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PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells

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Memory B and plasma cells (PCs) are generated in the germinal center (GC). Because follicular helper T cells (T_{FH} cells) have high expression of the immunoinhibitory receptor PD-1, we investigated the role of PD-1 signaling in the humoral response. We found that the PD-1 ligands PD-L1 and PD-L2 were upregulated on GC B cells. Mice deficient in PD-L2 (*Pdcd1lg2*$^{-/-}$), PD-L1 and PD-L2 (*Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$) or PD-1 (*Pdcd1*$^{-/-}$) had fewer long-lived PCs. The mechanism involved more GC cell death and less T_{FH} cell cytokine production in the absence of PD-1; the effect was selective, as remaining PCs had greater affinity for antigen. PD-1 expression on T cells and PD-L2 expression on B cells controlled T_{FH} cell and PC numbers. Thus, PD-1 regulates selection and survival in the GC, affecting the quantity and quality of long-lived PCs.

Adaptive immune responses generate long-lived, antigen-experienced cells that can protect the host during subsequent infections. This immune system memory consists of cells derived from both activated B cells and activated T cells. Various intrinsic and extrinsic signals that control the survival, differentiation, function and maintenance of T cell subsets have been elucidated[1]. In contrast to that extensive body of work, the signals that regulate the formation of long-lived plasma cells (PCs) and memory B cells remain largely unknown.

Long-lived PCs that secrete antibodies with high affinity for antigen, as well as memory B cells, are generated mainly during the germinal center (GC) response[2]. GCs are organized sites of proliferating cells in which antigen-specific B cells undergo extensive multiplication and somatic hypermutation[3]. During multiple rounds of division, high-affinity GC cells are selected and differentiate into either memory cells or PCs[4–6]. Dysregulation of proliferation, mutation and differentiation in GCs can lead to detrimental outcomes, including tumorigenesis[7], autoimmunity[8] and immunodeficiency[9]. Therefore, the regulation of survival, proliferation and differentiation signals in this dynamic environment is important for determining both the quality and size of PC and memory cell populations. Accordingly, many genes identified as being upregulated in memory cells compared with their expression in naive B cells were first upregulated in GC cells[10–12]. We hypothesized that these genes might be important for regulating the GC–to–memory cell transition and thus focused on them for further analysis. Comparison of gene expression in memory and naive B cells showed upregulation of the B7 (CD28 ligand) family member PD-L2 (B7-DC) in memory cells[11]. Further analysis demonstrated that PD-L1 (CD274 or B7-H1) and PD-L2 and their receptor, PD-1 (refs. 13,14), are also upregulated on B cells in the GC. Thus, this set of important immune regulators is being modulated in concert during GC and memory cell development, but their functions had yet to be explored.

PD-1 signaling has a major role in inhibiting T cell responses[13]. It is induced after T cell activation, and much higher expression of PD-1 is associated with exhausted memory T cells in chronic viral infection[15]. Although PD-1 has only two known ligands, PD-L1 and PD-L2, it has been shown that PD-L1 and CD80 interact to transduce bidirectional signals[16]. These multiple interactions most probably evolved to allow the immune system to constantly adapt, fine tune and eventually down-regulate the response. PD-L1 is expressed on many cells, including activated B cells, whereas PD-L2 was originally thought to be expressed only on DCs and macrophages[13,14,17] and has subsequently been shown to be expressed on B-1 cells[18]. Accordingly, research on these ligands has focused on DC–T cell interactions, with little known about their roles in B cells. Notably, T cells in the follicle have prominent expression of PD-1 (ref. 19). Follicular helper T cells (T_{FH} cells), along with T cells at the T cell–B cell border, are a source of extrinsic factors that promote GC formation, expansion and isotype switching[20–22]. It has been suggested that PD-1 is increased during viral infection to inhibit chronically activated T cells from causing immunopathology or becoming autoreactive[23,24]. Similarly, during humoral responses, regulation of proliferating GC cells is required for the production of high-affinity effector cells without causing the expansion of autoreactive or low-affinity clones. Yet, although PD-1 and its ligands have been investigated well in T cell responses, their role in regulating GC responses and the consequent output of long-lived B cells has not yet been explored.

Here we used a combination of mutant mice and cell-transfer approaches to test the hypothesis that B cells expressing PD-L1 and/or PD-L2 interact with PD-1$^+$ T_{FH} cells in the GC to regulate the

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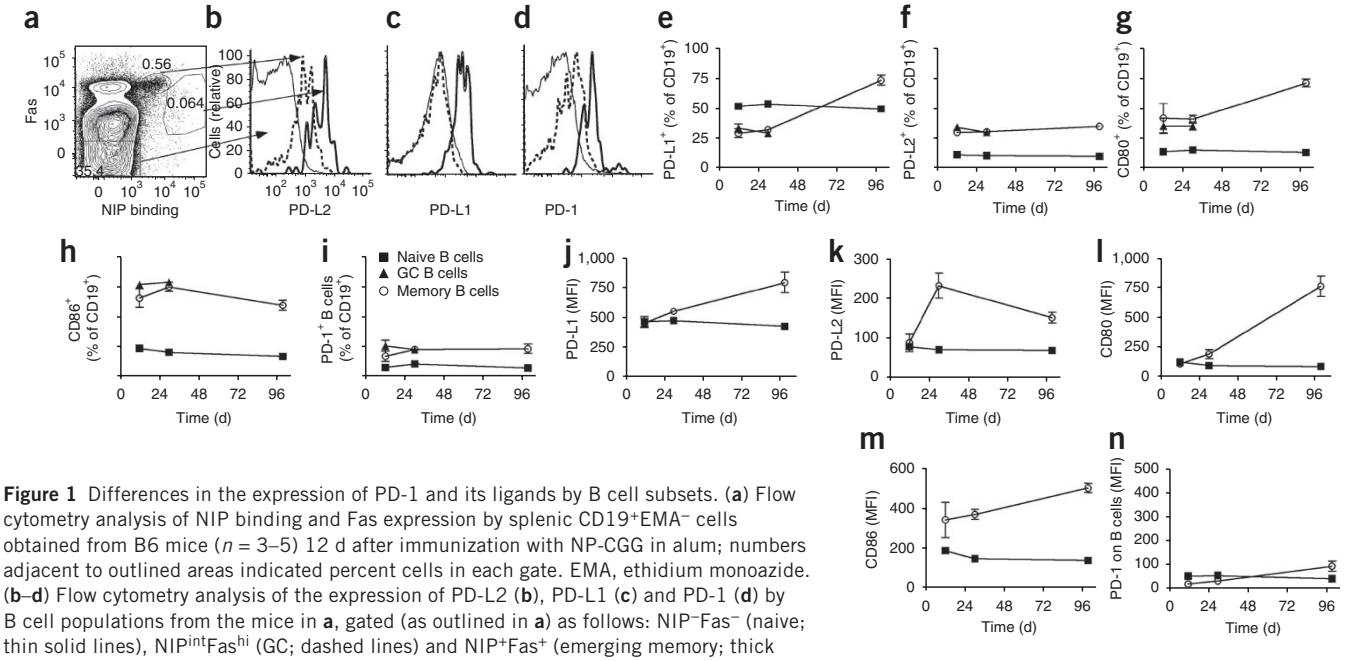


Figure 1 Differences in the expression of PD-1 and its ligands by B cell subsets. (**a**) Flow cytometry analysis of NIP binding and Fas expression by splenic CD19$^+$EMA$^-$ cells obtained from B6 mice (n = 3–5) 12 d after immunization with NP-CGG in alum; numbers adjacent to outlined areas indicated percent cells in each gate. EMA, ethidium monoazide. (**b**–**d**) Flow cytometry analysis of the expression of PD-L2 (**b**), PD-L1 (**c**) and PD-1 (**d**) by B cell populations from the mice in **a**, gated (as outlined in **a**) as follows: NIP$^-$Fas$^-$ (naive; thin solid lines), NIP^{int}Fas^{hi} (GC; dashed lines) and NIP$^+$Fas$^+$ (emerging memory; thick solid lines). (**e**–**n**) Frequency (**e**–**i**) and kinetics (**j**–**n**) of the expression of B7 family members and PD-1 on B cell subsets at 12 d and 4 and 14 weeks after immunization as described in **a**. The CD19$^+$NIP$^+$IgG1$^+$CD38$^+$κ^{lo} phenotype (κ, immunoglobulin κ-chain) was used to identify memory B cells 14 weeks after immunization because of the very low frequency of detectable cells. Data are representative of at least three independent experiments (mean ± s.e.m. in **e**–**n**).

formation of long-lived B cells. Indeed, we found that in the absence of PD ligands or PD-1, the formation of long-lived PCs was considerably diminished, although neither molecule was absolutely required for this. We traced the decrease in PCs back to the late stages of the GC, in which PD-1 signals substantially influence the rate of GC cell death, a major determinant of GC outcomes such as affinity maturation, selection and differentiation[25]. The PCs that did form in the absence of PD-1 signaling were of a higher affinity than were control cells, which further suggests that PD-1 has a role in selection during the GC response. This result was linked to lower production of interleukin 4 (IL-4) and IL-21 mRNA by T_{FH} cells in the absence of PD-1. Finally, given the identification of the PD-L1–CD80 pathway[16], it was important to investigate the cell-intrinsic requirements of PD-1 and PD-L2. Experiments with cell transfer and mixed–bone marrow chimeras demonstrated that the expression of PD-L1 and PD-L2 on B cells and the expression of PD-1 on T cells determined PC numbers. Therefore, PD-1 controls the development and function of T_{FH} cells, which most probably in turn controls the optimal formation of long-lived PCs by regulating the survival and selection of B cells in the mature GC.

RESULTS

Expression of PD-L1 and PD-L2 on GC and memory cells

Mouse memory B cells upregulate PD-L2 mRNA more than 25-fold compared with naive B cells[11]. We further assessed the expression of PD-L2, as well as that of PD-L1 and PD-1, on B cell subsets during a primary response in C57BL/6 (B6) wild-type mice (**Fig. 1a–d**). Naive cells expressed PD-L1 (**Fig. 1c**) but did not express PD-L2 (**Fig. 1b**) or PD-1 (**Fig. 1d**). GC cells had intermediate expression of all three molecules, although their expression of PD-L1 was similar to that of naive B cells. Emerging memory B cells[26] (so called because they express surface markers similar to those of memory cells 3 months after immunization) expressed more PD-L1, PD-L2 and PD-1 than did naive or GC cells, similar to long-lived memory B cells and consistent with microarray and quantitative PCR data obtained with memory cells at 12 weeks[11].

Expression of PD-1 on T cells is modulated during an immune response[23]. To determine whether expression of B7 family receptors and ligands is also modulated on B cells over the course of a primary response, we immunized wild-type mice with NP-CGG (4-hydroxy-3-nitrophenyl)acetyl conjugated to chicken-γ-globulin and evaluated B cell subsets during the middle phase (day 12) and late phase (week 4) of the GC response, as well as at a memory time point (week 14). Initially we assessed the percentage of each B cell type positive for the selected marker (**Fig. 1e–i**). The frequency of cells positive for PD-L1 and CD80 increased over time for memory B cells (**Fig. 1e**,**g**), whereas the frequency of positive cells remained unchanged for other molecules tested. We also measured the mean fluorescence intensity of these molecules over time (**Fig. 1j–n**). The intensity of expression on memory B cells generally increased over time; however, in the case of PD-L2, expression peaked at 4 weeks after immunization, although it remained higher than that of naive cells 3 months after immunization, consistent with microarray data[11].

Because PD-1 and its ligands were all expressed by GC B cells, we investigated their role in the formation of GCs at day 12. We examined mice deficient in PD-L2 (which abrogated PD-L2–PD-1 interactions; *Pdcd1lg2*$^{-/-}$ mice) or PD-1 (which abrogated the interactions of PD-1 with both PD-L1 and PD-L2; *Pdcd1*$^{-/-}$ mice) or both PD-L1 and PD-L2 (which abrogated interactions with both PD-1 and CD80; *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice). The size of GCs in *Pdcd1lg2*$^{-/-}$, *Cd274*$^{-/-}$ *Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice was similar to that of wild-type mice, and we readily detected T cells in the GCs (**Supplementary Fig. 1a**). GC B cell numbers were similar in *Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice (**Supplementary Fig. 1b**,**c**), which indicated that PD-1 signaling was not required for the formation of GCs or T_{FH} cells (the latter being defined as T cells present in the GC). *Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice were able to generate antibody-forming cells (AFCs) positive for (4-hydroxy-5-iodo-3-nitrophenyl)acetyl and immunoglobulin G1 (NIP$^+$IgG1$^+$) and developed titers of IgM and IgG1 antibodies to NP similar to those of wild-type B6 mice

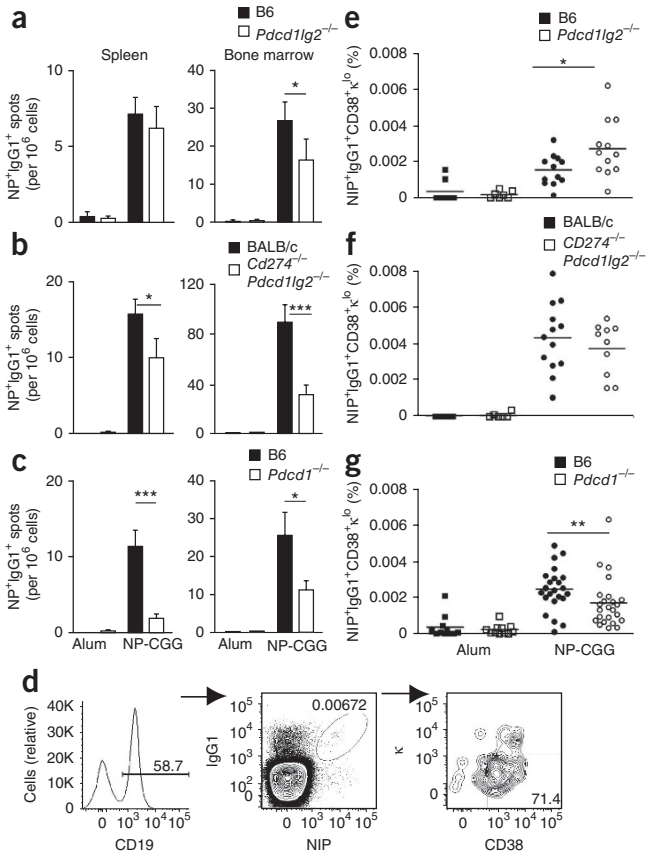


Figure 2 Long-lived PCs are lower in abundance in the absence of PD-1 signaling. (**a**–**c**) Enzyme-linked immunospot (ELISPOT) analysis of long-lived PC and memory responses in spleen and bone marrow from wild-type control mice (B6), *Pdcd1lg2*$^{-/-}$ mice (**a**; $n \geq 16$), *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice (**b**; $n \geq 10$) and *Pdcd1*$^{-/-}$ mice (**c**; $n \geq 14$) at least 12 weeks after immunization with NP-CGG or alum ($n \geq 5$ mice per genotype). Data are combined from two (*Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$) or at least seven (*Pdcd1lg2*$^{-/-}$ or *Pdcd1*$^{-/-}$) independent experiments (error bars, s.e.m.). (**d**) Memory B cell frequency after immunization as in **a**, assessed in live splenocytes gated as CD19$^+$NIP$^+$IgG1$^+$CD38$^+$κ^{lo}. The CD38 marker was included to gate out any residual GC (CD38$^-$) B cells; because naive B cells are also CD38$^+$, IgG1 was included to separate memory B cells from naive B cells, although this precludes analysis of any IgM$^+$ memory B cells present. Number above bracketed line (left) indicates percent CD19$^+$ cells; numbers adjacent to outlined areas indicate percent IgG1$^+$NIP$^+$ cells (middle) or κ^{lo}CD38$^+$ cells (right). Data are representative of at least 15 experiments. (**e**–**g**) Frequency of memory B cells in wild-type control mice (B6 or BALB/c), *Pdcd1lg2*$^{-/-}$ mice (**e**), *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice (**f**) and *Pdcd1*$^{-/-}$ mice (**g**). Each symbol represents an individual mouse; small horizontal lines indicate the mean. Data are combined from two (*Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$) four (*Pdcd1lg2*$^{-/-}$) or six (*Pdcd1*$^{-/-}$) independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann-Whitney nonparametric, two-tailed test).

(**Supplementary Fig. 1d**,**e**). Therefore, PD-1 was not required for the formation of a GC or for the early antibody response.

PD-1 signaling regulates the formation of long-lived PCs

We immunized wild-type mice, PD ligand–deficient mice and PD-1-deficient mice and then allowed them to 'rest' until the primary response had dissipated, which allowed us to assess long-lived B cell–derived populations. After 3 months, we observed fewer NP$^+$IgG1$^+$ AFCs in the bone marrow of *Pdcd1lg2*$^{-/-}$ mice and in both the spleen and bone marrow of *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice and *Pdcd1*$^{-/-}$ mice than in that of their wild-type control counterparts (**Fig. 2a**–**c**). In the absence of PD-1, antigen-specific IgG1$^+$CD38$^+$ memory B cells were able to form (**Fig. 2d**–**g**); however, the percentage of memory cells in mice lacking PD-1 was modestly lower than that of the wild-type controls (**Fig. 2g**), but we did not observe this effect in the ligand-deficient mice (**Fig. 2e**,**f**). Memory B cell numbers were slightly higher in *Pdcd1lg2*$^{-/-}$ mice than in wild-type controls, which suggested a possible divergence in the roles of PD-L1 and PD-L2 in memory cell development[16]; this could relate to broad tissue expression of PD-L1 and/or the function of CD80 as a second binding partner[16]. Therefore, in the absence of PD-1, both long-lived B cell–derived populations were smaller; however, PD-1 signaling seems to have only a minor role in memory cell formation relative to its role in PC formation.

Studies have shown that high-affinity, long-lived PCs are formed late in the GC response[27]. As AFC numbers were similar in the wild-type and PD-L2 and PD-1 strains at day 12, but not at later time points, we considered that PD-1 might have a more prominent role in the late GC response. IgG1$^+$ AFCs were significantly less abundant in the spleen in the absence of PD-1 signaling at 4 weeks after immunization in *Pdcd1lg2*$^{-/-}$ mice (**Fig. 3a**), *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice (**Fig. 3b**) and *Pdcd1*$^{-/-}$ mice (**Fig. 3c**). Long-lived PCs in the bone marrow were also significantly less abundant in all three strains (**Fig. 3a**–**c**), and differences in frequency of memory phenotype B cells in knockout mice compared with those of controls paralleled the differences seen at 3 months after immunization (data not shown). To determine when the differences between wild-type and PD-deficient mice in AFC numbers emerged, we immunized mice and assessed AFCs at many additional time points. *Pdcd1lg2*$^{-/-}$, *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice had fewer AFCs by days 15–16 (**Fig. 3d**–**f**). This was consistent for all time points assessed after days 15–16 for the *Pdcd1*$^{-/-}$ and *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice; however, the differences in the *Pdcd1lg2*$^{-/-}$ mice were variable depending on the time point. A lower number of AFCs was also concordant with lower circulating titers of both NP-specific IgG1 and IgM (**Fig. 3g**,**h**). The lower serum IgM further suggested that the lower number of IgG1 AFCs was not simply due to a defect in isotype switching but was the result of less AFC formation itself.

Lower GC B cell survival in the absence of PD-1

Why were PCs able to form early during the response but were fewer in number at later time points? It was possible that a block in differentiation from plasmablast to PC was occurring, allowing short-lived plasmablasts to be generated but not to terminally differentiate. However, examination of the progression of this process with several markers that change during PC differentiation (for example, downregulation of surface B cell antigen receptor and expression of the PC marker CD138) showed that there was no difference among PD-L2-deficient mice, PD-1-deficient mice and their wild-type controls (data not shown). As PC differentiation itself was not affected by the absence of PD-1 signals, we suspected there might be a defect in the GC that affected the development of these long-lived cells. Therefore, we investigated whether less proliferation or more apoptosis during GC responses in the absence of PD-1 signaling was causing the decrease in the number of long-lived B cells. We assessed this by detecting caspase activation with a fluorescent irreversible inhibitor of caspase that binds to activated caspase (CaspGLOW assay) and measuring incorporation of the thymidine analog 5-bromodeoxyuridine (BrdU), respectively (**Fig. 4a**). GC B cell frequency was lower in *Pdcd1*$^{-/-}$ mice than in wild-type mice (**Fig. 4b**) from day 15 on, even though at day 12, GC formation was similar to that of wild-type mice. Commensurate with the decrease in GC

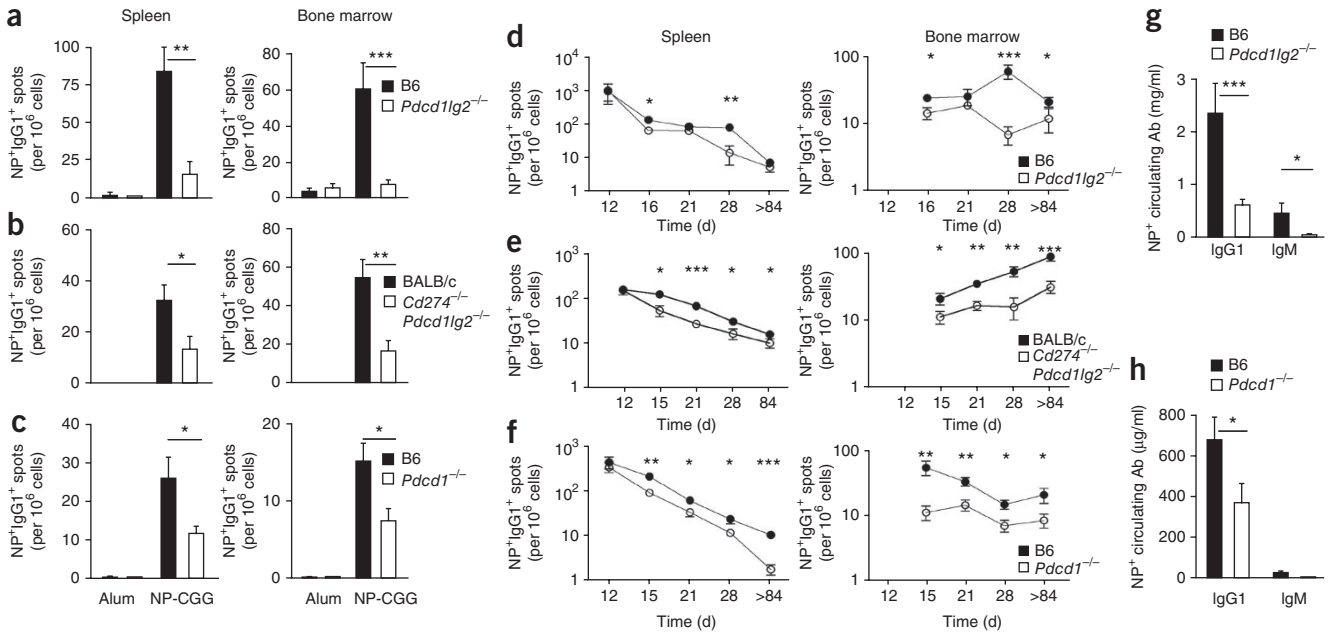


Figure 3 The decrease in PC numbers occurs during the late GC response and affects both IgG1 and IgM. (**a**–**c**) ELISPOT analysis of NP^+IgG1^+ AFCs in spleen and bone marrow from wild-type control mice (B6 or BALB/c; $n \geq 6$), *Pdcd1lg2*$^{-/-}$ mice (**a**; $n \geq 6$), *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice (**b**; $n \geq 10$) and *Pdcd1*$^{-/-}$ mice (**c**; $n \geq 12$) ~4 weeks after immunization with NP-CGG or alum ($n \geq 3$ mice per genotype). Data are combined from at least two independent experiments (error bars, s.e.m.). (**d**–**f**) NP^+IgG1^+ AFCs in spleen and bone marrow of wild-type control mice, *Pdcd1lg2*$^{-/-}$ mice (**d**), *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice (**e**) and *Pdcd1*$^{-/-}$ mice (**f**) at various times after immunization with NP-CGG (day 28 includes days 27–31, as also presented in **a**–**c**; day 12 is also in **Supplementary Fig. 1**; data beyond day 84, **Fig. 2**). Data are combined from at least two independent experiments, except data for days 18 and 21 for *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice represent one independent experiment per time point. (**g**,**h**) Circulating IgG1 and IgM in wild-type mice, *Pdcd1lg2*$^{-/-}$ mice (**g**; $n = 6$–8) and *Pdcd1*$^{-/-}$ mice (**h**; $n = 6$) at week 4 (**g**) and week 3 (**h**) after immunization with NP-CGG. Each symbol represents an individual mouse; small horizontal lines indicate the mean. Data are representative of at least two independent experiments (error bars, s.e.m.). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann-Whitney nonparametric, two-tailed test).

B cell frequency, we observed an increase in CaspGLOW$^+$ GC cell frequency in *Pdcd1*$^{-/-}$ mice (**Fig. 4c**). We also observed a decrease in GC B cell frequency and an increase in CaspGLOW$^+$ GC cell frequency in *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice (**Supplementary Fig. 2**). We observed this increase beginning on day 12, at the height of the GC response and 3 d before we first noted the decrease in AFCs (**Fig. 3**). There was no difference in BrdU incorporation by GC cells of B6 and *Pdcd1*$^{-/-}$ mice (**Fig. 4d**). In contrast to the death of GC cells, the death of emerging memory B cells and of the plasmablast and PC population in *Pdcd1*$^{-/-}$ mice was similar to that of wild-type mice (**Fig. 4e**), as was proliferation (data not shown). Hence, we could narrow the effect of PD-1 signaling to prevention of GC cell death in the mature GC; there was no detectable effect of lack of PD-1 signaling on GC or memory cell proliferation on PC differentiation. During the GC response, high-affinity variants are selected to differentiate, whereas low-affinity cells undergo cell death. Because of the enhanced death of GC B cells in *Pdcd1*$^{-/-}$ mice, we assessed the affinity of bone marrow PCs at least 12 weeks after immunization. By assessing the ratio of high-affinity AFCs to low-affinity AFCs, we observed greater affinity of the remaining PCs in the absence of either PD ligands or PD-1 (**Fig. 4f**). We also compared the variable heavy-chain *Igh* V_H186.2 sequences in memory B cells from *Pdcd1*$^{-/-}$ mice and wild-type controls (**Supplementary Fig. 3**). The average number of mutations per sequence was similar in the two data sets (B6, 8.8 mutations per sequence, with 59 sequences analyzed; *Pdcd1*$^{-/-}$, 8.5 mutations per sequence, with 32 sequences analyzed). Affinity maturation was also similar (B6, 0.53; *Pdcd1*$^{-/-}$, 0.5), assessed as the ratio of replacement in the complementarity-determining region (R_{CDR}) to silent mutations in both the complementarity-determining and framework regions (S_{total}) according to the following equation: $R_{CDR}/(S_{total} + R_{CDR})$. The frequency of replacement of tryptophan by leucine at position 33 was similar as well (B6, 36%; *Pdcd1*$^{-/-}$, 41%)[28]. However, we noted that although both wild-type B6 and *Pdcd1*$^{-/-}$ memory B cells showed positive selection in the IgH complementarity-determining regions, there was substantial negative selection in the framework regions in the *Pdcd1*$^{-/-}$ mice only, which suggested that responding B cells in these mice may be under more stringent selection and thus are more sensitive to deleterious mutations. As the late GC response is focused more on generating long-lived AFCs[27], we propose that excessive cell death in the absence of PD-1 signaling specifically affects the selection of cells entering the long-lived B cell compartments, which leads to a deficit in the generation of long-lived PCs and, to a lesser extent, memory B cells.

PD-1 affects the function and number of T_{FH} cells

Because T_{FH} cells have high expression of PD-1, we investigated whether the number and function of T_{FH} cells were affected during the GC response in the absence of PD-1 signaling. We identified T_{FH} cells by their downregulation of the chemokine receptor CCR7 in combination with PD-1 expression in *Pdcd1lg2*$^{-/-}$ and *Cd274*$^{-/-}$ *Pdcd1lg2*$^{-/-}$ mice or in combination with expression of the inducible costimulator ICOS in *Pdcd1*$^{-/-}$ mice. Although at day 12 T_{FH} cell frequency (**Fig. 5a**) and T_{FH} cell numbers (**Fig. 5b**) were similar in *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ and wild-type mice, this population was greater in *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice at later time points. We also saw this in both *Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice (**Fig. 5c**). To investigate how

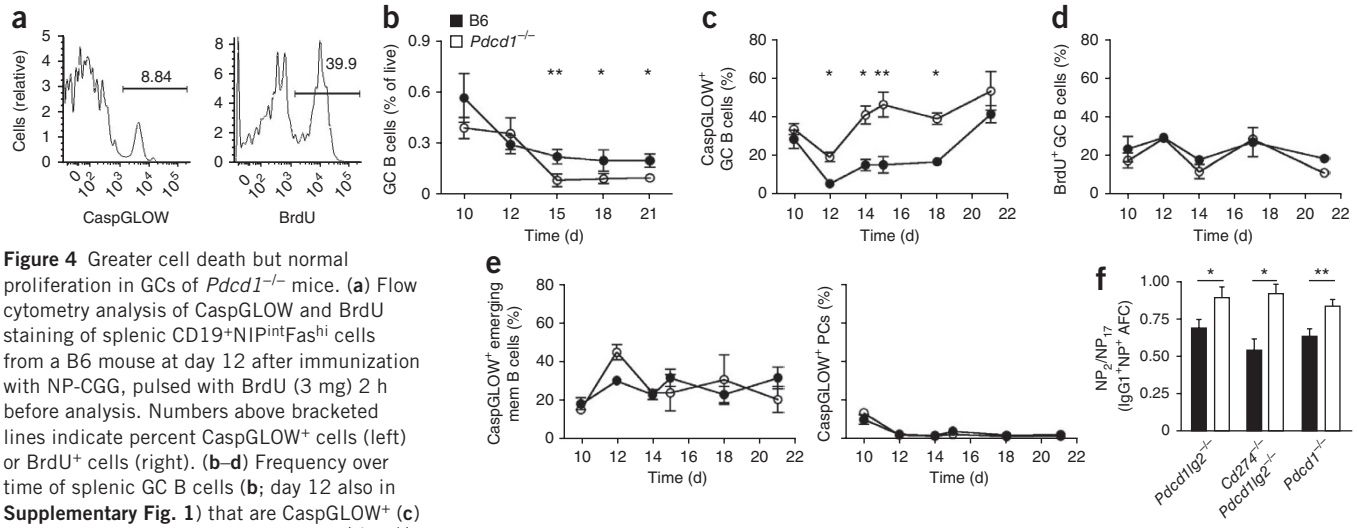


Figure 4 Greater cell death but normal proliferation in GCs of *Pdcd1*$^{-/-}$ mice. (**a**) Flow cytometry analysis of CaspGLOW and BrdU staining of splenic CD19^{+}NIP^{int}Fas^{hi} cells from a B6 mouse at day 12 after immunization with NP-CGG, pulsed with BrdU (3 mg) 2 h before analysis. Numbers above bracketed lines indicate percent CaspGLOW^{+} cells (left) or BrdU^{+} cells (right). (**b**–**d**) Frequency over time of splenic GC B cells (**b**; day 12 also in **Supplementary Fig. 1**) that are CaspGLOW^{+} (**c**) or BrdU^{+} (**d**) among splenic CD19^{+}NIP^{int}Fas^{hi} cells from wild-type and *Pdcd1*$^{-/-}$ B6 mice immunized with NP-CGG and pulsed with BrdU 2 h before analysis (3 mg/mouse); results are flow cytometry of cells visualized by detection with CaspGLOW or BrdU immediately after collection at days 10, 12, 14, 16, 18 and 21 after immunization. (**e**) Flow cytometry analysis of CaspGLOW^{+} emerging memory (mem) cells (left) and plasmablasts-PCs (intermediate–positive for NIP and positive for syndecan) from mice treated as described in **b**–**d** ($n \geq 4$ per time point). (**f**) ELISPOT analysis of NP_2-specific versus NP_{17}-specific IgG1^{+} bone marrow AFCs from knockout mice (open bars; $n \geq 6$) and wild-type mice (filled bars); one molecule of bovine serum albumin conjugated to two (NP_2-BSA) or seventeen (NP_{17}-BSA) NP molecules was used as capture antigen. $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney nonparametric, two-tailed test). Data are combined from or representative of at least two independent experiments (error bars, s.e.m.).

PD-1 signaling affected cytokine-production capacity (**Fig. 5d**), we sorted T_{FH} cells and collected mRNA. T_{FH} cells from *Pdcd1*$^{-/-}$ mice had less mRNA for IL-4 and IL-21, two cytokines secreted by T_{FH} cells that promote GC development[29]. We observed lower abundance of mRNA for both cytokines at all time points, with a greater decrease in the later GC, commensurate with the late defects in GC B cell survival. When we combined the similar data obtained on day 15 and day 18, we found these differences were significant for both *Il4* and *Il21* ($P < 0.01$). However, mRNA for interferon-γ (IFN-γ) was similar in *Pdcd1*$^{-/-}$ and wild-type mice; although there was slightly greater transcript abundance in the absence of PD-1, this difference was less than twofold and did not reach statistical significance, even when we combined the two similar time points of day 12 and day 15. IL-17 mRNA was not present in T_{FH} cells from either wild-type or knockout mice (data not shown). Therefore, although lack of PD-1 signaling resulted in a greater quantity of T_{FH} cells, it impaired the quality of T_{FH} cells by diminishing their capacity to synthesize important cytokines while not promoting the development of an alternatively polarized T cell type.

PD-L1 and PD-L2 on B cells regulates AFC production

In addition to their expression on B cells, PD-L1 and PD-L2 are expressed on other cells of the immune response. PD-1 is expressed on both B cells and T cells. Therefore, it was not clear on which cell type expression was critical for the promotion of proper AFC numbers. To investigate this, we assessed the cell-intrinsic expression requirements of both PD-L2 and PD-1 by purifying and transferring B cells or T cells deficient in ligand receptors (**Fig. 6a**,**b**). In wild-type mice, long-lived PCs persisted at very low frequencies (**Fig. 2**). Therefore, to facilitate the sensitivity and interpretation of transfer experiments, we crossed *Pdcd1lg2*$^{-/-}$ mice with B1-8 *Igh* knock-in mice[30], which express an NP-specific B cell antigen receptor; we thereby increased

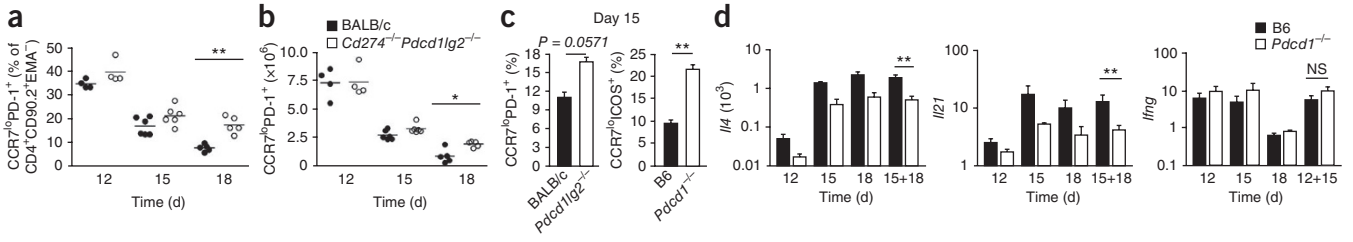


Figure 5 More cells of a T_{FH} phenotype correlates with lower cytokine production in the absence of PD-1 signaling. (**a**,**b**) Flow cytometry analysis of T_{FH} cell frequency among CD4^{+} cells (**a**) and number of T_{FH} cells (**b**) from wild-type (BALB/c) and *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice on days 12, 15 and 18 after immunization with NP-CGG; CD4^{+}CD90.2^{+}EMA^{-} cells were gated for the T_{FH} cell markers of PD-1 expression and CCR7 downregulation. Each symbol represents an individual mouse; small horizontal lines indicate the mean. Data are representative of two independent experiments at all time points (*Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$), days 15 or 16 (*Pdcd1lg2*$^{-/-}$) or days 15 and 18 (*Pdcd1*$^{-/-}$). (**c**) T_{FH} cell frequency among cells from wild-type (BALB/c or B6), *Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice ($n = 3$–5) at day 15 after immunization with NP-CGG, for cells gated as in **a**,**b**. Data are representative of at least two independent experiments during the late GC response. (**d**) Quantitative PCR analysis of the expression of *Il4*, *Il21* and *Ifng* by cells from wild-type and *Pdcd1*$^{-/-}$ mice immunized with NP-CGG in alum, sorted for ICOS expression and CCR7 downregulation ($n = 3$, day 12), in conjunction with CXCR5 expression ($n = 2$, day 15; $n = 3$, day 18; n indicates mice per genotype). Results were calculated by the comparative threshold method relative to actin expression as follows: $2^{(\text{actin CT} - \text{cytokine CT})}$. Data are from three independent experiments (one per time point). NS, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney nonparametric, two-tailed test).

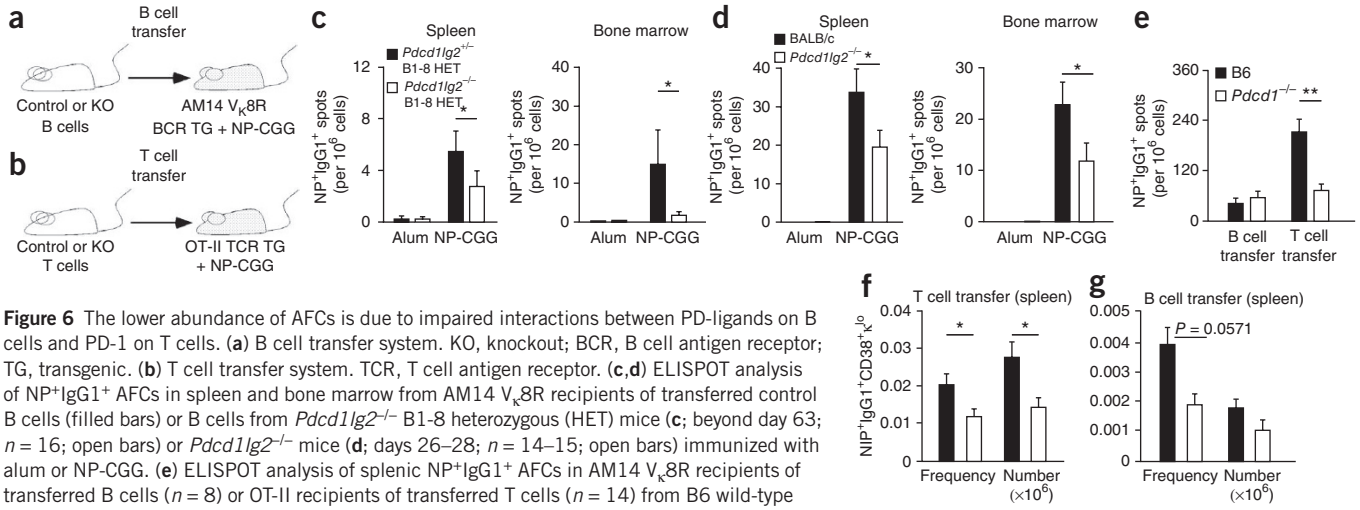
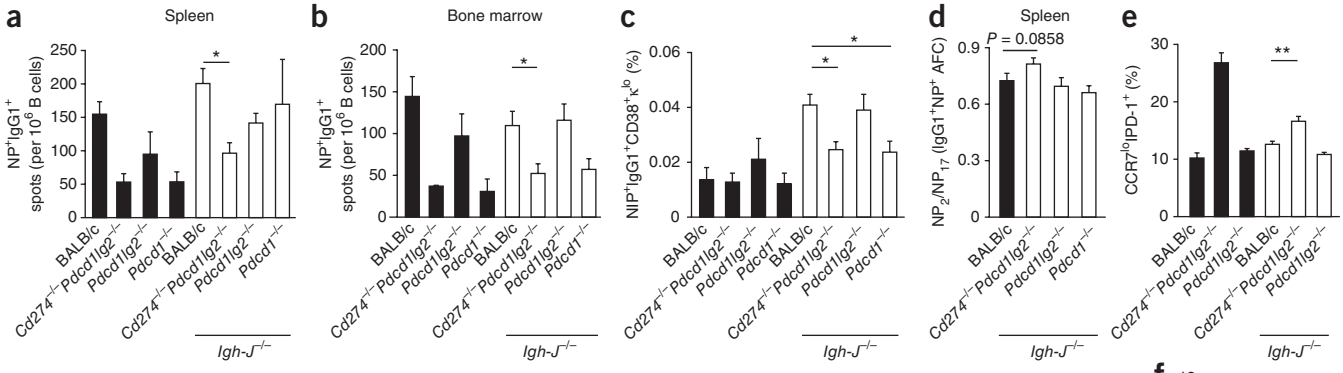


Figure 6 The lower abundance of AFCs is due to impaired interactions between PD-ligands on B cells and PD-1 on T cells. (**a**) B cell transfer system. KO, knockout; BCR, B cell antigen receptor; TG, transgenic. (**b**) T cell transfer system. TCR, T cell antigen receptor. (**c**,**d**) ELISPOT analysis of NP^+IgG1^+ AFCs in spleen and bone marrow from AM14 V_κ8R recipients of transferred control B cells (filled bars) or B cells from *Pdcd1lg2*$^{-/-}$ B1-8 heterozygous (HET) mice (**c**; beyond day 63; $n = 16$; open bars) or *Pdcd1lg2*$^{-/-}$ mice (**d**; days 26–28; $n = 14$–15; open bars) immunized with alum or NP-CGG. (**e**) ELISPOT analysis of splenic NP^+IgG1^+ AFCs in AM14 V_κ8R recipients of transferred B cells ($n = 8$) or OT-II recipients of transferred T cells ($n = 14$) from B6 wild-type or *Pdcd1*$^{-/-}$ mice, assessed at 4 weeks after immunization. (**f**,**g**) Frequency and number of $NIP^+IgG1^+CD38^+\kappa^{lo}$ B cells after transfer of wild-type (filled bars) or *Pdcd1*$^{-/-}$ (open bars) T cells (**f**) or B cells (**g**), assessed at 4 weeks after immunization. $^*P < 0.05$, $^{**}P < 0.001$ (Mann-Whitney nonparametric, two-tailed test). Data are combined from three (**c**) or four (**d**) independent experiments or are combined from two (B cell transfer) or three (T cell transfer) independent experiments (**e**–**g**; error bars, s.e.m.).

NP-specific precursor frequency among transferred cells and ensured that a 'readout' of long-lived PCs from transferred B cells would be possible. After transfer there was still a very low precursor frequency of antigen-specific *Pdcd1lg2*$^{-/-}$ B cells. We transferred B cells from either *Pdcd1lg2*$^{+/-}$ B1-8 heterozygous mice or *Pdcd1lg2*$^{-/-}$ B1-8 heterozygous mice on a B6 background into AM14 V_κ8R-transgenic recipients. AM14 V_κ8R-transgenic mice have a nearly completely homogenous B cell population of irrelevant specificity[31], so host B cells should not respond to NP-CGG (data not shown). At 63 or more days after immunization, there was a significantly lower abundance of both splenic and bone marrow PCs in the mice that received PD-L2-deficient B cells than in those that received PD-L2-sufficient B cells (**Fig. 6c**). Similarly, when we transferred B cells from *Pdcd1lg2*$^{-/-}$ or wild-type mice lacking the B1-8 knock-in allele into AM14 V_κ8R-transgenic recipients, AFC numbers were also lower 3 weeks after immunization in recipients of *Pdcd1lg2*$^{-/-}$ B cells than in recipients of wild-type BALB/c B cells (**Fig. 6d**). Together these results demonstrate that the lack of PD-L2 expression on B cells, rather than the lack of PD-L2 expression on DCs or macrophages, resulted in less generation of PCs. To examine the role of PD-1 expression on B cells, we transferred *Pdcd1*$^{-/-}$ or wild-type B6 B cells into AM14 V_κ8R-transgenic recipients. Reciprocally, we transferred *Pdcd1*$^{-/-}$ or wild-type B6 T cells into mice transgenic for the ovalbumin-specific OT-II T cell antigen receptor, again to restrict the host repertoire (**Fig. 6b**). We also immunized control recipient mice that did not receive donor cells to confirm that the recipient mice did not respond to NP-CGG (data not shown). Transferred *Pdcd1*$^{-/-}$ and wild-type B6 B cells yielded similar AFC production 4 weeks after immunization (**Fig. 6e**). In contrast, when we transferred *Pdcd1*$^{-/-}$ or wild-type B6 T cells, there were significantly



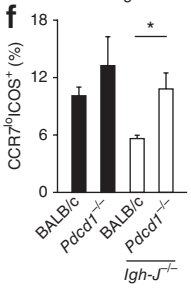


Figure 7 AFC production, memory B cell formation and T_{FH} cells are altered in mixed–bone marrow chimeras. (**a**,**b**) ELISPOT analysis of spleen (**a**) and bone marrow (**b**) from mixed–bone marrow chimeras generated by injection of a mixture (80:20) of *Igh-J*$^{-/-}$ bone marrow plus BALB/c, *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$, *Pdcd1lg2*$^{-/-}$ or *Pdcd1*$^{-/-}$ bone marrow (open bars) or wild-type or knockout bone marrow only (without *Igh-J*$^{-/-}$ bone marrow; filled bars) into lethally irradiated BALB/c recipients, which were allowed to rest for 6 weeks before immunization with NP-CGG, then were assessed 25–26 days later. *Igh-J*$^{-/-}$ + BALB/c, $n = 19$; *Igh-J*$^{-/-}$ + *Pdcd1lg2*$^{-/-}$, $n = 12$; *Igh-J*$^{-/-}$ + *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$, $n = 8$; *Igh-J*$^{-/-}$ + *Pdcd1*$^{-/-}$, $n = 5$. (**c**) Flow cytometry analysis of memory B cell frequency among $CD19^+$ cells in the mixed–bone marrow chimeras in **a**,**b**. (**d**) ELISPOT analysis of spleen from the mixed–bone marrow chimeras in **a**,**b**, as in **Figure 4f**. (**e**,**f**) Flow cytometry analysis of T_{FH} cell frequency among $CD4^+CD90.2^+$ cells in the mixed–bone marrow chimeras in **a**,**b**. *Igh-J*$^{-/-}$ + BALB/c, $n = 15$; *Igh-J*$^{-/-}$ + *Pdcd1lg2*$^{-/-}$, $n = 9$; *Igh-J*$^{-/-}$ + *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$, $n = 4$; *Igh-J*$^{-/-}$ + *Pdcd1*$^{-/-}$, $n = 5$. $^*P < 0.05$, $^{**}P < 0.001$ (Mann-Whitney nonparametric, two-tailed test). Data are combined from or are representative of two independent experiments (error bars, s.e.m.).

fewer AFCs in the recipients of PD-1-deficient cells (**Fig. 6e**). Notably, with transfer of both *Pdcd1*$^{-/-}$ B cells and T cells, B cells of a memory phenotype were less abundant (**Fig. 6f**,**g**).

To confirm the results obtained with the transfer systems described above, we also studied mixed–bone marrow chimeras. We mixed bone marrow from *Igh-J*$^{-/-}$ mice (which lack B cells) with bone marrow from *Pdcd1lg2*$^{-/-}$, *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$, *Pdcd1*$^{-/-}$ or wild-type mice at a ratio of approximately 80:20 and transferred the mixture into lethally irradiated BALB/c recipients, which we then immunized after reconstitution. We assessed the degree of chimerization of the mice, which approximated the ratio noted above (data not shown). In such chimeras, all B cells would lack the PD molecule of interest, whereas most non–B cells would express PD molecules. As a control, we transferred 100% bone marrow from gene-deficient mice. These control mice generally replicated the phenotype of the intact gene-deficient mice (**Fig. 7**), which demonstrated that the bone marrow transfer system worked and further showed that most of the phenotype of intact PD-deficient mice was attributable to hematopoietic expression of the relevant PD molecule. At days 25–26 after immunization, mice lacking PD-L1 and PD-L2 on B cells had significantly fewer AFCs and less memory B cell formation (**Fig. 7a–c**), and the affinity of AFCs was greater (**Fig. 7d**). Splenic AFCs were not significantly less abundant in chimeras in which only B cells lacked only PD-L2, though there was a trend in this direction (**Fig. 7a**). There was no less memory cell formation in such mice (**Fig. 7c**), consistent with results obtained with intact *Pdcd1lg2*$^{-/-}$ mice (**Fig. 2**). Chimeric mice lacking PD-1 expression on B cells showed no deficiency in splenic AFC numbers, although there was a trend toward fewer bone marrow AFCs that did not reach statistical significance; these mice did have significantly less memory cell formation (**Fig. 7a–c**), consistent with the transfer data reported above (**Fig. 6**). T_{FH} cells were more abundant in both the *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mixed–bone marrow chimeras but not in *Pdcd1lg2*$^{-/-}$ mixed–bone marrow chimeras (**Fig. 7e**,**f**). Therefore, we conclude that B cells expressing PD-L1 and PD-L2 and T cells expressing PD-1 interact via this pathway to regulate the production of both PCs and memory B cells, whereas PD-1 expressed by B cells, which could mediate B cell–B cell or B cell–T cell interactions (in which the ligand is expressed on the T cell), affects the formation of memory and possibly bone marrow PCs but not the formation of splenic PCs.

DISCUSSION

We have reported here that PD-1 and its ligands, PD-L1 and PD-L2, have a previously unknown role in regulating the primary humoral immune response and have provided insight into how this is mediated. The most prominent effect of the loss of PD-1 signaling was a lower number of long-lived PCs. One element of the complexity of B7-family immunoregulation is the variety of potential interactions. By using mice deficient in PD-1 or PD-L2 or both PD-L1 and PD-L2, along with cell transfer and mixed–bone marrow chimeras, we were able to delineate some of the key interactions and cell-intrinsic requirements for regulation of the response. In particular, our results directly indicate the involvement of interactions between PD-1 on T cells and PD-L1 and/or PD-L2 on B cells in the formation of long-lived PCs. A pivotal observation was that B cell–intrinsic expression of PD-L2 by itself was required for optimal AFC generation, directly indicating a nonredundant role for this molecule, which is not expressed on B cells until they differentiate in the GC. Nonetheless, in many ways, the phenotypes of the *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice were more pronounced than those of the *Pdcd1lg2*$^{-/-}$ mice, possibly reflecting constitutive PD-L1 expression on B cells, which can also contribute to PD-1 signals. However, our results do not rule out the possibility of roles for additional interactions mediated by these molecules, such as involvement of the binding of PD-1 on B cells to PD-L1 or PD-L2 on B cells in the promotion of memory cell formation, as suggested by our mixed–bone marrow chimera data. We observed significant differences between wild-type and PD-1 signaling–deficient mice in PC numbers at many time points, in different tissues and in each type of gene-targeted mouse on different backgrounds, which establishes the fact that PD-1 signaling regulates the formation of long-lived PCs.

We delineated several aspects of the mechanism by which signals through PD-1 regulate the primary humoral response. In the absence of PD-1 signaling, greater GC B cell death corresponded to a quantitative defect in PC numbers; however, the remaining PCs were of higher affinity than were wild-type PCs. This effect was most evident late in the response, and consistent with this, memory B cell numbers were not affected as severely as PCs, probably because their formation occurs early in the response[26,27,32]. How does PD-1 signaling on T cells promote GC B cell survival? The lack of PD-1 signaling resulted in more T_{FH} cells but less synthesis of IL-4 and IL-21 cytokine mRNA by these cells. Therefore, ligation of PD-1 on T cells by PD-L2 and/or PD-L1 expressed on B cells seems to qualitatively affect T_{FH} cell homeostasis and function. Reciprocally, signals in T cells influence the probability of B cell survival by delivering contact-based signals or cytokines, such as the ligand for the cell surface receptor Fas, as well as IL-4 and IL-21. IL-21 is required for optimal antibody production[33] and, notably, IL-21 is able to induce PD-1 expression[34]. Both IL-4 receptor-α–deficient and IL-21 receptor–deficient mice have phenotypes similar to that of the *Pdcd1*$^{-/-}$ mice, with both showing lower GC B cell numbers[35]. Furthermore, it has been shown that IL-21 acts directly on GC B cells through the IL-21 receptor to affect GC B cell maintenance and the subsequent formation of PCs and is not required for the formation of T_{FH} cells[36–38]. Together with our data, those findings provide a mechanism by which the smaller amount of T cell–derived IL-4 and IL-21 in the *Pdcd1*$^{-/-}$ mice is linked to the inability to sustain GC B cells in the late response, which possibly results in the competitive survival of AFCs of higher affinity.

Although they were diminished, AFC responses were not abrogated in the absence of PD-1-signaling, which indicates that interactions between PD-1 and its ligands control the extent of the response rather than whether it happens at all. Less production of PCs in the absence of PD-1 or other molecules, such as IL-21 (ref. 33), is more consistent with a model of division-linked differentiation, in which multiple survival and differentiation signals are integrated at each division, thereby affecting the probability of different potential outcomes[39]. Fine tuning of B cell activity per division allows small changes, such as those governed by PD-1 or IL-21 signals in the GC, to produce a large cumulative effect on the quality and quantity of humoral responses over time[40].

Our data have also provided insight into the functional importance of the much higher expression of PD-1 on T_{FH} cells, demonstrating a specific function in the control of GC B cell survival and selection. Notably, the data have demonstrated a paradoxical role for PD-1 in that the presence of PD-1 promoted the humoral response, whereas previously PD-1 has been seen only as an inhibitor of both autoimmune and immunopathogenic antiviral responses. Although concepts of PD-1 function in T cells have been provided mainly by studies of $CD8^+$ cells[23], the role of PD-1 probably depends on the type of T cell[41]. T_{FH} cells seem to be very different even from other types of $CD4^+$ T cells in terms of their ontogeny and the transcription factors that define them[19]. Although PD-1 is commonly thought of as a marker of exhaustion, T_{FH} cells are not exhausted in that they have copious secretion of IL-21 and other cytokines during the humoral response. In the absence of PD-1, T_{FH} cells did accumulate more *in vivo*, suggestive of relief of an inhibitory signal. However, this correlated with a decrease in T_{FH} cell function, notably less IL-21 production. Therefore, it is possible that high expression of PD-1 may freeze T cells in a T_{FH} cell state, which

allows sustained production of IL-4 and IL-21 in the B cell follicle, whereas smaller to negligible amounts of PD-1 on T cells may allow proliferation and differentiation into another type of effector helper T cell. Although the roles of PD-1 in inhibiting and mediating exhaustion in $CD8^+$ T cells while enabling T_{FH} cells to prevent GC cell death seem in conflict, we propose a unifying view that PD-1 could function to favor aspects of the humoral response over the cellular response. This could be an adaptation for certain chronic inflammatory situations; if acute cytotoxic responses prove ineffectual, these are suppressed, perhaps limiting immunopathology, whereas long-lived antibody responses are enhanced, perhaps limiting further pathogen spread.

Overall, although the roles of PD-1 signaling in the T cell response have been the subject of many reports[23], the role of this signaling pathway in the B cell response has remained almost completely unstudied. Our report helps to fill this gap, defining an unexpected role for PD-1 signaling in promoting the long-lived PC response. These pathways could have many consequences for both humoral immunity and autoimmunity, whose outcomes are often determined by the exact balance and timing of the response.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

K.L.G.-J., M.M.T and M.J.S. designed research; K.L.G.-J. and C.G.S did research; L.C. and A.H.S. generated and contributed knockout mice; and K.L.G.-J. and M.J.S. analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and immunization. *Pdcd1lg2*$^{-/-}$ were generated as described[42] and were backcrossed onto the B6 background for at least ten generations. *Pdcd1lg2*$^{-/-}$ and *Cd274*$^{-/-}$*Pdcd1lg2*$^{-/-}$ mice on the BALB/c background were generated as described[43]. *Pdcd1*$^{-/-}$ mice were generated as described and were backcrossed onto the B6 background[44] or BALB/c background[45]. B6 and BALB/c controls, as well as OT-II T cell antigen receptor–transgenic mice, were from Jackson Laboratories. AM14 V_κ8R mice[46,47], *Igh-J*$^{-/-}$ mice[48] and B1-8 knock-in mice[30] were generated as described. For primary responses, mice were immunized intraperitoneally with 50 μg alum-precipitated NP-CGG, at a ratio of NP to CGG ranging from 25 to 31, or with precipitated alum alone as a control. All mice were maintained under specific pathogen–free conditions and were immunized or given cell transfer at 6–12 weeks of age. All animal experiments were approved by the Yale Institutional Animal Care and Use Committee.

Antibodies and detection reagents. The following staining reagents were used: NIP-binding reagents (NIP-phycoerythrin and NIP-allophycocyanin) and monoclonal antibody to CD3ε (anti-CD3ε; 2C-11), monoclonal anti-CD4 (GK1.5), monoclonal anti-CD90.2 (30H12), monoclonal anti-CD80 (1610A1) and monoclonal antibody to κ-chain (187.1), prepared as described[49]. Anti-IgG1 (A85-1), anti-CD19 (1D3), anti-CD38 (90), anti-CD138 (281-2), anti-CD86 (GL1), anti-CD45R (RA3-6B2), anti-CD95 (Jo-2), anti-CD25 (PC61), anti-CXCR5 (2G8) and streptavidin-phycoerythrin-indotricarbocyanine (557598) were from BD Biosciences; anti-CCR7 (4B12), anti-PD-L1 (MIH5), anti-PD-L2 (TY25), anti-PD-1 (J43) and anti-CD62L (Ly-22) were from eBiosciences; streptavidin–Alexa Fluor 680 (S-3235A) was from Invitrogen; anti-BrdU (PRB-1) was from Molecular Probes; and anti-ICOS (C398.4A), anti-PD-1 (29F.1A12) and IgD (11-26c.2a) were from Biolegend.

Flow cytometry. Single-cell suspensions of splenocytes were stained on ice with the appropriate antibodies in PBS containing 3% (vol/vol) calf serum and 0.05% (wt/vol) sodium azide. Nonspecific staining was blocked by monoclonal antibody 24G2 to CD16/32 (prepared as described[49]) or by rat serum for intracellular staining. For intracellular staining, samples were fixed and made permeable before being incubated with antibody in Cytoperm (BD). Live cells and dead cells were distinguished with ethidium monoazide (Molecular Probes), and gating strategies were used for doublet discrimination. For purification of populations, cells were sorted on a FACSAria (BD Immunocytometry Systems). For analysis, samples were fixed with 1% (vol/vol) paraformaldehyde before being analyzed on a BD LSR II. Data were analyzed with FlowJo software (TreeStar).

BrdU detection and apoptosis assays. For BrdU labeling, mice were given intraperitoneal injection of 3 mg BrdU (Sigma-Aldrich) 2 h before being killed. BrdU was detected in cells as described[25]. For detection of apoptosis *in situ*, splenocytes were incubated for 30–45 min at 37 °C with z-VAD-fmk–fluorescein (Casp-Glow; benzyloxycarbonyl–Val-Ala-Asp–fluoromethylketone; BioVision) in RPMI medium. Cells were washed according to the manufacturer's protocol and then were labeled with the appropriate antibodies for surface phenotyping.

ELISPOT assay and ELISA. For analysis of the production of AFCs by ELISPOT assay or antibody by ELISA, plates were coated overnight at 4 °C with 5 μg NP-BSA conjugated at the appropriate ratio (NP_5-BSA for IgM, $NP_{16–27}$-BSA for IgG1, and NP_2-BSA for IgG1 affinity studies). Nonspecific binding was blocked with 1% (wt/vol) BSA in PBS and samples were incubated at 37 °C. Alkaline phosphatase–conjugated secondary antibodies (to IgG1 (1070-04) or IgM (1020-04); Southern Biotech) were detected with bromo-4-chloro-3-indolyl phosphate substrate (Southern Biotech) or *p*-nitrophenyl phosphate (Southern Biotech) for ELISPOT assay or ELISA, respectively.

Quantitative PCR. Total RNA was isolated from sort-purified populations and cDNA synthesis and quantitative PCR were done as described[13]. Primer sequences were as follows: *Il4* sense, 5′-AAAGACTTCCTGGAAAGCCTA-3′, and antisense, 5′-CCTTATGGCCAAATGAAGTGA-3′; *Il17* sense, 5′-TCCAGAAGGCCCTCAGACTA-3′, and antisense, 5′-TCATGTGGTGGTCCAGCTT-3′; *Il21* sense, 5′-TGAAAGCCTGTGGAAGTGCAAACC-3′, and antisense, 5′-AGCAGATTCATCACAGGACACCCA-3′; *Ifng* sense, 5′-ATGAACGCTACACACTGCATC-3′, and antisense, 5′-CCATCCTTTTGCCAGTTCCTC-3′; and actin as described[11].

Sequencing analysis. Cell pellets from sort-purified populations were digested overnight as described[50]. V_H186.2-J_H2 sequences were amplified by nested PCR with external primers 5′-CCTGACCCAGATGTCCCTTCTTCTCCAGCA-3′ (sense) and 5′-GGTGTCCCTAGTCCTTCATGACC-3′ (antisense) and internal primers 5′-AGGTCCAACTGCAGCC-3′ (sense) and 5′-TGTGAGAGTGGTGCCT-3′ (antisense). Amplified DNA was cloned, PCR analysis of colonies was done and DNA was purified and sequenced as described[50] with the M13 sequencing primers. The Clustal W method of alignment was used with MegAlign (DNASTAR). Selection analysis, including analysis of mutations in the complementarity-determining and framework regions, was done by published methods[28].

Adoptive transfer. B cells from wild-type or knockout mice were obtained with the EasySep Mouse B Cell Enrichment kit according to the manufacturer's instructions (StemCell Technologies). Single-cell suspensions were transferred into the tail veins of recipient mice. Approximately 1×10^7 B cells were transferred per mouse for wild-type transfers, with NIP^+ cells accounting for ~0.02–0.05% of B cells; approximately 1×10^5 to 2×10^5 B cells were transferred per mouse for B1-8 transfer. The purity of B cells was typically 90%. Mice were allowed to 'rest' for 1–2 d and then were immunized as outlined above.

Bone marrow chimeras. Bone marrow from wild-type or knockout mice and bone marrow *Igh-J*$^{-/-}$ mice were mixed at a ratio of 20:80. Cell suspensions were injected into lethally irradiated BALB/c recipients (which received total-body irradiation, two doses of 450 cGy, separated by 3 h, from a ^{137}Cs source). Mice were allowed to 'rest' for at least 6 weeks before immunization and were assessed for the extent of chimerism at the time cells were collected.

Immunofluorescence histology. Sections were stained with peanut agglutinin–biotin (L-1075; Vector Laboratories) and fluorescein isothiocyanate–conjugated antibody to T cell antigen receptor-β (H57-597; BD Pharmingen), with streptavidin–Alexa Fluor 555 (S32355; Invitrogen) and antibody to fluorescein isothiocyanate–Alexa Fluor 488 (A11096; Molecular Probes), respectively, as secondary reagents. Images were obtained at a magnification of 20×.

Statistics. The Mann-Whitney nonparametric, two-tailed test was used for statistical analyses.

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