 is required for the generation of antibody responses (e.g., Delogu et al., 2006), its involvement has been almost entirely attributed to helper T cell activation (e.g., Shahinian et al., 1993; Ferguson et al., 1996), even though any effect on PCs cannot be separately distinguished in these studies. The first clear indication of an intrinsic B cell role was only recently suggested by the finding that CD28 deficiency in B lineage blunted early (day 14 after vaccination) primary antibody responses even with adequate T cell help (Delogu et al., 2006), although the underlying mechanism (defects in B→PC differentiation, homing, PC survival, antibody production, or some other mechanism) and effect on durable antibody responses was not determined. We have found that CD28 on LLPC functions as a two-way molecular bridge, transducing a survival signal to the LLPC as well as back-signaling through CD80/CD86 to modulate stromal niche DC to support LLPC (and possibly SLPC) function via IL-6 production. This ability to transduce the prosurvival signal appears limited to LLPC and suggests that survival within the BM niches is restricted to PC that can signal through CD28. Thus, the molecular competency (Manz and Radbruch, 2002) of a PC to reside in a LLPC BM niche is in part set by its CD28 signaling threshold, with SLPC unable to use these niches because of a higher activation threshold more characteristic of T cells. This setpoint may be determined by the type of B cell being activated and/or the context in which the activation takes place. For example, memory B cell activation would be indicative of a recurring pathogen against which long-lived antibody titers would be beneficial, and a highly inflammatory setting caused by an acutely destructive pathogen for which persistent protective antibodies against reinfection would also be beneficial. This would be consistent with observations that repeated antigen exposure is necessary for most vaccines to elicit durable antibody titers, and that the inflammation elicited by specific vaccines correlates with the ability to generate long-lived humoral immunity (Pulendran, 2009). The molecular basis for where the CD28 activation threshold is set is unknown but is likely to be a key determinant of whether a newly differentiated PC is fated to become a LLPC or SLPC.

These findings also underscore that CD28 has several significantly different characteristics in LLPC compared with T cells. First, there appears to be no "co-" in the stimulation induced by CD28 in LLPC, and this signal alone is sufficient to support LLPC survival in the absence of other exogenous factors (i.e., in serum-free conditions). How CD28 activation supports LLPC survival is not clear as, unlike T cells, we have not identified a role for Bcl-x_L up-regulation in PCs. Ongoing studies suggest other antiapoptotic factors and enhanced metabolic fitness are playing a role. Another difference is the nonredundancy of CD80 and CD86 in maintaining the LLPC population compared with their relative redundancy in activating CD28 on T cells. Our data suggests that this nonredundancy is not the result of another CD80/CD86-binding receptor (CTLA-4 and PD-1), although more definitive studies are needed to be conclusive. Whether this nonredundancy is a result of some characteristic of CD28 signaling on the PC side or CD80/CD86 signaling on the stromal side is unclear and is currently being examined.

The requirement for CD80 and CD86 for LLPC survival and colocalization of BM PC and DC in vivo, as well as the ability of DC to support LLPC survival in vitro, strongly suggests that DCs (along with other myeloid professional antigen-presenting cells) are stromal components of the LLPC BM niche. The physical colocalization of LLPC with DC in the BM closely parallels the direct association of plasmablasts with DC and monocyte/macrophages in the lymph node, which induces the myeloid cells to generate the IL-6/April-rich microenvironment necessary for plasmablast survival and maturation (Mohr et al., 2009). Furthermore, the ability of CD28 to induce DC production of IL-6 via CD80/CD86 binding (which has been shown in DC–T cell interactions [Orabona et al., 2004] but not with PC) provides a molecular mechanism for how PCs induce the stromal microenvironment to produce this cytokine. Consistent with previous studies, we find that this IL-6 production is less important for LLPC survival when in contact with DC but is necessary for sustained antibody production (Martins et al., 2006; Radbruch et al., 2006), and it is possible that the decrease in IL-6 production within the BM microenvironment caused by loss of CD28 or CD80/86 results in a disproportionately greater drop in serum NIP-IgG1 levels compared with the loss of NIP-specific PC in vivo. It is interesting to speculate that regulation of Ig production is via CD28-mediated induction of high level BLIMP-1 expression that is needed for immunoglobulin production (Shapiro-Shelef and Calame, 2005) and is characteristic of LLPC after they enter the BM niches (Kallies et al., 2004). Of note, BLIMP-1 expression in T cells is increased by CD28 co-stimulation (Martins et al., 2006), which suggests another pathway by which CD28 may modulate LLPC function.

Finally, an intrinsic role for CD28 in LLPC survival/function suggests that therapeutically targeting this receptor may be directly effective in manipulating humoral immunity in human health and disease. In the context of vaccine development, strategies to augment CD28 signaling (for example, traditional, i.e., not super-agonist) anti-CD28 antibodies that trigger signaling in PC but not T cells may lead to greater LLPC survival and higher/more persistent antibody titers. Conversely, inhibition of CD28 signaling may compromise the survival of pathogenic LLPCs that are still dependent on the BM niche for survival. These include the malignant LLPC in multiple myeloma and autoreactive LLPC in many autoimmune syndromes and organ graft rejection. In this regard, it is relevant to note that agents that enhance or block CD28-mediated T cell co-stimulation are already in clinical use (e.g., CTLA4-Ig [abatacept] for the treatment of rheumatoid arthritis) and may have new (or perhaps newly recognized) application in normal and pathogenic humoral immunity.

MATERIALS AND METHODS

Animals. Female and male C57BL/6J (WT), B6.129S2-Igh-6^{tm1Cgn}/J (μMT), B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (SJL), and B6.129S2-Cd28^{tm1Mak}/J (CD28$^{-/-}$) mice were purchased from The Jackson Laboratory at 5–6 wk of age. Female C57BL/6J retired breeders at ~9 mo old were purchased from The Jackson Laboratory. Upon receipt, animals were housed and bred at the Division of Laboratory Animal Resources (Roswell Park Cancer Institute [RPCI], Buffalo, NY) in a pathogen-free barrier facility. All animal experiments were approved by the RPCI Institutional Animal Care and User Committee.

Antibodies and flow cytometry. Antibodies for NF-κB p50 (clone NLS) and p65 (clone F-6) were purchased from Santa Cruz Biotechnology, Inc. Imiquimod was purchased from Sigma Aldrich. Anti-CD80 mAb (clone 16.10.A1) and anti-CD86 (clone GL-1) were generated from hybridomas. Cells were stained with anti-CD45.1 (clone A20), anti-CD45.2 (clone 104), anti–B220-PE/Cy7 (clone RA3-6B2), anti–I-A/I-E-PerCP/Cy5.5 (clone M5/114.15.2), anti-CD19 (clone 6D5), and anti–CD3-PE (clone 17A2; BioLegend); anti–hamster IgG (H + L)–FITC and isotype control rat IgG2a-PE (Beckman Coulter); anti-CD28 (clone PV1; Beckman Coulter; gift from C. June and B. Levine, University of Pennsylvania, Philadelphia, PA); anti–CD138-PE (clone 281–2; BD); anti–mouse IL-6R (R&D Systems); and anti–goat IgG-FITC (United States Biochemical Corporation). Polyclonal control hamster-IgG was purchased from Genetex, Inc. Polyclonal control hamster IgG and anti-CD28 mAb were conjugated to Dynabeads goat anti–mouse IgG (Invitrogen) per the manufacturer's instructions and were cultured with cells at a 2:1 bead to cell ratio, respectively. Cells were incubated with staining reagents in staining media (PBS-1% FCS, 5 mM Hepes, and 5 mM 10% sodium azide) for 30 min in 4°C. Analysis was performed by flow cytometry (LSR II and FACScan 2; BD).

PC isolation. PCs from WT and CD28$^{-/-}$ mice were isolated using a MACS (Miltenyi Biotec) CD138^{+} PC isolation kit. Cells were labeled with non-PC depletion cocktail and anti-biotin microbeads for non-PC depletion. Cells were then labeled with CD138 microbeads and run over the magnetic column twice to remove any CD138^{-} cells (Minges Wols and Witte, 2008). The purity of the CD138^{+} population was >83%.

CellVue labeling and cell cultures. BMDCs were generated from BM of WT, B6.129S4-Cd80^{tm1Shr}/J (CD80$^{-/-}$), B6.129S4-Cd86^{tm1Shr}/J (CD86$^{-/-}$), B6.129S4-Cd80^{tm1Shr} Cd86^{tm2Shr}/J (CD80/86$^{-/-}$), and B6.129S2-Il6^{tm1Kopf}/J (IL-6$^{-/-}$) mice (gift from A. Grakoui and H. Scarborough, Emory University, Atlanta, GA). BM cells were differentiated in culture with 20 ng/ml GM-CSF (derived from supernatant; gift from J.L. Clements, RPCI) for 7 d

(Luckashenak et al., 2006). BMDCs were stained with CellVue Claret Fluorescent Cell Linker (Molecular Targeting Technologies, Inc.) per the manufacturer's instructions to distinguish BMDC from PC by FACS. 2 or 2.5 × 10^4 PCs with or without 2 × 10^5 BMDCs were cultured in a 48-well flat-bottom tissue culture plate in 0.8 ml of culture medium/well at 37°C with 5% CO_2 with 20 ng/ml GM-CSF for 30 d (Minges Wols et al., 2002). Serum starvation assays were completed as described in Bahlis et al. (2007).

BM reconstitution and immunizations. BM chimeras were generated as previously described (Delogu et al., 2006). In brief, the chimeras were generated by retroorbitally injecting 10^6 BM cells, depleted of T cells (Miltenyi Biotec), at a 1:1 ratio of either SJL and WT or SJL and $CD28^{-/-}$ BM for the competitive repopulation studies into lethally irradiated SJL mice. BM chimeras were generated as described at a 40:60 ratio of μMT and WT or μMT and $CD28^{-/-}$ BM into lethally irradiated WT mice. Mice were immunized subcutaneously with 1:1 ratio of 100 μg NIP-ovalbumin (Biosearch Tech) in complete Freund's adjuvant (Thermo Fisher Scientific) on day 0 and boosted on day 7 with a 1:1 ratio of 100 μg NIP-ovalbumin in incomplete Freund's adjuvant (Thermo Fisher Scientific).

ELISAs. Murine IgM, IgG, IgA, IgG1, and NIP-IgG1 Ab titers were determined by ELISA per the manufacturer's instructions (Bethyl Laboratories, Inc.). In brief, NUNC 96-well plates were precoated with capture antibody in coating buffer or NIP–bovine serum albumin in 15 μg/ml PBS–0.2 M NaCl (Biosearch Technologies) overnight in 4°C. Murine IL-6 was assayed by ELISA per manufacturer's instructions (R&D Systems).

ELISPOT assays. NIP-specific and total IgG ASC were quantified by ELISpot assay as per manufacturer's instructions (Mabtech). In brief, 96-well plates (Millipore) were precoated with 15 μg/ml anti-IgG capture antibody or 20 μg/ml NIP–bovine serum albumin in PBS.

Electromobility shift assay (EMSA). EMSAs were done for NF-κB as previously described (Bahlis et al., 2007). In brief, purified BM and splenic PC were cultured with or without polyclonal control hamster Ig or anti-CD28 mAb beads in 10% FCS media for 30 min. Whole lysates were made, and equal amounts of protein were incubated with ^{32}P-labeled primer containing consensus NF-κB–binding sites (5′-GATCCAACG-GCAGGGGAATTCCCCTCTCCTTA-3′) and separated on a 4% polyacrylamide gel.

Immunohistochemical staining and confocal microscopy. For section staining, samples were fixed in 10% neutral buffered formalin. Sternum and femurs were sectioned at 5 μm. For antigen retrieval, slides were heated in the microwave for 20 min in citrate buffer, pH 6.0, followed by a 15-min cool down and a PBS/Tween wash. Slides were then loaded on the DAKO autostainer and the following program was run: casein 0.03% (in PBS/T) was used to block for 30 min, blown off, and fascin (neomarkers), primary rat monoclonal antibody CD138 (clone 281–2; BD), or rat IgG2a (isotype control) was on slides for 1 h. A PBS/Tween wash was followed by labeled Polymer (Dako envision+) for 30 min on fascin slides and biotinylated rabbit anti–rat IgG for 30 min on CD138/rat IgG2a slides, another PBS/Tween wash, and Elite ABC kit (VECTASTAIN; Vector Laboratories) for 30 min. Slides were again washed with PBS/Tween, the DAB chromagen (Dako) was applied for fascin (brown), and Fast-red was applied for CD138 (pink) for 5 min. The slides were dehydrated, cleared, and cover-slipped. An entire section of sternum and femur were used to quantify $CD138^+$ cells adjacent to $fascin^+$ BMSC. CD138 positively stained cells alone or adjacent to $fascin^+$ BMSC were counted. Percentage of adjacent $CD138^+$ cells to $fascin^+$ BMSC was determined by the number of $CD138^+$ cells adjacent to $fascin^+$ BMSC cells/total number of $CD138^+$ cells.

Intact BM cores were removed from femurs of WT mice and embedded in OCT, snap-frozen in liquid nitrogen, and stored in −80°C. 9-μm tissue sections were prepared and fixed with acetone at −16°C for 20 min. Nonspecific binding was blocked by preincubation with PBS + Tween/Casein for 30 min. Staining with antibodies and secondary reagents was performed for 60 and 30 min, respectively, at room temperature. The following antibodies were used: anti–hamster IgG-PE (clone HTK888; BioLegend), anti–CD80-PE (clone 16-10AI; BioLegend), anti-CD138 (clone 281–2; BD), and anti–rat IgG-FITC (eBioscience). Sections were analyzed by a confocal system microscope (DM IRE2 and TCS SP2; Leica) with software (version 2.61). Four BM sections were used to quantify $CD138^+$ cells adjacent to $CD80^+$ BMSC. CD138 positively stained cells alone or adjacent to $CD80^+$ BMSC were counted. Percentage of adjacent $CD138^+$ cells to $CD80^+$ BMSC cells was determined by the number of $CD138^+$ cells adjacent to $CD80^+$ BMSC cells/total number of $CD138^+$ cells.

NF-κB luciferase assay. BALB/c-Tg(IκBα-luc)-Xen (IκBα-luc) mice (gift from I. Gitlin and A.V. Gudkov, RPCI) were used in the NF-κB luciferase assay. NF-κB activity was assayed using Dual Luciferase Reporter Assay (Promega) per the manufacturer's instructions. In brief, purified BM and splenic PC were cultured in 10% FCS media alone or with polyclonal hamster Ig or anti-CD28 mAb beads for 1 h. Cells were lysed in 1× passive lysis buffer (PLB), and the PLB lysate was then resuspended in LARII buffer and firefly luciferase activity was measured by a luminometer (Monolight 3010; BD). Relative NF-κB activity was determined as follows: (luciferase activity)/(cell number for each sample/volume of lysis buffer) × (μl of LARII added to sample).

Statistical analysis. A Student's *t* test was performed for statistical analysis using two-tailed nonequal variances and 95% CI. For comparison of NIP-specific ASC of WT:μMT versus $CD28^{-/-}$:μMT in spleen and BM linear regression, analysis was performed for each mouse to give a single estimated slope value. Rate of decay was performed by ANOVA and the following equation was used to determine half-life: (elapsed time × log2)/[log(beginning amount/ending amount)].

Online supplemental material. BM and splenic PC were characterized by CD138 and CD19 expression by flow cytometry prior and after CD138 purification. Supernatant from PC were analyzed for IgM and IgG production by ELISA (Fig. S1). Splenic PC survival was assessed by co-culture studies with BMDCs (Fig. S2). Induction of Ig from $CD28^{-/-}$ BM PC was assessed with the addition of recombinant IL-6 (Fig. S3). BM PC survival was assessed with the addition of recombinant IL-6 (Fig. S4). Serum from chimeras was analyzed for IgA and NIP-specific IgA or IgM and NIP-specific IgM by ELISA (Fig. S5). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110040/DC1.

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C.H. Rozanski, R. Arens, and L.M. Carlson designed and performed experiments. C.H. Rozanski and K.P. Lee analyzed data and prepared the manuscript. S.P. Schoenberger helped design experimental strategy and provided critical reagents. J. Nair, L.H. Boise, A.A. Chanan-Khan, and S.P. Schoenberger helped analyze the data and provided significant input to the manuscript.

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