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B7-H1 Promotes Germinal Center B cell Survival and Inhibits T cell Activation

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SUMMARY

The immune system has developed several regulatory mechanisms to maintain homeostasis of adaptive immune responses. T cell PD-1 recognition of B7-H1 (PD-L1) expressed on antigen presenting cells and non-lymphoid tissue regulates T cell activation. We show that B7-H1^{-/-} mice exhibit exacerbated proteoglycan-induced arthritis (PGIA) and increased T helper-1 CD4⁺ T cell responses. Unexpectedly, the PG-specific antibody response in B7-H1^{-/-} mice was diminished. A reduction in the number of PNA⁺ germinal centers (GCs) coincided with a decrease in CD19⁺GL-7⁺CD95⁺GC B cells that was a result of increased caspase-induced apoptosis. The percent of CD38⁺CD138⁺ emerging plasma cells was decreased. B7-H1^{-/-} mice exhibited an increased frequency of CD4⁺PD-1^{hi}CXCR5^{hi}ICOS^{hi}CD62L^{lo} T follicular helper (T_{FH}) cells that displayed a hyperactive phenotype with increased expression of mRNA transcripts for Bcl6, IL-21 and the apoptosis-inducer molecule FasL. In cell-transfer of B7-H1^{-/-} cells into SCID mice, non-B non-T cells were sufficient to normalize the antibody response, T cell hyperactivity and the development of PGIA. These studies indicate that B7-H1 on non-B non-T cells signal PD-1 on T effector cells to prevent excessive activation and reduce autoimmune arthritis. Furthermore, these studies demonstrate a novel role for B7-H1 expression in promoting B cell survival by regulating the activation of T_{FH} cell.

Keywords

B-cells; B7-H1; T_{FH}-cells; Inflammation; Antibodies

INTRODUCTION

Chronic inflammation associated with autoimmunity, characteristic of rheumatoid arthritis (RA), is reliant on the activation of self-reactive CD4⁺ T cells and autoantibody secreting B cells [1]. Along with initial signals through the BCR or TCR, secondary signals in the form

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CONFLICTS OF INTEREST

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of membrane bound co-stimulatory and co-inhibitory molecules modulate the intensity and type of adaptive immune response [2, 3].

Programmed death (PD)-1 is a co-inhibitory molecule expressed by B cells, dendritic cells (DCs), natural killer T cells, activated monocytes, activated T cells and also defines T-follicular helper (T_{FH}) and “exhausted” T cells [4, 5]. Signals through PD-1 inhibit T cell activation and are critical for the suppression of autoimmunity [6, 7]. PD-1 ligands include B7-H1 (PD-L1), B7-DC (PD-L2), and most recently B7-1 (CD80) [2, 8]. Current work suggests bidirectional signaling capabilities of B7-H1 and B7-1 when expressed on CD4⁺ T cells which leads to impaired T cell proliferation, IFN- γ and IL-2 production [8]. Expression of B7-DC is inducible on DCs, macrophages, mast cells, and some peritoneal B1 B cells, while B7-H1 and B7-1 are expressed on most APCs including activated B cells. B7-H1 is also present on non-hematopoietic cells [2, 9].

The recent efficacy of B cell depletion therapy in RA patients highlights the importance of B cells as mediators of chronic inflammation [10]. Similarly, B cell deficient mice are completely resistant to arthritis [11, 12]. Once activated by T cells at the T-B cell border, antigen-stimulated B cells rapidly proliferate in the follicles of secondary lymphoid organs and form germinal centers (GCs) where they undergo somatic hypermutation and isotype switch resulting in memory B cell generation and long-lived plasma cells (PC) that secrete high affinity antibody [13, 14]. T_{FH} cells found within the B cell follicle and GCs are required for the differentiation of B cells into memory B cells or long-lived antibody secreting PCs through several extrinsic factors including ICOS and IL-21 and the transcription factor Bcl6 [15]. Without stringent regulation, the rapid proliferation and increased mutation rate of B cell immunoglobulin genes within the GC could result in autoreactive B cell development and the survival of low affinity clones with cross-reactivity for self-antigen [16, 17]. The suppression of self-reactive T_{FH} cells is also necessary for prevention of systemic autoimmunity [18]. Aside from the expression of the E3 ubiquitin ligase roquin and the presence of follicular T-regulatory cells, few mechanisms for suppressive of T_{FH} cells have been described [19, 20]. The prominent expression of PD-1 on T_{FH} cells combined with the upregulation of B7-H1 on activated B cells suggests the possibility that PD-1/B7-H1 interactions may regulate survival of self-reactive B cells during GC reactions [21–23]. Good-Jacobson et al. recently demonstrated that expression of PD-L2 on B cells and PD-1 on T cells regulates plasma cell production and memory B cell formation [24]. However, these studies do not assess directly a role for B7-H1 (PD-L1) and PD-1 interaction in B cell responses.

In the present study, we have examined the role of B7-H1 in the development of PGIA and determined the contribution of B7-H1 expression to PG-specific T cell and B cell responses. In B7-H1^{-/-} mice a more aggressive form of PGIA developed than in WT mice that coincided with a significant increase in CD4⁺ T helper (Th) 1 cell numbers and cytokine production. However, despite the increase in arthritis and T effector responses, antibody responses were inhibited. Reduced antibody titers in B7-H1^{-/-} mice correlated with a decreased frequency of GCs and PCs. The reduced GCs and PCs in B7-H1 mice were linked to an increase in the frequency and hyperactive phenotype of T_{FH} cells. An increase in FasL RNA transcripts in T_{FH} along with enhanced apoptosis in the GC B cells suggest that B7-H1/PD-1 interactions are modulating differentiation or selection of B cells in the GC. Cell-transfer into SCID mice showed that the diminished B cell response was not an intrinsic effect of B7-H1 expression on B cells or T cells. In addition, B7-H1 expression by non-B non-T cells was sufficient to prevent the exacerbation of arthritis and CD4⁺ T cell activation. These studies confirm the well established role for B7-H1/PD-1 interaction as inhibiting T cell activation as well as identifying a novel role for systemic B7-H1 expression in modifying the production of B cells through the maintenance of GC and PC B cells.

RESULTS

B7-H1 regulates autoimmune arthritis by suppressing autoreactive CD4⁺ T cell responses

To determine if B7-H1 expression is sufficient to regulate the development of arthritis, we assessed induction of arthritis in BALB/c WT and B7-H1^{-/-} mice immunized with PG-DDA and evaluated the onset and severity of arthritis. In B7-H1^{-/-} mice, development of arthritis was significantly more aggressive and severe than in WT mice (Fig. 1A). By 11 weeks, B7-H1^{-/-} mice reached an average arthritis score of 13.35 ± 0.2 SEM while the average WT mice arthritic score was 7.89 ± 0.14 SEM ($p < 0.05$). In addition, the onset of PGIA in B7-H1^{-/-} mice was earlier than WT mice (Fig. 1B). To determine the extent of inflammation, we examined hind paw histology in WT and B7-H1^{-/-} mice early and late in the development of disease (Fig. 1C–G). The histologic picture at the early time point in B7-H1^{-/-} mice was characteristic of acute arthritis. Abundant mononuclear and polymorphonuclear infiltration in tissue and joint space with edema of the synovial and periarticular tissues were accompanied by synovial hyperplasia. In the articular surface, cartilage erosion and disintegrating chondrocytes were observed. Conversely, in WT mice at this early time point, cellular infiltration and histopathologic signs of arthritis were significantly reduced. Eventually, WT mice succumb to similar disease as B7-H1^{-/-} mice as there were no significant differences in histological score at later time points.

The increased severity of PGIA in B7-H1^{-/-} mice suggested a dysregulation in T cell or B cell autoreactivity. We therefore assessed PG-specific T cell responses. CD4⁺ T cells were purified from spleens of arthritic mice and restimulated in vitro with naïve splenocytes and PG. B7-H1^{-/-} CD4⁺ T cell proliferation was significantly enhanced in comparison to WT CD4⁺ T cells (Fig. 1H). CD4⁺ T cells from B7-H1^{-/-} mice had normal percentages of Tregs, but displayed a more activated phenotype as indicated by CD62L expression (Fig. 1I & J). PGIA is defined as a Th1-mediated disease dependent on IFN- γ and not on IL-17 [25]. T cells from B7-H1^{-/-} mice produced significantly more IFN- γ and IL-4 (Fig. 1K & M) and the frequency of IFN- γ and IL-4-specific T cells were increased (Fig. 1L & N). The amount of IL-17 and the frequency of IL-17 producing CD4⁺ T cells detected from B7-H1^{-/-} mice, however, were significantly reduced (Fig. 1O & P). These data indicate that the severity of PGIA in B7-H1^{-/-} mice is concomitant with an elevated PG-specific CD4⁺ T cell reactivity that produces an enhanced IFN- γ response. Since PGIA is reliant on autoantibody production, we anticipated in the presence of exacerbated T cell effector function that the PG-specific antibody response would increase.

Germinal center and plasma B cell generation are reduced in B7-H1^{-/-} mice

We next tested the PG-specific antibody response in B7-H1^{-/-} mice. The titers of IgG1 and IgG2a antibodies specific for human and mouse PG were measured at several different time points over the course of disease; serum samples were collected at 1, 4, 7 and 10 weeks after the initial PG-DDA immunization. The IgG2a response to human PG was significantly reduced whereas the IgG1 response was reduced, but not significantly (Fig. 2A & B). The PG-specific IgG1 and IgG2a titers to mouse PG were significantly reduced in B7-H1^{-/-} mice (Fig. 2C & D).

Since antibody secreting B cells are a product of late GC reactions, the diminished autoantibody titers may have resulted from reduced GCs and emerging PCs [26]. Histological sections of spleens obtained from B7-H1^{-/-} mice 1 week after the second PG-DDA immunization displayed significantly decreased numbers of PNA-positive GCs in comparison to WT mice (Fig. 3A). In addition, the percent of CD19⁺ cells that were double positive for the germinal center markers GL-7 and CD95 were reduced in B7-H1^{-/-} mice (Fig. 3B). The decrease in PNA⁺ GCs correlated with a reduction in the percent of CD38⁺

CD138⁺ emerging PCs present in spleens of B7-H1^{-/-} mice (Fig. 3C). These data indicate that B7-H1 expression modulates the B cell antibody response through the maintenance of GCs and the subsequent emergence of antibody-secreting PCs. Therefore, in light of a recent report describing bidirectional signals of B7-H1 in T cells, the decrease in the humoral immune response of B7-H1^{-/-} mice may be a result of intrinsic properties of B7-H1^{-/-} B cells [8]. However, naïve B7-H1^{-/-} mice exhibited similar percentages of splenic T1, T2, marginal zone and follicular B-cells and also displayed a similar capacity to proliferate in response to LPS or anti-CD40 and anti-IgM stimulation as naïve WT mice (Fig. 3D & E). Thus, these data indicate that B7-H1^{-/-} B cells develop and respond to external stimuli normally.

T_{FH} cells are hyperactive in B7-H1^{-/-} mice

We next determined whether the reduction in GC and PC responses correlated with an alternation in T_{FH} frequency and phenotype. Spleens harvested 10 days following the second PG-DDA immunization from B7-H1^{-/-} mice contained significantly higher numbers of CD4⁺PD-1^{hi}CXCR5^{hi}ICOS^{hi}CD62L^{lo} T_{FH} cells, which coincided with a decrease in T_{FH} cell death as measured by caspase activation that was detected using a fluorescent pan-caspase inhibitor designated as CaspGlow (Fig. 4A–C). In addition, surface expression of ICOS was increased on T_{FH} cells from B7-H1^{-/-} mice (Fig. 4D). We further assessed the expression of mRNA transcripts on T_{FH} cells sorted on the basis of CD4⁺CXCR5^{hi}CD62L^{lo} for several molecules associated with T_{FH} cell function [27]. B7-H1^{-/-} T_{FH} cells expressed increased mRNA transcripts for both Bcl6 and IL-21 (Fig. 4E & F). Interestingly, mRNA transcripts for the proapoptotic molecule FasL was also significantly elevated compared to WT T_{FH} cells (Fig. 4G). Elevated expression of FasL by T_{FH} cells was commensurate with an increase in caspase-induced GC B cell death (Fig. 4H & I). Since GC B cells are highly sensitive to FasL mediated negative selection, these data indicate that a deficiency in B7-H1 leads to increased activation of T_{FH} cells and FasL expression that results in increased death of GC B cells [26].

Non-B non-T cells provide B7-H1 signals required for B cell antibody responses

To determine if diminished autoantibody responses were a direct or indirect signal through B7-H1 to B cells, we reconstituted SCID mice with B cells or B and T cells specifically lacking B7-H1. To create a deficiency of B7-H1 specifically on B cells, SCID mice were reconstituted with WT B-depleted spleen cells plus B7-H1^{-/-} B cells (T^{WT} B^{B7-H1^{-/-}}) and to create a deficiency in both B and T cells SCID mice were reconstituted with spleen cells from B7-H1^{-/-} mice (T^{B7-H1^{-/-}} B^{B7-H1^{-/-}}). Transfer of WT spleen cells (T^{WT} B^{WT}) served as positive control mice. Seven days after reconstitution, SCID mice were immunized with PG and spleens were obtained 1 week after the second immunization with PG. SCID mice reconstituted with T^{WT} B^{B7-H1^{-/-}} displayed a similar percent of GC CD19⁺ GL-7⁺ CD95⁺ cells and PC CD38⁺ CD138⁺ cells as T^{WT} B^{WT} control mice (Fig. 5A & B). A restoration of serum titers of anti-human PG IgG1 and IgG2a and anti-mouse PG IgG1 in T^{WT} B^{B7-H1^{-/-}} was also observed (Fig. 5C–E). Anti-mouse IgG2a was below the detection of our assay. These data demonstrate that GC and PC responses were not regulated directly through expression of B7-H1 on B cells. The contribution of B7-H1 by T cells was also not a contributing factor to the reduced antibody response as T^{B7-H1^{-/-}} B^{B7-H1^{-/-}} mice had normal antibody responses and the percent of GC and PC was similar to T^{WT} B^{WT} controls (Fig. 5A–E). Collectively, these data indicate that expression of B7-H1 on non-B cell non-T in the SCID recipient was sufficient to deliver a positive signal for B cell autoantibody production and GC formation.

Non-B non-T cells provide B7-H1 signals required for development of arthritis

We next determined if B cell expression of B7-H1 modulates the T cell response in PGIA. Naïve and LPS activated B cells from WT and B7-H1^{-/-} mice were used to stimulate CD4⁺ T cells from PG-immunized WT mice in vitro. As expected, LPS activation of B cells from both WT and B7-H1^{-/-} mice induced significantly more CD4⁺ T cell proliferation than naïve B cells (Fig. 6A). However, both naïve and activated B7-H1^{-/-} B cells stimulated significantly more CD4⁺ T cell proliferation than either naïve or activated WT B cells (Fig. 6A). Thus, B7-H1 expression by B cells delivers an inhibitory signal to PG-specific CD4⁺ T cells in vitro.

To determine if specific deficiency of B7-H1 on B cells could account for the enhanced arthritis and autoreactive T cell response in B7-H1^{-/-} mice, we reconstituted SCID mice with B7-H1^{-/-} B cells (T^{WT} B^{B7-H1-/-}) as described above. Arthritis developed in T^{WT} B^{WT}, T^{WT} B^{B7-H1-/-}, and T^{B7-H1-/-} B^{B7-H1-/-} mice with similar incidence and severity (Fig. 6B & C). The CD4⁺ PG-specific T cell proliferative response was also similar in T^{WT} B^{WT}, T^{WT} B^{B7-H1-/-}, and T^{B7-H1-/-} B^{B7-H1-/-} mice (Fig. 6D). These data indicate that expression of B7-H1 on non-B cell non-T cells was sufficient to control the elevated T cell activity and arthritis severity observed in B7-H1^{-/-} mice.

DISCUSSION

PD-1/B7-H1 (PD-L1) interactions are critical in inducing T cell tolerance in autoimmunity [28]. Our data in the PGIA model confirm these findings and show that B7-H1^{-/-} mice develop early onset and severe arthritis with enhanced PG-specific CD4⁺ T cell responses with elevated Th-1 IFN- γ production. There appears to be reciprocal regulation between IFN- γ and B7-H1 as IFN- γ is reported to induce B7-H1 expression and B7-H1/PD-1 interactions inhibit IFN- γ expression [4, 29]. Since PGIA is a Th1-mediated disease, the increase in IFN- γ in B7-H1^{-/-} mice is expected to contribute to the increase in disease severity [25]. In contrast to IFN- γ , IL-17 (Th17) is reduced in B7-H1^{-/-} mice. We have reported that IFN- γ regulates IL-17 production in PGIA [30]. Hence, the increase in IFN- γ also contributes to the reduction in the IL-17 response in B7-H1^{-/-} mice in vivo.

Interestingly, we found autoantibody production was inhibited in B7-H1^{-/-} mice which correlated with a decrease in the number of PNA⁺ GCs and a reduction in the percent of GL-7⁺CD95⁺ GC B cells. Since GCs are not completely absent in B7-H1^{-/-} mice, the deficiency is probably in the maintenance and not the generation of the GC. The modest effect of B7-H1 may be due to additional molecules that cumulatively contribute to the B cell antibody response. Each individual interaction may only have a modest effect, but together, may dramatically determine the formation of the B cell antibody response. The percent of CD38⁺CD138⁺ PCs, which are produced late in the GC reaction, were also reduced in B7-H1^{-/-} mice. Good-Jacobson et al. reported similar results in PD-1, PD-L2, and PD-L1/PD-L2-deficient mice [24]. Induction of GCs and PCs could be an intrinsic property of B7-H1 expressing B cells during antigen stimulation or early B cell development [8]. However, we found no difference between B7-H1^{-/-} and WT B cell development or proliferative responses.

The expression of PD-1 on T_{FH} cell suggests that B7-H1/PD-1 interaction may be important in the regulation of GC B cells [21]. The increase in frequency of T_{FH} cells along with their expression of ICOS and several mRNA transcript including IL-21, Bcl6, and FasL indicated that T_{FH} and T effector cells are both hyperactive in the absence of B7-H1. T_{FH} cells produce IL-21 in response to ICOS stimulation [31]. ICOS and IL-21 are both critical for antibody production as ICOS^{-/-} and IL-21R^{-/-} mice have severely impaired antibody and GC development similar to B7-H1^{-/-} mice [32, 33]. Conversely, IL-21 upregulates PD-1

expression and is capable of inducing B cell death through down-regulation of the anti-apoptotic molecules Bcl-2 and Bcl-x_L [34, 35]. In addition, GC B cells highly express Fas and are sensitive to Fas-mediated negative selection through the activation of caspase cascade and subsequent apoptosis [26, 36]. The increase in IL-21 production and expression of FasL on T_{FH} cells correlates with the increase in the apoptosis of GC B cells in B7-H1^{-/-} mice. Thus, B7-H1/PD-1 interactions may impart important B cell survival signals for the maintenance of GC B cells and PCs. In contrast, Good-Jacobson et al show, despite a greater number of T_{FH} cell in PD-1^{-/-} mice, IL-4 and IL-21 mRNA expression was reduced [24]. Thus, it is possible that signaling PD-1 by both B7-H1 and PD-L2 may increase the capacity of T_{FH} to synthesize cytokines, whereas signaling PD-1 by B7-H1 alone decreases T_{FH} cytokine production.

Signaling through B7-H1 expressed on B cells could potentially direct the differentiation of B cells into plasma cells. However, reconstitution of SCID mice that lack B7-H1 specifically on B cells exhibited comparable levels of autoantibodies, GCs and PCs as SCID mice receiving WT B cells. The recovery of GCs and PCs reveals that the positive signal through B7-H1 is not intrinsic to the B cell. Although B7-H1 is expressed on T cells, signaling through B7-H1 on T_{FH} cells is also not responsible for promoting adequate antibody responses as SCID mice with a deficiency in B7-H1 on B and T cells display normal B cell responses. Therefore, the B7-H1 signal required for optimal antibody responses may be provided by FDCs or other non-B non-T cells to PD-1 expressing T_{FH} cells. Our data does not rule out a role for B7-H1 expression on B cells as a recent report shows that B cell expression of B7-H1 and PD-L2 is necessary for survival of GC B cells [24]. Thus, there may be a contribution from PD-L2 in conjunction with B7-H1 that was not observed in mice deficient only in B7-H1. These data suggest that there are two separate pathways for B7-H1/PD-1 regulation of humoral immunity; a B cell extrinsic effect of B7-H1 expression by non-B non-T cells that regulate T_{FH} cell activation and survival of GC B cells, and a B cell intrinsic effect of PD-L2 alone or PD-L2 and B7-1 expression by B cells that interacts with PD-1 on T_{FH} cells to optimize IL-21 and IL-4 production by T_{FH} cells to similarly maintain GC B cell survival. It is possible that TFH are initially activated by B7-H1 expressed by non-B non-T cells to synthesize cytokines and upregulate ICOS expression and in turn deliver activation signal to PD-L2 on the B cell. B cells through PD-L2 expression on the TFH are activated

B cell expression of B7-H1 may regulate the aggressive CD4⁺ T cell response in arthritic B7-H1^{-/-} mice. However, reconstitution of SCID mice with B7-H1^{-/-} or WT B cells resulted in the development of similar arthritis severity and T cell proliferative responses. We attributed the reduction in disease severity to a restored regulation of the T cell responses by B7-H1 on a non-B non-T cell population. This suppression may be due to several cell types as B7-H1 is broadly expressed on both hematopoietic and non-hematopoietic cells [2]. Similar regulation by tissue expression of B7-H1 was observed for T cell responses and the development of diabetes [28]. B7-H1 contributes to reduced responsiveness by limiting cell numbers, inducing cell cycle arrest, increasing apoptosis and activating Tregs [37–39]. In our studies expression of B7-H1 on T cells is also not responsible for the increase in T cell activity and arthritis as cell-transfer of B7-H1^{-/-} T cells into SCID mice restores normal T cell activation and arthritis severity.

The exacerbated arthritis observed in B7-H1^{-/-} mice indicates that the titer of autoantibodies was above the threshold necessary to initiate PGIA, which is dependent on autoantibodies, but insufficient to inhibit disease severity [12]. However, lack of B7-H1 or blockade of B7-H1 signaling may be more influential in other antibody-mediated diseases. Accordingly, in a model of autoimmune nephritis PD-L1^{-/-} (B7-H1) mice do not experience an increased incidence or severity of glomerular IgG deposits [40]. However, in T cell mediated diseases,

B7-H1 functions to regulate disease as loss or neutralization of B7-H1 results in an exacerbation of disease [28, 41].

In summary, this work highlights a novel role of PD-1/B7-H1 signaling in promoting B cell antibody production by enhancing the survival of GCs in contrast to its function in suppressing T effector cell activation and cytokine production.

MATERIALS AND METHODS

Mice, Antigen, and Immunizations

WT BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, Maine). B7-H1^{-/-} mice were engineered as originally described and backcrossed 8 generations onto the BALB/c background [42]. Female (>3 months) mice were immunized intraperitoneally (i.p.) with 150µg of human PG in 2mg of dimethyl-dioctadecyl-ammonium bromide (DDA) (Sigma-Aldrich, St. Louis, MO) 3 times at 3 week intervals [43]. Human cartilage was obtained from the Orthopedic Tissue, Transplant, and Implant Repository of Rush University Medical Center from patients undergoing joint replacement surgery. PG (aggrecan) was extracted and purified from pooled cartilage samples [43]. All animal experiments were approved by the Animal Care and Use Committee at Rush University Medical Center (Chicago, IL).

Assessment of Arthritis

Mice were scored for the development of arthritis 3 times a week in a blinded manner by evaluating the amount of erythema and swelling of each individual paw following established guidelines [43]. For histology, hind limbs were dissected, decalcified, embedded in paraffin, and sectioned at 6 µm. Saggittal sections were stained with H&E, and scored on the amount of cellular infiltration and joint architectural destruction on a scale of 0–4 with 0 being no infiltration.

T cell Proliferation Assessment

Spleens were isolated from BALB/c WT or B7-H1^{-/-} naïve or arthritic mice 10 weeks or from reconstituted SCID mice 8 weeks after initial PG-DDA immunization. B cells from naïve WT or B7-H1^{-/-} mice were purified by AutoMAC separation (Miltenyi-Biotec, Bergisch Gladbach, Germany). Purified B cells were either pre-activated with LPS (10µg/ml) overnight in culture or used as naïve unstimulated APCs. WT and B7-H1 CD4⁺ T cells (2.5×10⁵) from arthritic mice were purified by AutoMACS negative selection and co-cultured with PG (10µg/ml) and purified mitomycin-C treated ((25 µg/1×10⁷ cell)/ml for 30 min) naïve or LPS activated B cells (2.5×10⁵) for 5 days in RPMI-1640 media containing 5% FCS, 100 µg/ml penicillin, 100µg/ml streptomycin and 2 mM L-glutamine in quadruplicate. ³H-thymidine was added for the final 24 hours and assessed for proliferation.

Assessment of Th1, Th2, and Th17 cytokine profile

CD4⁺ T cells (3 × 10⁵ cells/well) isolated 10 weeks after the initial PG-DDA immunization were co-cultured with mitomycin-C treated (25µg/1 × 10⁷ cells) naïve spleen cells (2 × 10⁵ cells/well) and PG (10µg/ml). Numbers of IFN-γ or IL-17 producing cells were detected after 72 hour cultures by ELISPOT kits (eBiosciences, San Diego, CA) per manufactures' instructions [44]. Supernatants were collected and examined for cytokine concentrations by IFN-γ (BD Biosciences, San Jose, CA) and IL-17 (R&D Systems, Minneapolis, MN) ELISA kits on 96 "half-well" plates [44].

Detection of anti-PG antibodies by ELISA

Serum was obtained by bleeding mice from the orbital plexus at 1, 4, 7 and 10 weeks after the initial PG-DDA immunization. Reconstituted SCID mice were bled at 8 weeks. The presence of anti-mouse PG and anti-human PG antibodies in serum samples was determined by ELISA in serial diluted (PBS/0.5% Tween-20) samples and internal standard samples (pooled sera from arthritic mice)[44].

Splenic B cell assessment

Spleens of naïve WT or B7-H1^{-/-} mice were assessed for B-cell populations as previously described [44]. Naïve B-cells were isolated by AutoMACS negative selection and stimulated for 5 days with or without LPS (0.4 or 2 µg/ml) (Sigma-Aldrich) or anti-CD40 (3 µg/ml) and anti-IgM (3 µg/ml) (BD Biosciences). Final 24 hour ³H-thymidine incorporation was measured to determine proliferation. For IHC, spleens were harvested 1 week following the second PG-DDA immunization and frozen in liquid nitrogen, embedded and 8µm frozen sections cut. GCs were detected with biotinylated-PNA (1:500) followed with immunoperoxidase ABC (Vector Laboratories, Burlingame, CA) and DAB peroxidase substrate (Vector Laboratories). PNA⁺ GCs were counted in six randomly selected x64 view fields from 3 sections of each spleen at least 50µm apart. Percentages of GC B cells were quantified using anti-GL7 FITC, anti-CD95 PE and anti-CD19 APC Abs (BD Biosciences) along with percentages of PCs using anti-CD38 FITC and anti-CD138 PE Abs (BD Biosciences) and a FACSCanto II with FACSDiva software (BD Biosciences). Apoptosis was detected by incubating splenocytes with zVAD-FMK-Fluorescein (Casp-Glow, BioVision, Mountain View, CA) in RPMI for 30 minutes at 37°C prior to staining with surface antibodies.

SCID repopulation experiments

B-cells from spleens of naïve WT and B7-H1^{-/-} were either purified by negative selection using microbeads or depleted (B-depleted) using anti-CD19 microbeads using AutoMACS separation (Milty Biotech). Mixtures of B cells (1×10^7) and B-depleted cells (1×10^7) in 100 µl of PBS were injected intravenously into SCID (NCI, Frederick, MD) recipients. Positive controls received both WT B-depleted cells and WT B cells (T^{WT} B^{WT}). To create mice lacking B7-H1 specifically on B cells (T^{WT} B^{B7-H1^{-/-}}) SCIDs received a mixture of WT B-depleted cells and purified B7-H1^{-/-} B cells. For mice lacking B7-H1 on B and T cells (T^{B7-H1^{-/-}} B^{B7-H1^{-/-}}), SCIDs received B7-H1^{-/-} B-depleted cells and B cells. One week after transfer, peripheral blood B and T cells were phenotyped for reconstitution and SCID mice immunized 3 times with PG-DDA and arthritis monitoring.

Flow cytometry and quantitative RT-PCR of naïve T-cells and T_{FH} cells

Spleens were harvested from naïve WT mice or from WT and B7-H1^{-/-} mice 10 days following the second PG-DDA immunization. For flow cytometry, spleen cells were labeled with: anti-CXCR5-biotin, anti-CD62L-FITC, Streptavidin-PE, anti-CD3-PerCp-Cy5.5 (BD Biosciences), anti-ICOS-APC, anti-PD-1-PE-Cy7 (Biolegend, San Diego, CA) and anti-CD4-APC-eFluora 780 (eBiosciences) for detection of T_{FH} cells. Apoptosis was determined as described above. For qPCR, negatively selected CD4⁺ T cells were sorted using a FACSARIA (BD Biosciences) for CD4⁺CD44^{lo}CD62L^{hi} naïve T cells, CD4⁺CD62L^{lo}CXCR5^{hi} T_{FH} cells, or CD4⁺CD62L^{lo}CXCR5^{lo} non-T_{FH} activated T cells. Total RNA was isolated and cDNA was generated as previously described [25]. Real-time PCR was performed using an IQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) and QuantiFast SYBR Green PCR Supermix (Qiagen, Valencia, CA) per manufacturer's guidelines. Expression of Bcl6, IL-21 and FasL were determined using specific primers and normalized to expression of the "housekeeping" gene of β-actin.

Statistical analysis

The Mann-Whitney U test was used to compare nonparametric data for statistical significance, with two-tailed p-values indicated throughout as: * $p < 0.05$

Supplementary Material

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Abbreviations

DDA	dimethyl-dioctadecyl-ammonium bromide
FDC	follicular dendritic cell
PC	plasma cells
PG	proteoglycan
PGIA	proteoglycan-induced arthritis
PNA	peanut agglutinin
T_{FH}	T follicular helper cells
RA	rheumatoid arthritis

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Figure 1. Exacerbation of PGIA and autoreactive CD4⁺ T cell responses in B7-H1^{-/-} mice WT BALB/c (n=10–15) and PD-L1^{-/-} (n=10–15) mice were monitored for arthritis severity (A) and incidence (B) following three i.p. immunizations of human PG in DDA at 3-week intervals. Hindlimbs were harvested from WT (C & E) or B7-H1^{-/-} (D & F) mice 6 and 10 weeks after the initial PG immunization. Sections were stained with H&E. Magnifications of 4× (C & D) and 10× (E & F) were used for histological scores (G) were averaged from both hind joints from 3–6 mice per group and are representative of two independent experiments. Proliferation in response to PG (10μg/ml) restimulation (H) of CD4⁺ cells collected at 10 weeks was measured by [³H]-thymidine incorporation during the last 24 hours of 5 day cultures. Flow cytometry was used to determine the percentage of CD25⁺ FoxP3⁺ Tregs (I) and expression level of CD62L (J) of splenic CD4⁺ T cells. IFN-γ (K & L), IL-4 (M & N) and IL-17 (O & P) were measured by ELISA (K, M & O) of supernatants from 4 day cultures or by ELISPOT assays (L, N & P). Values are means ± SEM and are representative of three independent experiments. *, p< 0.05, Mann-Whitney U.

Figure 2. Autoantibody titers are diminished in B7-H1^{-/-} mice

WT (n=5–10) and B7-H1^{-/-} (n=5–10) mice were immunized i.p. with PG-DDA 3 times at three week intervals as indicated by arrows. Serum samples collected from at weeks 1, 4, 7 and 10 after the initial PG-DDA immunization were measured for the presence of autoantibody. Titers of both human-PG-specific IgG1 (**A**) and IgG2a (**B**) and mouse-PG-specific IgG1 (**C**) and IgG2a (**D**) antibodies were measured by ELISA. Values are means $\pm$ SEM and are representative of four independent experiments. *, $p < 0.05$, Mann-Whitney U.

Figure 3. Germinal center B cells and plasma cells are decreased in B7-H1^{-/-} mice

Spleens from WT and B7-H1^{-/-} mice (n=3-4) were isolated 1 week after the second PG-DDA immunization. Germinal center B cells were assessed by PNA staining of frozen sections (**A**) and by dual staining of CD19⁺ cells with GL-7 and CD95 (**B**). Plasma cells were also identified using flow cytometry by CD38 and CD138 double positive cells (**C**). Spleens from naïve WT or B7-H1^{-/-} mice were assessed for T1, T2, marginal zone (MZ) and follicular (FO) B cell subsets (**D**) or for the proliferative capacity of total splenic B cells to response to LPS (0.4 or 2 μg/ml) or anti-CD40 (3 μg/ml) + anti-IgM (3 μg/ml) (**E**). Values are means ± SEM and are representative of two independent experiments. *, p<0.05, Mann-Whitney U.

Figure 4. B7-H1 negatively regulates T_{FH} cells

Spleens from WT (n=4-5) or B7-H1^{-/-} (n=4) mice were harvested 10 days following the second PG-DDA immunization. Absolute number (A), percent CaspGLOW⁺ (B & C) and ICOS mean fluorescence intensity (MFI) (D) of splenic T_{FH} cells (CD3⁺CD4⁺PD-1^{hi}CXCR5^{hi}ICOS^{hi}CD62L^{lo}) were assessed by flow cytometry. mRNA was isolated from sorted naïve, T_{FH} and non-T_{FH} cells from WT or B7-H1^{-/-} and assessed for Bcl-6 (E), IL-21 (F) and FasL (G) transcripts by qPCR. GL-7⁺CD95⁺ GC B cell death was assessed by CaspGLOW and flow cytometry (H & I). Values are means ± SEM and are representative of two independent experiments. *, p< 0.05, Mann-Whitney U.

Figure 5. Stimulatory B7-H1 signals are not provided by B or T cells

Isolated splenic B cells (2×10^7) from WT or B7-H1^{-/-} mice were coinjected with B-depleted (2×10^7) splenic cells from WT or B7-H1^{-/-} intravenously into SCID mice recipients. One week following cell transfer, mice began PGIA immunization regime. SCID mice groups were reconstituted as follows: WT B-depleted cells + WT B cells (T^{WT} B^{WT}) (n=5); WT B-depleted cells + B7-H1^{-/-} B cells (T^{WT} B^{B7-H1-/-}) (n=5); B7-H1^{-/-} B-depleted cells + B7-H1^{-/-} B cells (T^{B7-H1-/-} B^{B7-H1-/-}) (n=5). Spleens were collected 1 week following the second PG-DDA immunization and analyzed by flow cytometry for GC B cells as GL-7⁺ CD95⁺ or CD19⁺ (A) along with PCs as CD38⁺ CD138⁺ of total cells (B). Autoantibodies titers (C-E) were evaluated by ELISA from serum samples collected at 8 weeks. Values are means $\pm$ SEM and are representative at least two independent experiments. *, p < 0.05, Mann-Whitney U.

Figure 6. B cell B7-H1 regulates T cell activation *in vitro*, but non-B and T cell tissue expression of B7-H1 can regulate excessive PGIA *in vivo*

Purified splenic, naïve or LPS pre-activated WT or B7-H1^{-/-} B cells were cocultured with purified CD4⁺ T cells from PG immunized WT mouse spleens for 5 days with or without PG (10μg/ml) restimulation. Proliferation (**A**) was measured through [³H]-thymidine in the final 24 hours. SCID mice were reconstituted with splenic B and T cells from WT and B7-H1^{-/-} mice as described previously. Following cell transfer and PG-DDA immunizations mice were monitored for PGIA severity (**B**) and incidence (**C**). Spleens were harvested at week 8. Proliferation (**D**) of splenic CD4⁺ T cells in responses to PG (10μg/ml) restimulation was measured by ³H-thymidine incorporation for the final 24 hours of 5 day cultures. Values are means ± SEM and are representative of three independent experiments. *, p < 0.05, Mann-Whitney U.