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Programmed Cell Death 1 Suppresses B-1b Cell Expansion and Long-Lived IgG Production in Response to T Cell-Independent Type 2 Antigens

Karen M. Haas

B-1b cells play a key role in producing Abs against T cell-independent type 2 Ags. However, the factors regulating Ab production by this unique B cell subset are not well understood. In this study, a detailed analysis of the B cell response to 2,4,6-trinitrophenol (TNP)-Ficoll was performed using normal mice. TNP-Ficoll delivered i.p. or i.v. induced rapid Ag-specific B-1b cell activation, expansion, isotype switching, and plasmablast/plasma cell differentiation. Ag-specific B-1b cell numbers peaked at day 5 and then gradually declined in the spleen but remained elevated in the peritoneal cavity beyond 40 d postimmunization. In addition to expressing CD43, CD44, and CD86, Ag-activated B-1b cells transiently expressed programmed cell death 1 (PD-1), which functionally suppressed BCR-induced B-1b cell in vitro proliferation when additional costimulatory signals were lacking. Inhibiting PD-1:PD-1 ligand interactions during TNP-Ficoll immunization significantly enhanced Ag-specific B-1b cell expansion and the frequency of IgG isotype switching and plasmablast/plasma cell differentiation. Remarkably, PD-1 mAb blockade during the first week following immunization resulted in significantly increased numbers of both splenic and bone marrow Ag-specific IgG3-secreting cells, but not IgM-secreting cells, at both early (day 5) and late (week 6) time points. Moreover, Ag-specific serum IgG3 levels, as well as IgG2c, IgG2b, and IgA levels, remained significantly elevated in PD-1 mAb-treated mice relative to control Ab-treated mice for ≥6 wk postimmunization. Thus, PD-1:PD-1 ligand interactions occurring shortly after initial T cell-independent type 2 Ag encounter play a critical role in suppressing Ag-specific B-1b cell expansion and the development of long-term IgG-producing bone marrow and spleen cells. The Journal of Immunology, 2011, 187: 000–000.
Ag, DNP-Ficoll, and exhibit multiple immune abnormalities, including moderate myeloid and lymphoid hyperplasia, hyperresponsive B cells, and decreased CD5 expression on peritoneal B-1 cells that may be due to dysregulated CD5 expression and/or increased B-1b cell numbers (29). It is unclear whether increased TI-2 Ab responses in PD-1−/− mice are due to one or more of these preexisting abnormalities or due to PD-1 regulatory effects that occur at the time of immunization. Thus, the role of PD-1: PD-1 ligand (PDL) interactions in regulating TI-2 Ab responses remains unknown.

Studies investigating factors regulating TI-2 Ag responses have used pathogen-derived Ags, including pneumococcal polysaccharides, as well as synthetic Ags, such as haptenated Ficoll, which, in contrast to pathogen-derived Ags, are free of contaminating pathogen-associated molecular patterns (PAMPs) that can supply additional immunomodulatory signals. The synthetic Ag, 2,4,6-trinitrophenol (TNP)-Ficoll, an inert copolymer of sucrose and epichlorohydrin conjugated to TNP, has been used for decades as a prototypic TI-2 Ag. Recent studies using knockout mice with deficiencies in select B cell populations suggested that MZ B cells play a key role in the humoral immune response to TNP-Ficoll (30, 31). Nonetheless, because mice lacking this subset remain able to produce anti–TNP-Ficoll Ab responses (27), alternative populations may participate in humoral responses to this commonly used Ag.

In this study, Ag-specific B cell activation, expansion, differentiation, and Ab production in response to TNP-Ficoll were examined using normal mice. In contrast to BCR transgenic mice, normal mice are advantageous in that they express a broad Ab repertoire and unaltered B cell subset distribution, both of which may be important factors in shaping TI-2 Ab responses. Importantly, in the current study, Ag-specific B-1b cells were found to be a major B cell population that responded to TNP-Ficoll, regardless of immunization route. In response to immunization, Ag-specific B-1b cells selectively increased in number; expressed multiple markers of activation, including PD-1; and underwent isotype switching to produce inhumoral responses to this commonly used Ag.

CD5+ B cell subsets were purified from peritoneal cavity lavage by negative-depletion procedure. Macrophages were removed by plate adherence in RPMI 1640 containing 5% FCS (1 h at 37°C, 5% CO2). Nonadherent cell populations were depleted of Thy1.2+ cells using magnetic bead negative-depletion procedure. Macrophages were removed by plate adherence in RPMI 1640 containing 10% FCS (Life Technologies BRL). Cells were washed two times with PBS, and blocked for 1 h at 37°C with complete RPMI (crP RPMI) 1640 containing 10% FCS (Life Technologies BRL). Cells were plated at a concentration of 10^6 to 10^7 cells/ml in crP RPMI 1640 containing 10% FCS and cultured for 18 h. Alkaline phosphate-conjugated polyclonal goat anti-mouse IgM and IgG3 Abs (Southern Biotechnology Associates) were used in conjunction with NBT/5-bromo-4-chloro-3-indolyl phosphate substrate (Promega, Madison, WI), according to the manufacturer’s instructions. Membranes were dried, and spots were enumerated.

**Abs and immunofluorescence analysis**

Blood collected in heparin and spleen homogenate pellets were lysed in RBC lysis buffer (0.15 M NH4Cl/0.1 M KClO3). Peritoneal cell populations were isolated by lavaging the peritoneal cavity with 10 ml PBS. Single-cell blood, spleen, lymph node, and peritoneal cavity leukocyte suspensions (2 × 10^6/ml) were incubated in PBS containing 2% bovine calf serum with 20 μg/ml TNP30–Ficoll–Fluoresceinα (Biosearch Technologies) for 30 min at room temperature, followed by subsequent staining with fluorochrome-labeled mAbs and secondary detection reagents used included anti-mouse IgM and IgG3 (Southern Biotechnology Associates); Abs reactive with mouse CD1d (1B1), CD5 (53-7.3), B220 (RA3-6B2), CD23 (B3B4), CD19 (6D5), CD44 (IM7), and CD86 (GL1) (all from BioLegend); CD19 (ID3), CD80 (16-10A1), CD21/35 (7G6), and PD-1 (J43) mAb (all from BioLegend and eBioscience); and CD138 (BD Biosciences). Cells were harvested using FACSCalibur and FACSCantoII flow cytometers (BD Biosciences, San Jose, CA). Positive and negative cell populations were determined using unreactive isotype-matched Abs (BioLegend and eBioscience), and data were analyzed using FlowJo analysis software (Tree Star).

**B cell-proliferation assays**

LPS (Escherichia coli 0111:B4; Sigma) and anti-mouse Ab (RM ImmunoResearch, West Grove, PA). In some cultures, 2 μg/ml biotinylated PD-1 mAb (J43; eBioscience) or biotinylated Armenian hamster IgG (eBioscience) was added, along with 5 μg/ml streptavidin (Sigma). LPS (Escherichia coli 0111:B4; Sigma) and anti-mouse Ab (RM ImmunoResearch, West Grove, PA). In some cultures, 2 μg/ml biotinylated PD-1 mAb (J43; eBioscience) or biotinylated Armenian hamster IgG (eBioscience) was added, along with 5 μg/ml streptavidin (Sigma).
FIGURE 1. Ag-specific B cell phenotype, activation, differentiation, and expansion kinetics in response to TNP-Ficoll immunization. A–G. Flow cytometric analysis and enumeration of TNP₆₅-Ficoll–binding (Ag-specific) cells from naive and immune mice (50 μg TNP₆₅-Ficoll administered i.p.). A. Left panels. Representative flow cytometric analysis of Ag-specific B220⁺ splenic and peritoneal B cells in naive and immune (day-3) mice. Right panels. Frequencies and numbers of splenic and peritoneal Ag-binding B cells from days 0–35 postimmunization (n ≥ 5 mice/group). B. Phenotype of splenic and peritoneal Ag-specific B cells in naive (shaded graphs) and immune (day-5; thick line) mice. Isotype control binding for Ag-specific B cells from immune mice is indicated by the dashed line. C. B220 and CD11b expression by blood, spleen, peritoneal cavity, and lymph node Ag-specific cells at days 0 and 5 postimmunization. D. CD5 and CD11b expression on spleen Ag-specific B cells at days 0 and 35 postimmunization. E. Frequencies and numbers of Ag-specific splenic and peritoneal B-2 (B220⁺CD11b⁻CD5⁺), B-1b (B220⁺CD11b⁺CD5⁻), and B-1a (B220⁺CD11b⁺CD5⁺) cells in naive and immune mice. Activation marker (CD43, CD44, CD80, and CD86; F) and IgG3 (day-5; G) expression by Ag-specific CD11b⁺ and CD11b⁻ B cells in spleen (F, G) and peritoneal cavity (G) following TNP-Ficoll immunization. Isotype-control binding is shown for Ag-specific cells from immune mice (F). All results are representative of data obtained with at least three mice/group. Data in A and E represent mean (± SEM). *p < 0.05, naive versus immune mice.
However, by 35 d postimmunization, splenic Ag-specific B cell numbers were only increased ~20% over numbers in naive animals (Fig. 1A). In contrast, elevated Ag-specific peritoneal B cell frequencies and numbers did not decrease following the day-5 time point, but they remained significantly increased over naive levels beyond 35 d postimmunization. The increases observed in TNP-Ficol–binding B cells following immunization were likely due to Ag-specific binding as opposed to FcR binding of TNP-specific Ab, because stripping B cells of any FcR-bound Ab by 3 min of incubation with 50 mM glycine buffered saline (pH = 3) (33) did not significantly alter the frequency of TNP-Ficol–binding B cells (99 ± 4% of no-treatment control, n = 4). Thus, TNP-Ficol immunization rapidly increased Ag-specific B220+ B cell numbers in the spleen and peritoneal cavity, with numbers gradually contracting in spleen but remaining elevated in the peritoneal cavity 5 wk after immunization.

The phenotype, activation, and differentiation status of Ag-specific B cells were assessed following TNP-Ficol immunization. Relative to Ag-binding B cells in naive mice, Ag-specific splenic and peritoneal B cells in immune mice (day 5) had increased forward scatter and side scatter, as well as increased CD86, CD44, and CD43 expression, indicative of activation (Fig. 1B, data not shown). In addition, Ag-specific splenic B cells in immune mice had unchanged CD1dint expression, reduced levels of CD21/35, CD23, and B220, and increased levels of CD19. A fraction of Ag-specific B220+ B cells in the spleen had also undergone isotype switching to IgG3 and expressed CD138, indicative of plasmablast differentiation (Fig. 1B, data not shown). Thus, TNP-Ficol immunization induces Ag-specific peritoneal and splenic B cell activation and differentiation, with a substantial population of splenic Ag-specific B cells expressing a B220hiCD19hiCD1dintCD21/35lo phenotype.

**Ag-specific B-1b cells are a major B cell population responding to TNP-Ficol immunization**

The B220hiCD19hiCD1dintCD21/35lo phenotype is common to B-1 cells. Thus, the expression pattern of additional B-1 markers, CD11b and CD5, was examined for Ag-specific cells. In naive mice, Ag-specific peritoneal B cells were mostly CD11b+ and expressed either intermediate (CD5int) or very low levels of CD5 (CD5neg-lo), characteristic of B-1a and B-1b cells, respectively (Fig. 1B), whereas naive splenic TNP-specific B cells were CD5+ and CD11b+. Following i.p. immunization, CD11b+ Ag-specific B cell frequencies increased in the peritoneal cavity, spleen, blood, and lymph nodes (Fig. 1B, 1C). These cells had decreased B220 expression levels. In addition, increases in Ag-specific B cells in the spleen and peritoneal cavity expressing a CD5neg-lo phenotype were observed following immunization (Fig. 1B). Importantly, the level of CD5 expressed by Ag-specific B cells from immune mice was lower than that expressed by peritoneal B-1a cells and was comparable to levels present on peritoneal B-1b cells (Fig. 1B, Supplemental Fig. 1A). Interestingly, Ag-specific B cells expressing CD5 were not found in naive CD11b+ mice, but they were identified in TNP-Ficol–immunized CD19−/− mice (Supplemental Fig. 1B), which lack B-1a cells (14). Thus, B cells responding to TNP-Ficol express a CD11b+CD5neg-lo phenotype.

The phenotype of Ag-specific B cells (CD11b+ B220hiCD19hi CD1dintCD21/35CD5neg-lo) responding to TNP-Ficol was similar to that of peritoneal B-1b cells. Therefore, changes in Ag-specific B-1b cell frequencies, activation, isotype switching, and differentiation were specifically evaluated following TNP-Ficol immunization. Relative to naive numbers, Ag-specific B-1b cell numbers were significantly increased in spleen (16-fold) and peritoneal cavities (3-fold) 5 d following immunization, in contrast to Ag-binding B-1a and B-2 cells, which were not significantly altered (Fig. 1E). Ag-specific peritoneal B-1b cell numbers were increased further (12-fold) at day 35. Ag-specific CD11b+ expressing CD5− cells were still detectable in the spleen 35 d postimmunization (Fig. 1D) and were still significantly greater in frequency and number (2.5-fold) than in naive mice at this time point (Fig. 1E). Ag-specific CD11b+ (B-1b) B cells were the predominant subset activated by immunization, as evidenced by increased levels of CD43, CD44, CD86, and CD80 expression on this subset relative to CD11b+ B cells (Fig. 1F). Moreover, Ag-specific B-1b cells selectively underwent isotype switching to IgG3, and these cells were found in both the spleen and peritoneal cavity (Fig. 1G). Finally, Ag-specific CD138+CD11b+ plasmablasts were observed in the spleen but not the peritoneal cavity. Nonetheless, a large fraction of Ag-specific B220hiCD138+ cells were CD11b− (data not shown), consistent with the loss of this marker upon differentiation to Ab-secreting cells (ASCs) (34–36). Thus, Ag-specific B-1b cells become activated, expand, undergo class switching, and differentiate into splenic ASCs in response to TNP-Ficol immunization.

**Ag-specific B-1b cells are the major B cell population responding to TNP-Ficol immunization, regardless of the route of Ag delivery**

To assess whether the Ag-specific B cell responses to TNP-Ficol were dependent on the route of Ag delivery, mice were immunized with TNP-Ficol by i.v., s.c., or i.p. injection. Five days postimmunization, Ag-specific peritoneal and spleen B cell frequencies, numbers, and phenotypes were analyzed. Increases in total Ag-specific splenic B cell frequencies over naive mice (0.47 ± 0.02%; p < 0.05) were not significantly different between mice immunized i.v. (1.15 ± 0.13%) or i.p. (1.00 ± 0.04%; data not shown). Similarly, the increases observed in total Ag-specific peritoneal B cell frequencies over naive mice (0.75 ± 0.08%; p < 0.05) were not significantly different between mice immunized i.v. (1.60 ± 0.22%) or i.p. (1.67 ± 0.31%; data not shown). Subcutaneous immunization elicited weaker, but nonetheless significant, increases in Ag-specific splenic B cell frequencies (0.63 ± 0.11% versus 0.47 ± 0.02% for naive; p < 0.05) and increases in Ag-specific peritoneal B cell frequencies (1.05 ± 0.12% versus 0.75 ± 0.08% for naive). Thus, i.p. and i.v. immunizations elicit similar increases in Ag-specific splenic and peritoneal B cell frequencies 5 d following immunization, whereas s.c. immunization elicits a weaker response.

Ag-specific B cell phenotypes were assessed to determine whether the Ag-specific B cell subset(s) responding to TNP-Ficol were altered by the route of Ag delivery. As shown in Fig. 2A and 2B, the frequencies of CD21intCD1dint, CD21hiCD1dint, CD21lo-int CD1dint, and CD21hiCD1dlo Ag-binding splenic B cells were examined. Significant increases in CD21hiCD1dint Ag-specific B cells (>4-fold) and CD21hiCD1dlo Ag-specific B cells (>2-fold) were observed relative to naive mice (Fig. 2B). These increases were comparable between mice immunized i.p. or i.v. (p > 0.05). Ag-specific CD21hiCD1dint (mainly follicular B cells) and CD21hiCD1dlo (MZ B cells) frequencies were not significantly changed 5 d following i.p. or i.v. TNP-Ficol immunization. Moreover, CD11b+ Ag-specific cells appeared in CD21hiCD1dint and CD21hiCD1dlo populations following immunization, regardless of whether TNP-Ficol was delivered by i.p. or i.v. route (Fig. 2C). In contrast, Ag-specific CD21hiCD1dint and MZ B cell populations exhibited minimal CD11b expression following immunization. Notably, no significant difference was observed in the frequencies of Ag-specific CD11b+ splenic B cells in mice immunized i.p. versus i.v. (data not shown). Thus, i.p. and i.v. immunizations altered the frequency of Ag-specific TNP-Ficol–binding B cells, but the route of Ag delivery had a modest effect on the magnitude of this increase.
TNP-Ficoll immunization elicits similar increases in CD11b+ Ag-specific splenic B cells that coexpress a CD21intCD1dint or CD21loCD1dlo phenotype. Consistent with the results above, i.v. and i.p. immunization resulted in similar increases in CD19+CD11b+CD5neg-lo Ag-specific splenic B-1b cell frequencies and numbers (Fig. 2D; 10-fold) over naive mice. Subcutaneous immunization also increased CD11b+ Ag-specific B-1b cell frequencies and numbers over naive mice, albeit to a lesser extent (data not shown). Moreover, regardless of the route of immunization, the majority of Ag-specific IgG3+ B cells coexpressed CD11b+ (Fig. 2D). In addition, similar frequencies of the Ag-specific splenic B-1b cell pool expressed IgG3 in i.p. and i.v. immunized mice (Fig. 2F), and no differences were observed in the frequencies of IgG3+ Ag-specific B-1b cells in the peritoneal cavity. These results indicate that PD-1 expression is induced on Ag-specific B-1b cells in vivo.

PD-1 expression is induced on Ag-specific B-1b cells in vivo. As shown in Fig. 3, PD-1 upregulation is largely

**FIGURE 2.** Ag-specific B-1b cells participate in the response to TNP-Ficoll, regardless of whether Ag is delivered i.p. or i.v. A–F, Flow cytometric analysis and enumeration of splenic and peritoneal Ag-specific B cells from naive and immune mice (50 µg TNP65-Ficoll administered i.p. or i.v.; day 5). Representative flow cytometric analysis (A) and frequencies (B) of Ag-specific splenic B cells in naive and immune mice expressing a CD21intCD1dint, CD21loCD1dlo, and CD21hiCD1dhi (MZ B) phenotype. C, CD11b expression by CD21intCD1dint, CD21loCD1dlo, and CD21hiCD1dhi Ag-specific cells. D, Frequencies and numbers of splenic and peritoneal Ag-specific B-1b (CD19+CD11b+CD5neg-lo) cells in naive and immune mice. E, IgG3 expression by Ag-specific splenic B (CD19+) cells (upper panels) and CD11b expression by IgG3+ Ag-specific cells (middle panels). F, CD138 expression by Ag-specific splenic B cells (upper panels) and CD11b and B220 expression by CD138+ Ag-specific cells (middle panels). Frequencies of CD19+ Ag-specific cells ("Total") and CD19+CD11b+ Ag-specific cells ("CD11b+") expressing IgG3 are shown for immune mice. F, CD138 expression by Ag-specific splenic B cells (upper panels) and CD11b and B220 expression by CD138+ Ag-specific cells (middle panels). Frequencies of B220+ Ag-specific cells ("Total") and B220+CD11b+ Ag-specific cells ("CD11b+") expressing CD138 are shown for immune mice. Results are representative of data obtained with at least three mice/group. Data in B and D–F represent mean (± SEM). *p < 0.05, naive versus immune mice.
confined to CD11b-expressing (B-1) cells. PD-1 upregulation on Ag-specific peritoneal B cells was observed as early as 1 d post-immunization and appeared on blood and spleen B-1b cells by 2 d post-immunization. PD-1 expression levels were highest between days 2 and 3, decreased by day 5, and were undetectable by day 9 postimmunization. A similar trend was observed for CD44 expression (data not shown). Thus, PD-1 is selectively and transiently induced on Ag-specific B-1b cells following TNP-Ficoll immunization.

**PD-1–BCR coengagement suppresses BCR-induced B-1b cell proliferation**

Similar to in vivo-expression kinetics, PD-1 is induced on cultured purified peritoneal B-1b cells and spleen B cells between days 1 and 2 post-BCR activation, with peak expression observed on day 3 (Fig. 4A). CD5 expression was similarly induced on BCR-activated spleen B cells and B-1b cells purified by negative bead selection (Fig. 4B, Supplemental Fig. 1C, data not shown) or FACS sorting (Supplemental Fig. 1D, 1E). CD5 expression was also induced on FACS-purified CD19−/− peritoneal B-1b cells (Supplemental Fig. 1E). Culturing cells in the presence of 5 μg/ml LPS had little effect on BCR-induced PD-1 expression levels in B-1b cells and only slightly reduced expression on splenic B cells (Fig. 4C). Similar to these results with LPS, TNFR superfamily members (i.e., BlyS receptors and CD40) do not modulate BCR-induced PD-1 upregulation on B-1b cells (27). In contrast, LPS suppressed CD5 upregulation on BCR-activated B-1b cells (Fig. 4B), consistent with that previously reported for spleen B cells (37). Thus, BCR signaling induces PD-1 expression on B-1b cells, with secondary signals supplied by LPS and TNFR family members having little effect on PD-1 upregulation.

To determine the functional consequences of PD-1 engagement on B-1b cell proliferation induced by BCR signaling, a biotinylated PD-1 mAb was used in combination with streptavidin to crosslink PD-1 independently or with the BCR using biotinylated F(ab′)2 goat anti-mouse IgM. Independent PD-1 cross-linking during BCR activation had little effect on B-1b cell or spleen B cell proliferation elicited either by anti-IgM or LPS (data not shown). However, cocross-linking PD-1 with IgM during B cell activation significantly reduced B-1b cell and spleen B cell proliferation, as measured by reduced division indices (average number of cell divisions of entire population) relative to cultures in which a biotinylated isotype control mAb was used in place of PD-1 mAb (Fig. 4D, 4E). The frequencies of B-1b cells characterized as viable (Annexin V−/7AAD−), early (Annexin V+/7AAD−), or late

**FIGURE 3.** PD-1 expression is induced on Ag-specific B-1b cells in vivo. B220+ Ag-specific peritoneal, spleen, and blood CD11b+ and CD11b− cells were evaluated for PD-1 expression (thick line) by flow cytometry on days 0, 1, 2, 3, 5, 9, and 14 postimmunization with 50 μg TNP65-Ficoll. Isotype control binding by Ag-binding cells is indicated by the shaded area.

**FIGURE 4.** PD-1–BCR coengagement suppresses B-1b cell proliferation but not survival. A, BCR-induced PD-1 surface expression on purified peritoneal B-1b cells and spleen B cells. B cells were cultured with 5 μg/ml goat anti-mouse IgM F(ab′)2 for the indicated number of days, harvested, stained with PD-1 mAb, and analyzed by flow cytometry. The shaded graphs indicate isotype control binding by activated B cells (d2). PD-1 expression by unstimulated (d3) medium (Med.)-cultured B cells (far left panels). BCR-induced CD5 (B) and PD-1 (C) upregulation on B-1b cells (B, C) and splenic B cells (C) activated with 5 μg/ml goat anti-mouse IgM F(ab′)2 in the presence or absence of 5 μg/ml LPS. The shaded graphs indicate isotype control staining in C. Proliferation and survival in BCR-activated B-1b cells (D, F) and spleen B cells (E) in the presence and absence of BCR-PD-1 cocross-linking. Purified CD5− peritoneal B or spleen B cells were labeled with 0.5 μM CFSE and cultured in the presence of 1 μg/ml biotinylated goat anti-mouse IgM F(ab′)2 along with 2 μg/ml biotinylated Armenian hamster anti-mouse PD-1 (J43) or biotinylated Armenian hamster IgG control. Streptavidin (5 μg/ml) was added to cultures as a cross-linking agent. At day 4, cells were harvested and stained with fluorochrome-labeled mAbs against CD11b and B220, with CFSE loss assessed in the B220−CD11b+ peritoneal population (D, F) or splenic B220+ population (E). Apoptosis was assessed using 7AAD and Annexin V-PE staining. Mean (± SEM) division indices were calculated from at least three experiments using FlowJo analysis software (D, E). *p < 0.05, differences between means. Proliferation for CFSE-labeled peritoneal B-1b (G) and spleen B cells (H), cultured as in D and E, along with anti-CD40 (0.5 μg/ml; HM40-3) or LPS (1 μg/ml). Symbols in H represent division indices for individual mice.
(Annexin V+/7AAD³) apoptotic cells were similar between cultures in which PD-1 was cocross-linked with the BCR compared with cultures in which biotinylated isotype control mAb was used in place of PD-1 mAb (Fig. 4F). Thus, reduced proliferation was not due to decreased survival, because the viabilities of B-1b cell cultures (as well as spleen B cell cultures) subjected to anti-IgM-PD-1 mAb cross-linking versus anti-IgM-control mAb cross-linking were not significantly different within experiments (B-1b cell cultures: 45.6 ± 16% versus 41.9 ± 13.8%, n = 3 experiments, p > 0.05, paired t test; spleen B cell cultures: 22.4 ± 4.7% versus 23.6 ± 6%, n = 5; p > 0.05, paired t test). Finally, co-stimulation supplied by either CD40 (Fig. 4G, 4H) or LPS (Fig. 4H) prevented PD-1 inhibitory effects on BCR-induced proliferation in both B-1b (Fig. 4G, data not shown) and spleen cells (Fig. 4H). Thus, PD-1 coengagement with the BCR exerts an inhibitory effect on BCR-induced B cell proliferation, but not survival, which can be overcome by co-stimulation.

**PD-1 mAb blockade significantly increases Ag-specific B-1b cell numbers and differentiation following TI-2 Ag immunization**

Given the expression pattern of PD-1 by Ag-specific B-1b cells following TNP-Ficoll immunization (Fig. 3) and its inhibitory effects on primary B cell proliferation (Fig. 4) (38), the effect of blocking PD-1 from interacting with its ligands during TI-2 Ag immunization was assessed. This was accomplished using the PD-1–blocking mAb, RMP1-14. Following TNP-Ficoll immunization, mice receiving rat IgG control Ab had significantly increased Ag-specific peritoneal B cell frequencies (1.7-fold) and numbers (2.6-fold) relative to naive mice (Fig. 5A). As expected, this increase was largely attributed to significantly increased Ag-specific B-1b cells, because significant increases were not observed in B-1a or B-2 subsets (Fig. 5B). However, mice receiving the PD-1–blocking mAb following immunization had significantly greater increases in Ag-specific peritoneal B cell frequencies (2.4-fold) and numbers (4.4-fold) relative to control mice (Fig. 5A). PD-1 mAb treatment significantly increased Ag-specific peritoneal B-1b cells over mice treated with control Ab 5 d postimmunization, but it had no effect on other Ag-binding B cell subsets (Fig. 5B). Although Ag-specific B-1b cell frequencies increased 2.6-fold in control immune mice, they increased 4-fold in mice receiving PD-1 mAb. As observed in earlier experiments (Fig. 1E), increases in Ag-specific peritoneal B-1b cells were still observed out to 40 d following immunization (Fig. 5C). However, mice receiving PD-1–blocking mAb (at days 1, 3, and 6) exhibited significantly greater increases in Ag-specific peritoneal B-1b cell frequencies and numbers relative to mice receiving control Ab (Fig. 5C). Notably, PD-1 mAb treatment did not influence overall total peritoneal B-1b cell frequencies or numbers on days 5 and 40 (Fig. 5D, data not shown). Finally, PD-1 mAb treatment resulted in significantly increased Ag-specific IgG3⁺ peritoneal B cell frequencies and numbers 5 d postimmunization (Fig. 5F), nearly all of which expressed CD11b (Fig. 5E). Thus, blocking PD-1 interactions with its ligands during TNP-Ficoll immunization significantly and selectively increased Ag-specific peritoneal B-1b cell numbers and IgG3⁺ B cells.

Immunization-induced increases in Ag-specific total B and B-1b cell frequencies and numbers were not significantly altered in blood or spleen by PD-1 mAb treatment relative to control Ab-treated mice 5 or 40 d postimmunization (Fig. 5G, 5H, data not shown). However, 5 d postimmunization, CD138 (marking plasmablast/plasma cell differentiation) was expressed by a significantly greater frequency of Ag-specific B cells in mice that received PD-1 mAb compared with mice that received control Ab (Fig. 5F). Approximately one third of these Ag-specific CD138⁺ plasmablasts expressed CD11b in both treatment groups at day 5 (data not shown). Moreover, although overall Ag-specific splenic B cell frequencies were not altered by treatment, the frequency of remaining Ag-specific splenic B cells that were IgG3⁺ at 40 d postimmunization was significantly increased (2-fold) in mice that had received PD-1 mAb blockade (0.41 ± 0.06%, n = 4) versus control Ab (0.2 ± 0.03%, n = 4; p < 0.05, data not shown). Thus, blocking PD-1-PDL interactions significantly increased isotype switching in Ag-specific B cells and the frequency of Ag-specific B cells committed to producing Ab.

**PD-1 mAb blockade significantly increases IgG production against TI-2 Ags**

Because blocking PD-1 interactions with its ligands significantly promoted increases in Ag-specific B-1b cell numbers, IgG switching, and differentiation following immunization, the effect of PD-1 mAb blockade on TI-2 Ab production was assessed. As shown in Fig. 6, PD-1 blockade had no effect on TNP-specific IgM levels following TNP-Ficoll immunization. However, PD-1 mAb blockade significantly enhanced Ag-specific IgG levels. IgG3 production, the dominant isotype produced in response to TNP-Ficoll, was significantly increased by PD-1 mAb blockade, as were Ag-specific IgG1, IgG2b, IgG2c, and IgA levels. Notably, Ag-specific IgG3 and IgG2b levels remained significantly augmented at 6 wk postimmunization in mice that had received transient early PD-1 mAb blockade. Thus, blocking PD-1-PDL interactions during the first week following immunization significantly enhanced the production of isotype-switched Ag-specific Abs, with IgG3 and IgG2b levels remaining increased up to 6 wk postimmunization.

**PD-1 mAb blockade significantly increases both early and long-term splenic and BM IgG3-producing cells in response to TI-2 Ag**

Evidence suggests that TI-2 Ag-activated, as well as TI-pathogen activated, B-1b cells may predominantly secrete long-term Ab as plasmablasts within the spleen (15, 16, 20, 39), although B-1b cells may also differentiate into long-lived BM plasma cells (40). ELISPOTs were performed to assess Ab production by splenic and BM plasma(blast) cells at both early and late time points following TNP-Ficoll immunization. At 5 d postimmunization, TNP-secreting IgM and IgG3 ASC spleen and BM numbers were significantly increased over naive mice (Fig. 7A). Relative to day-5 ASC numbers, day-40 IgM and IgG3 splenic ASC numbers were diminished, whereas BM ASC numbers were increased (Fig. 7B). Therefore, given the previous estimation of total BM cellularity in mice (1.5 × 10⁶) (41) relative to spleen (1 × 10⁶), BM ASCs likely make a substantial contribution to TNP-specific Ab levels. Notably, at both time points, IgG3 ASCs accounted for ≥75% of splenic IgG ASC numbers and ~50% of BM IgG ASCs, indicating this was the major IgG isotype produced in response to TNP-Ficoll (data not shown). Thus, TNP-Ficoll immunization induces rapid induction of splenic ASCs and long-term production of IgM and IgG3 Ab by both splenic and BM ASCs.

The effect of blocking PD-1-PDL interactions, during the first week following immunization, on ASC numbers at both early (day 5) and late (day 40) time points was assessed. PD-1 mAb treatment during the first week following TNP-Ficoll immunization significantly increased (≥ 2-fold) IgG3-secreting cells in spleen and BM at both time points relative to control Ab-treated mice (Fig. 7A, 7B). In contrast, PD-1 mAb blockade did not significantly alter Ag-specific splenic IgM-producing cell numbers relative to control Ab-treated mice at either time point (Fig. 7A, 7B).
FIGURE 5. Blocking PD-1:PD-L1 interactions during TI-2 Ag immunization increases the frequency of Ag-specific B-1b cells, IgG3+ cells, and CD138+ B cells. A–H, Ag-specific B220+ frequencies and numbers at days 5 and 40 in mice immunized with 50 μg TNP65-Ficoll i.p. and administered PD-1 (RMP1-14; black bars) or rat IgG control (gray bars) Abs (200 μg on day 1 and 100 μg on day 3; 100 μg was also given on day 5 for the 40-d experiment). Ag-specific cells and CD11b, CD5, IgG3, and CD138 expression were assessed by flow cytometry. Peritoneal Ag-specific B220+ B cell frequencies and numbers (A) and Ag-specific peritoneal B-1a, B-1b, and B-2 subset frequencies (B) in naive mice and 5 d postimmunization in mice treated with PD-1 mAb or rat IgG control. C, Ag-specific peritoneal B-1b cell frequencies and numbers in naive and immune mice 40 d postimmunization. D, Total peritoneal B-1b cell frequencies at days 0, 5, and 40 postimmunization. Ag-specific IgG3+ CD11b+ peritoneal B cell frequency plots (E) and Ag-specific B220+IgG3+ cell frequencies and numbers 5 d postimmunization (F). Blood (G) and spleen (H) Ag-specific B-1b cell frequencies and numbers at days 0 and 5 postimmunization. I, CD138+B220+ cell frequencies within the Ag-specific B cell population. The first plot demonstrates the gating strategy for Ag-binding cells. Data represent mean (± SEM) (n = 3–4 mice/group). *p < 0.05.
although there was a trend toward increased IgM-secreting BM cells at both time points. Moreover, anti-TNP–specific IgG and IgG3 levels, but not IgM levels, were significantly increased in 7-d cultures of spleen cells from PD-1 mAb-treated mice relative to control Ab-treated mice (Fig. 7C), consistent with increased IgG-secreting cell frequencies found in these mice. Thus, blocking PD-1:PDL interactions during the early stages of TI-2 Ag encounter significantly increased the number of IgG3-secreting splenic and BM ASCs found at both early (day 5) and late (day 40) time points following immunization.

**Discussion**

In this study, nontransgenic normal mice were used to analyze the activation, phenotypic alterations, and expansion kinetics of Ag-specific B cells in response to a defined prototypic TI-2 Ag. B cells responding to TNP-Ficoll expressed a phenotype consistent with activated B-1b cells, as was true for isotype-switched Ag-specific B cells (Fig. 1). Importantly, immunization induced Ag-specific B-1b cells to selectively upregulate PD-1 (Fig. 3). PD-1 was shown in multiple studies to suppress Ag-specific T cell responses. The transient blockade of PD-1:PDL interactions during TI-2 Ag immunization significantly increases the production of isotype-switched Ab. TNP-specific serum IgM, IgG, IgG1, IgG2b, IgG2c, IgG3, and IgA levels in mice immunized with 25 μg TNP-Ficoll and administered PD-1 or rat IgG control Abs, as in Fig. 5. Data represent mean (± SEM) (n = 3–4 mice/group). Similar results were obtained in an independent immunization experiment. *p < 0.05, control Ab versus PD-1 mAb.

**FIGURE 7.** Transient blockade of PD-1:PDL interactions increases both early and long-term Ag-specific IgG3-secreting cell numbers in spleen and BM. TNP-specific IgM and IgG3-secreting cells (ASCs) in spleen and BM 5 days (A) and 40 days (B) postimmunization, as determined by ELISPOT. C. TNP-specific IgM, IgG, and IgG3 levels secreted by total splenocytes (harvested 5 d postimmunization) cultured in cRPMI 1640 + 10% FCS for 7 d. Culture supernatants were diluted 1:3 in TBS containing 1% BSA and assessed for TNP-specific Abs by ELISA. Mice were immunized and administered PD-1 or rat IgG control Abs, as in Fig. 5. Data represent mean (± SEM) (n = 3–4 mice/group). *p < 0.05, control Ab versus PD-1 mAb. Although not indicated, in all cases Ag-specific ASC numbers and anti-TNP Ig levels were significantly higher in immune mice compared with naive mice. N.D., not detected.
expansion and function (42) and, in the current study, was shown to similarly inhibit AgR-induced proliferation of B-1b cells in vitro (Fig. 4). PD-1 expression by Ag-specific B-1b cells may similarly contribute to suppression of Ag-specific B-1b cell expansion and/or Ab production in vivo, because PD-1 mAb blockade significantly enhanced Ag-specific B-1b cell numbers, IgG3 switching, and Ab production (Figs. 5–7). Collectively, the results of this study demonstrated a key role for the PD-1:PD-L regulatory axis in controlling B-1b cell responses and IgG production to TI-2 Ags.

The division of labor among B cell subsets in Ab production against TI-2 Ags is not completely clear, with B-1b cells, B-1a cells, MZ B cells, and even follicular B cells (43), under certain circumstances, implicated in producing TI-2 Ab responses. Work by multiple laboratories demonstrated a key role for B-1b cells in producing Abs in response to defined TI-2 Ags, including type 3 pneumococcal polysaccharide (14, 40), TNP-Ficoll (15), and α-1,3 dextran (16); additional TI Ags (17–19); and the Gal αL3Galβ1–4GlNAc carbohydrate epitope involved in transplant rejection (34). Nonetheless, MZ B cells and B-1a cells may also contribute to Ab production against these and other TI-2 Ags (16, 30, 31), including phosphorylcholine when either displayed on bacteria (21) or on Ficoll as a TI Ag (14). These subsets may respond differentially to Ag and/or additional cues to produce Abs (23, 44). The present study demonstrates that the Ag-specific B cells that rapidly become activated, expanded, and/or mobilize in response to TNP-Ficoll are CD11b+CD19−CD21−CD44+CD86+CD5− CD80+CD1d+CD23+ B220−CD43+PD-1−CD5−/lo (Fig. 1), consistent with an activated B-1b cell phenotype (Fig. 4) (27). Importantly, CD5 was expressed by Ag-specific B-1b cells at a lower level than that found for B-1a cells (Fig. 1B, Supplemental Fig. 1A) and was present on Ag-specific B cells from immunized (but not naive) CD19−/− mice (Supplemental Fig. 1B), which lack B-1a cells but produce near-normal Ab responses to TNP-Ficoll (14, 27). Increased CD5 expression is observed on BCR-activated spleen B cells (Fig. 4B) (37) and bead-purified B-1b cells (Fig. 4B, Supplemental Fig. 1C). Because CD5iso B cells could have remained following bead selection, wild-type and CD19−/−−CD5− B-1b cells were also FACS sorted to high purity and were similarly found to express increased CD5 levels with BCR activation (Supplemental Fig. 1D, 1E). Thus, low CD5 expression on Ag-specific B-1b cells following immunization can be indicative of AgR-mediated B-1b cell activation. Alternatively, it remains possible that TNP-Ficoll induces expansion of Ag-specific CD5iso B-1 cells as opposed to increasing expression of CD5 on Ag-activated B-1b cells. Interestingly, Francisella tularensis LPS (Ft-LPS), an extremely weak TLR4 agonist, elicits a similar Ag-specific TI response to that observed with TNP-Ficoll, with i.p. Ft-LPS, an extremely weak TLR4 agonist, elicits a similar Ag-specific TI response to that observed with TNP-Ficoll, with i.p. Ft-LPS reported demonstrating that plasmablasts also reside in the BM (48). Infection models using Enterobacter cloacae (16) and Borrelia hermsii (17) similarly demonstrated that B-1b cells may yield a form of unconventional memory that may be attributed to the presence of long-lived splenic B-1b plasmablasts. Nonetheless, it is evident from the present study that, although splenic ASCs produce the majority of TI-2 Ab in the early stages of the immune response, BM ASCs make a substantial contribution to persistent TI-2 Ab levels. Importantly, the degree to which B-1b cells contribute to BM ASCs in response to TNP-Ficoll was not resolved by the current study. Notably, B-1b cells were shown to give rise to BM ASCs in response to PPS-3 (40), and BLIMP-1+ B-1 B cells can seed the long-lived ASC compartment (47). However, whether B-1b cell-derived BM ASCs represent plasmablasts or fully differentiated BM plasma cells is not clear, given a recent report demonstrating that plasmablasts also reside in the BM (48). In contrast to results with spleen B-1b cells, Ag-specific peritoneal B-1b cell numbers remained elevated at peak levels up to ∼6 wk following immunization (Figs. 1E, 5C). This long-term maintenance of peritoneal Ag-specific B-1b cells following immunization has not been observed (49) or examined (50, 51) in previous studies using BCR transgenic mouse strains to examine B cell expansion in response to purified TI-2 Ags, although it was reported for α-1,3 dextran-specific B-1b cells in VgJ558 Tg mice following E. cloacae challenge (16). Moreover, accumulation and maintenance of Ft-LPS–specific peritoneal B-1a cells 2 mo following Ft-LPS immunization were reported by Cole et al. (22). The explanation and significance of this finding are unclear, but studies examining the functionality and role of these peritoneal cells are underway. In summary, Ag-specific B-1b cell numbers significantly increased in multiple tissues during the first week following TI-2 Ag immunization and, with the exception of the peritoneal B cells, gradually declined thereafter. Further studies...
aimed at examining the signals controlling Ag-specific B-1b cell expansion, contraction, and differentiation, as well as long-term maintenance, are warranted.

The results of this study demonstrated a significant role for PD-1:PDL interactions in regulating B-1b cell function. First, BCR cross-linking induced PD-1 expression on B-1b cells, and PD-1 significantly inhibited BCR-induced proliferation when cocross-linked with the BCR, without enhancing apoptosis (Fig. 4), in a manner similar to that observed for splenic B cells (Fig. 4) (38). Second, Ag-specific B-1b cells in spleen, peritoneal cavity, and blood were specifically induced to express PD-1 following immunization, with the highest levels observed between days 2 and 5. Finally, interfering with PD-1:PDL interactions by administrating a PD-1–blocking mAb between days 1 and 6 post-immunization significantly increased Ag-specific B-1b cell numbers, the frequency of Ag-specific B-1b cells switching to IgG3, the frequency of Ag-specific splenic CD138+ cells, and Ag-specific IgG production by both splenic and BM ASCs (Figs. 5, 7). These increases were accompanied by significant increases in Ag-specific serum IgG, as well as IgA, levels (Fig. 6). Although it is possible that anti-TNP BM and spleen ASCs are largely derived from Ag-specific B-1b cells, it remains unresolved whether this is the case, as well as whether increased BM or spleen ASCs observed with PD-1 mAb blockade are due to expanded peritoneal B-1b cells. The fact that transient PD-1 mAb blockade applied during the first week of immunization resulted in significantly increased Ag-specific B-1b cell numbers and IgG-producing ASCs at both early and late time points suggested that PD-1:PDL interactions play a critical role in inhibiting the early immune response to TI-2 Ags and that the splenic and BM IgG ASCs generated during the early response to TI-2 Ags are long-lived.

PD-1:PDL interactions may limit Ag-specific B-1b cell expansion and IgG production by several mechanisms. Because in vitro-proliferation assays support an inhibitory role for PD-1 in regulating BCR-driven proliferation in B-1b cells, simultaneous interaction between a TI-2 Ag-activated (PD-1–expressing) B-1b cell and a PDL-expressing cell provides a likely mechanism by which PD-1:PDL interactions may limit AgR signals that drive B-1b cell proliferation in vivo. Importantly, it is not clear whether TNP-specific IgG production by additional B cell subsets is modulated by PD-1. Because B cell division is required for isotype switching (52, 53), PD-1 inhibitory signaling in TI-2 Ag-activated B cells would, thereby, limit both clonal expansion and isotype switching. Indeed this is what is observed. Notably, if TI-2 Ags are associated with PAMPs or elicit T cell help via protein association, costimulatory signals (e.g., LPS, CD40L) may help to overcome PD-1 inhibitory signals (Fig. 4G, 4H) or upregulation in certain cases (38). Although PD-1’s effects have most often been studied in the context of AgR signaling, PD-1 has effects on other non-AgR–bearing cell types and, therefore, must regulate additional signaling pathways. Thus, PD-1 may modulate B cell survival, proliferation, and/or differentiation independently of its effects on AgR signaling. Interestingly, PD-1 blockade in macaques during chronic SIV infection was proposed to increase SIV Env–binding Ab titers (54), as well as other humoral memory responses, by preventing deletion of activated PD-1+ memory B cells (55).

Although the in vitro results in this study (Fig. 4F) demonstrated that PD-1–BCR coligation suppressed proliferation as opposed to survival, it remains possible that PD-1 influences Ag-specific B-1b cell survival in vivo following activation. Finally, it remains possible that PD-1, expressed by some other cell type, regulates B-1b cell responses to TI-2 Ags. For example, PD-1 expression by follicular Th cells, as opposed to B cells, plays a significant role in promoting (as opposed to suppressing) germinatal center B cell responses and plasma cell formation and, hence, Ab production, in response to T cell-dependent Ags (56).

Future experiments using PD-1 conditional knockouts or mixed BM chimeras will be required to test whether PD-1 expression by B-1b cells contributes to suppression of TI-2 Ab responses.

PD-1 shares functional similarity to other B cell-inhibitory receptors expressed on B-1b cells, including CD5 and CD22, which recruit and activate SHP-1 to dampen BCR signaling. Although their expression patterns differ, these receptors are likely involved in maintaining B cell tolerance, because mice deficient in any of these receptors have hyperresponsive B cells that produce autoantibodies (57–66). Interactions between these B cell-expressed inhibitory receptors and host cell surface ligands likely function in suppressing Ab responses against self-Ags. Nonetheless, PD-1, CD5, and CD22 may have distinct roles in regulating Ab responses to immunogenic TI-2 Ags versus self-Ags, because TI-2 Ab responses are augmented in PD-1−/− mice (29), are normal in CD5−/− mice (67), and are decreased in CD22−/− mice (63–65, 68). However, recent work by Nemazee and colleagues (68) indicated that CD22 promotes tolerance to TI-2 Ags that are decorated with sialic acid (self) ligands. Thus, PD-1 and other B cell immunoinhibitory receptors may play complex roles in regulating B cell responses against self-Ags versus foreign TI-2 Ags.

In summary, this study revealed an immunoinhibitory role for PD-1:PDL interactions in regulating B-1b cell responses to TI-2 Ags, with particular significance in suppression of long-lasting Ag-specific IgG production. These findings may have important implications for current strategies targeting PD-1:PDL interactions as treatment modalities for multiple diseases and conditions. More importantly, these findings may have significance for TI-2 Ag-based vaccine development. TI-2 Ab responses in mice parallel those in humans (11), and a human B-1 cell counterpart was recently identified (69). Thus, it is possible that PD-1:PDL interactions similarly suppress TI-2 IgG responses in humans. In contrast to IgM, IgG is produced in limiting quantities against most TI-2 Ags. Nonetheless, because IgG may elicit enhanced protection against carbohydrate-bearing pathogens relative to IgM, future strategies transiently targeting the PD-1:PDL pathway may provide an opportunity to elicit enhanced IgG-mediated protection against TI-2 Ag-bearing pathogens.

Disclosures

The author has no financial conflicts of interest.

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3. Wang, T. T., and A. H. Lucas. 2004. The capsule of <i>Schistosoma mansoni</i> is decorated with sialic acid (self) ligands. Thus, PD-1 and other B cell immunoinhibitory receptors may play complex roles in regulating B cell responses against self-Ags versus foreign TI-2 Ags.

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12 PD-1 REGULATION OF B-1b CELL RESPONSES TO TI-2 Ags


Supplemental Figure 1
Supplemental Figure 1. CD5 expression on Ag-specific B cells from CD19^{−/−} and wild type mice following TNP-Ficoll immunization and on total FACS-purified peritoneal B-1b cells. A-B, CD5 expression on Ag-specific B220^{+} splenocytes from wild type (A) and CD19^{−/−} (B) mice immunized with 50 μg TNP-Ficoll (thick line; d3). CD5 expression on Ag-specific B cells from naïve CD19^{−/−} mice is indicated by the shaded histogram (B) and isotype control staining is shown by the dashed line (A-B). In (A), CD5 expression levels by total (non-Ag-specific) peritoneal B-1a cells (thin line) and T cells (shaded histogram) are also shown. C, Streptavidin-FITC-based detection of Thy1.2-depleted peritoneal B220^{+} B cells labeled with biotinylated CD5, GR1, DX5, and F4/80 mAbs before (shaded histogram) and after (thick line) streptavidin bead depletion. Purity of negatively-selected CD11b^{+}B220^{+}CD5^{−} cells is shown (right plot). D, Wild type peritoneal cells were stained with fluorochrome-labeled mAbs against B220, CD5, and CD11b. B220^{+}CD5^{−}CD11b^{+} (B-1b) cells were FACS sorted to 99% purity and were CD5^{−} as shown in the post-sort histogram. E, Wild type and CD19^{−/−} peritoneal B-1b cells FACS-purified as in (D) were cultured in medium or with goat anti-IgM mouse F(ab’)_{2} Ab for 2 days and assessed for CD5 expression by flow cytometry.