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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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). 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.

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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 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 vivo is PC intrinsic

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 10⁵ 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
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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 IκBκ 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 CD28 activation enhanced 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 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 chimera. 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.
The ability of DC to support PC survival was examined in vitro in co-cultures of purified WT BM PC + WT BMDC and demonstrated sevenfold 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 (see Fig. 5).

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. 

**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 sevenfold more viable PC compared with medium alone conditions out to 30 d of culture (Fig. 4 C). However,
**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 pro-inflammatory 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 pro-survival 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 (Tuallon, 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 pre-vaccination 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...
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<sup>−/−</sup> or CD86<sup>−/−</sup> mice (and WT controls) were vaccinated and analyzed in the same fashion as for the CD28<sup>−/−</sup>:μMT chimeras. Total serum IgG1 levels in the CD80<sup>−/−</sup> and CD86<sup>−/−</sup> 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<sup>−/−</sup> and CD86<sup>−/−</sup> mice compared with WT at all time points, persisting out past 2 mo (Fig. 7 A, right). The total numbers of CD138<sup>+</sup>B220<sup>−</sup>PC were comparable in the spleens of CD80<sup>−/−</sup>, CD86<sup>−/−</sup>, and WT mice (Fig. 7 B, left), but BM PCs were significantly decreased in CD80<sup>−/−</sup> and CD86<sup>−/−</sup> mice compared with WT (Fig. 7 B, right). Similarly, the frequency of NIP-specific ASC in the spleens of CD80<sup>−/−</sup>, CD86<sup>−/−</sup>, and WT mice were equivalent (Fig. 7 C) but significantly decreased over time in the BM of CD80<sup>−/−</sup> and CD86<sup>−/−</sup> 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 6. Loss of CD28 in the B lineage reduces long-lived antibody responses and long-lived PC numbers.](https://example.com/figure6.png)
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 responses, 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 Bel-2 up-regulation in PCs. Ongoing studies suggest other anti-apoptotic 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 in induce 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), B6.129S-Igh-6–tm1Cgn/J (mMT), B6.SJL-Pippc– Pepc–/BoyJ (SIL), and B6.129S2-Cd86–tm1Shr/J (CD86–/–) 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 purchased from BioLegend; anti-CD28 (clone 4A9) was purchased from Genetex, Inc. 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 anti-CD80 mAb (clone 4A9), anti-IgF/Cy7 (clone RA3-6B2), anti-I-A/I-E-PerCP/Cy5.5 (clone M5/114.15.2), anti-CD19 (clone 6D8), and anti–CD3–PE (clone 17A2; BioLegend); anti–hammer 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 Heps, and 5 mM 10% sodium azide) for 30 min in 4°C. Analysis was performed by flow cytometry (LSK II and FACScan 2; BD).

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–tm1Shr/J (CD80/CD86–/–), and B6.129S2–Igh-6–tm1Cgn/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

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BRM reconstitution and immunizations. BM chimeras were generated as previously described (Delogu et al., 2006). In brief, the chimeras were generated by retrovirally inject 10^7 BM cells, depleted of T cells (Miltenyi Biotec), at a 1:1 ratio of either SJL or 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 (Biosource Tech) in complete Freund's adjuvant (Thermo Fisher Scientific) on day 0 and boosted subcutaneously with 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 to coating buffer or NIP-bovine serum albumin in 15 µg/ml PBS-0.2 M NaCl (Biosource 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 (Bablis 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 32P-labeled primer containing consensus NF-κB-binding sites (5'-GATCCAAAAG-GCAAGGGGAATTCTCCCTCTCTTTA-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 for 5 min. The slides were dehydrated, cleared, and cover-slipped. An entire slide was mounted 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 in incomplete Freund's adjuvant (Thermo Fisher Scientific).

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^-/- BMPC 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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High-sensitive immunophenotyping and DNA ploidy studies for the investigation of minimal residual disease in multiple myeloma. Br. J. Haematol. 107:121–131. doi:10.1046/j.1365-2141.1999.01685.x

Amanj, I.J., and M.K. Slifka. 2010. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. Immunol. Rev. 236:125–138. doi:10.1111/j.1600-065X.2010.00912.x

Amanj, I.J., N.E. Carlson, and M.K. Slifka. 2007. Duration of humoral immunity to common viral and vaccine antigens. N. Engl. J. Med. 357:1903–1915. doi:10.1056/NEJMoa0606902

Bahls, N.J., A.M. King, D. Kolonias, L.M. Carlson, H.Y. Liu, M.A. Hussain, H.R. Terebelo, G.E. Byrne Jr., B.L. Levine, L.H. Bove, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.

Bogen, B. 2002. A mouse model for immunotherapy of myeloma. Hematol. J. 3:224–229. doi:10.1038/sj.hj.6200183

Bowe, L.H., A.J. Mann, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, L.H. Boise, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.

Bogen, B. 2002. A mouse model for immunotherapy of myeloma. Hematol. J. 3:224–229. doi:10.1038/sj.hj.6200183

Bowe, L.H., A.J. Mann, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, L.H. Boise, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.

Bogen, B. 2002. A mouse model for immunotherapy of myeloma. Hematol. J. 3:224–229. doi:10.1038/sj.hj.6200183

Bowe, L.H., A.J. Mann, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, L.H. Boise, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.

Bogen, B. 2002. A mouse model for immunotherapy of myeloma. Hematol. J. 3:224–229. doi:10.1038/sj.hj.6200183

Bowe, L.H., A.J. Mann, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, L.H. Boise, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.

Bogen, B. 2002. A mouse model for immunotherapy of myeloma. Hematol. J. 3:224–229. doi:10.1038/sj.hj.6200183

Bowe, L.H., A.J. Mann, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, L.H. Boise, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.

Bogen, B. 2002. A mouse model for immunotherapy of myeloma. Hematol. J. 3:224–229. doi:10.1038/sj.hj.6200183

Bowe, L.H., A.J. Mann, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, L.H. Boise, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.

Bogen, B. 2002. A mouse model for immunotherapy of myeloma. Hematol. J. 3:224–229. doi:10.1038/sj.hj.6200183

Bowe, L.H., A.J. Mann, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, L.H. Boise, and K.P. Lee. 2007. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 109:5002–5010. doi:10.1182/blood-2006-03-015242

Benson, M.J., S.R. Dillon, E. Castigi, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle. 2008. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J. Immunol. 180:3655–3659.
Shaffer, A.L., K.I. Lin, T.C. Kuo, X. Yu, E.M. Hurt, A. Rosenwald, J.M. Giltnane, L. Yang, H. Zhao, K. Calame, and L.M. Staudt. 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17:51–62. doi:10.1016/S1074-7613(02)00335-7

Shahinian, A., K. Pfeffer, K.P. Lee, T.M. Kündig, K. Kishihara, A. Wakeham, K. Kawai, P.S. Ohashi, C.B. Thompson, and T.W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609–612. doi:10.1126/science.7688139

Shapiro, V.S., K.E. Truitt, J.B. Imboden, and A. Weiss. 1997. CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites. *Mol. Cell. Biol.* 17:4051–4058.

Shapiro, V.S., M.N. Mollenauer, and A. Weiss. 2001. Endogenous CD28 expressed on myeloma cells up-regulates interleukin-8 production: implications for multiple myeloma progression. *Blood* 98:187–193. doi:10.1182/blood.V98.1.187

Shapiro-Shelef, M., and K. Calame. 2005. Regulation of plasma-cell development. *Nat. Rev. Immunol.* 5:116–126. doi:10.1038/nri1440

Shapiro-Shelef, M., K.I. Lin, L.J. McHeyzer-Williams, J. Liao, M.G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 19:607–620. doi:10.1016/S1074-7613(03)00267-X

Shapiro-Shelef, M., K.I. Lin, D. Savitsky, J. Liao, and K. Calame. 2005. Blimp-1 is required for maintenance of long-lived plasma cells in the bone marrow. *J. Exp. Med.* 202:1471–1476. doi:10.1084/jem.20051611

Shapeer, A., and J.G. Freeman. 2002. The B7-CD28 superfamily. *Nat. Rev. Immunol.* 2:116–126. doi:10.1038/nri7272

Shitaka, M.K., R. Antia, J.K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. *Immunity* 8:363–372. doi:10.1016/S1074-7613(00)80541-5

Stein, P.H., J.D. Fraser, and A. Weiss. 1994. The cytoplasmic domain of CD28 is both necessary and sufficient for costimulation of interleukin-2 secretion and association with phosphatidylinositol 3-kinase. *Mol. Cell. Biol.* 14:3392–3402.

Tangye, S.G., and D.M. Tarlinton. 2009. Memory B cells: effectors of long-lived immune responses. * Eur. J. Immunol.* 39:2065–2075. doi:10.1002/eji.2009394531

Thompson, C.B., T. Lindsten, J.A. Ledbetter, S.L. Kinkel, H.A. Young, S.G. Emerson, J.M. Leiden, and C.H. June. 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. USA* 86:1333–1337. doi:10.1073/pnas.86.4.1333

Tokoyoda, K., T. Egawa, T. Sugiyama, B.I. Choi, and T. Nagasawa. 2004. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* 20:707–718. doi:10.1016/j.immuni.2004.05.001

Tu, Y., A. Gardner, and A. Lichtenstein. 2000. The phosphatidylinositol 3-kinase/AKT kinase pathway in multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. *Cancer Res.* 60:6763–6770.

Tuaillon, N. 2000. Repertoire analysis in human immunoglobulin heavy chain minilocus transgenic, muMT/muMT mice. *Mol. Immunol.* 37:221–231. doi:10.1016/S0161-5890(00)00044-4

Winter, O., K. Moser, E. Mohr, D. Zotos, H. Kaminski, M. Szyksa, K. Roth, D.M. Wong, C. Dame, D.M. Tarlinton, et al. 2010. Megakaryocytes constitute a functional component of a plasma cell niche in the bone marrow. *Blood* 116:1867–1875. doi:10.1182/blood-2009-12-259457

Xiang, Z., A.J. Cutler, R.J. Brownlie, K. Fairfax, K.E. Lawlor, E. Severinson, E.U. Walker, R.A. Manz, D.M. Tarlinton, and K.G. Smith. 2007. FcgammaRIIB controls bone marrow plasma cell persistence and apoptosis. *Nat. Immunol.* 8:419–429. doi:10.1038/ni1440

Zhan, F., B. Barlogie, V. Arzoumanian, Y. Huang, D.R. Williams, K. Hollering, M. Pineda-Roman, G. Tricot, F. van Rhee, M. Zangari, et al. 2007. Gene-expression signature of benign monoclonal gammopathy evident in multiple myeloma is linked to good prognosis. *Blood* 109:1692–1700. doi:10.1182/blood-2006-07-037077

Zhang, N., M.H. Ahsan, L. Zhu, L.C. Sambucetti, A.F. Purchio, and D.B. West. 2005. Regulation of IkappaBalpha expression involves both NF-kappaB and the MAP kinase signaling pathways. *J. Inflamm. (Lond.)* 2:10. doi:10.1186/1476-9255-2-10
Figure S1. Characterization of purified BM and splenic PCs. (A and B) Total unpurified BM cells and splenocytes (A) and purified BM and splenic PCs (B) were analyzed for CD19 and CD138 expression by flow cytometry. (C) $10^5$ purified BM and splenic PCs were cultured in 96-well plate for 24 h, and supernatant was collected and analyzed for IgM and IgG production by ELISA. Data are one representative of three independent experiments. Mean ± SD is shown.

Figure S2. Long-term splenic PC survival is not supported by BMDCs. $2.5 \times 10^4$ purified splenic PCs were cultured with or without $2 \times 10^5$ BMDC. Total viable PC numbers were determined by 7AAD incorporation assessed by flow cytometry. Data are one representative of three independent experiments.
Figure S3. IL-6 induces immunoglobulin production from CD28−/− PCs. 2 x 10^4 purified CD28−/− BM PCs were cultured with or without 1 ng/ml rIL-6. Supernatant was analyzed for total IgG production by ELISA. Mean ± SD is shown. Data are one representative of three independent experiments. **, P < 0.01.

Figure S4. Exogenous IL-6 supports BM PC survival. rIL-6 was added to purified BM PC in media alone, with viable PC numbers. Data are presented as the mean ± SD and are representative of two independent experiments.
Figure S5. Maintenance of IgM and IgA immunoglobulin titers is not CD28 dependent. Serum was collected from the chimeras detailed in the figure and analyzed for total and NIP-IgA (A) or IgM (B) by ELISA. Each point represents one mouse. Mean is indicated by black bars.