Blockade of B7-H1 (PD-L1) enhances humoral immunity by positively regulating the generation of T follicular helper cells

Running title: ROLE FOR B7-H1 IN T FOLLICULAR HELPER CELLS

Emily Hams\textsuperscript{1*}, Mark J. McCarron\textsuperscript{1*}, Sylvie Amu\textsuperscript{*}, Hideo Yagita\textsuperscript{†}, Miyuki Azuma\textsuperscript{‡}, Lieping Chen\textsuperscript{§}, Padraic G. Fallon\textsuperscript{*\textsuperscript{¶\textsuperscript{2}}}

\textsuperscript{1}Institute of Molecular Medicine, St. James’s Hospital, Trinity College Dublin, Dublin 8, Ireland.; \textsuperscript{†}Department of Immunology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-8421, Japan; \textsuperscript{‡}Department of Molecular Immunology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan, \textsuperscript{§}Department of Oncology and the Institute for Cell Engineering, The Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA. \textsuperscript{¶}National Children’s Research Centre, Our Lady’s Children’s Hospital, Crumlin, Dublin 12, Ireland.

\textsuperscript{1}Equal contribution
\textsuperscript{2}Corresponding author

Mailing address: Padraic Fallon, Institute of Molecular Medicine, St. James’s Hospital, Trinity College Dublin, Dublin 8, Ireland. Email: pfallon@tcd.ie

Phone: 353 1 8963267; Fax: 353 1 8964040
Abstract:

T follicular helper (T\textsubscript{FH}) cells are critical initiators in the development of T cell dependent humoral immunity and the generation of protective immunity. We demonstrate that T\textsubscript{FH} cell accumulation and antibody production is negatively regulated by B7-H1 (PD-L1) in response to both helminth infection and active immunization. Following immunization of B7-H1\textsuperscript{−/−} mice with KLH or helminth antigens there is a profound increase in induction of T\textsubscript{FH} cells as a result of increased cell cycling and decreased apoptosis relative to WT mice. The increase in T\textsubscript{FH} cells in the absence of B7-H1 was associated with significant elevations in antigen-specific immunoglobulin response. Co-transfer experiments \textit{in vivo} demonstrated that B7-H1 expression on B cells was required for negatively regulating T\textsubscript{FH} cell expansion and production of antigen-specific immunoglobulin. Treatment of immunized WT mice with anti-B7-H1 or anti-PD1 mAbs, but not anti-B7-DC, led to a significant expansion of the T\textsubscript{FH} cell population and an enhanced antigen-specific immunoglobulin response. Our results demonstrate that the co-inhibitory B7-H1:PD-1 pathway can limit the expansion of T\textsubscript{FH} cells and constrain antigen-specific immunoglobulin responses. This finding has direct implications for investigations examining the feasibility of therapeutically manipulating this pathway and reveals new insights into the regulation of the humoral immune response.
**Introduction:**

In response to infections with various pathogens, the generation of adaptive immunity is orchestrated by the differentiation of CD4+ T cells to a range of distinct subpopulation with specific functionality (1). With respect to the generation of T cell dependent humoral immunity, CD4+ T follicular helper (TFH) cells have emerged as a specialized follicle-homing T cell subset that are specifically equipped to provide B cell help (2). TFH cells are identifiable based on high expression of various activation-induced cell surface receptors, such as chemokine receptor CXCR5 and molecules like PD-1, ICOS and BTLA, whose expression pattern is tailored to the functional activities of TFH cells (3). More recently, TFH cells were characterized by expression of a lineage-specific transcription regulator, BCL-6 (4-6).

TFH cells express PD-1 (CD279), with PD-1 expression acting as a pivotal co-inhibitory pathway in the regulation of T cell activation via interactions with programmed death ligand 1 (B7-H1; also known as PD-L1, CD274) and B7-DC (PD-L2, CD273) (7). The B7-H1:PD-1 pathway plays a multitude of roles in the maintenance of central and peripheral tolerance (8). Also, the B7-H1:PD-1 pathway is one mechanism behind CD8+ T cell exhaustion that results in ineffective T cell viral immunity during chronic viral infections, such as HIV (9). In addition to a role for B7-H1 in immune-mediated subversion by chronic viral infections, it is also implicated in inducing anergy in T cells in the context of helminth parasite infections. The human trematode parasite *Schistosoma mansoni* is a potent modulator of T cell polarization, which is coincident with a progressive diminution of the T cell response and development of a chronic state of immune hyporesponsiveness (10), including the parasite usurping B7-H1 expression on murine macrophages to induce T cell anergy (11).
In this study, we observed a significant increase in antibody production during *S. mansoni* infection of B7-H1<sup>-/-</sup> mice that was associated with marked expansion of T<sub>FH</sub> cells. Using different antigens we have shown that following immunization, B7-H1<sup>-/-</sup> mice have a significantly elevated T<sub>FH</sub> cell frequency and levels of antigen-specific antibodies. Co-transfer experiments revealed B cells as the source of B7-H1 that function to restrain *in vivo* T<sub>FH</sub> cell expansion. Furthermore, the use of mAbs *in vivo* demonstrated that blocking the B7-H1:PD-1 pathway, but not the B7-DC:PD-1 pathway, led to enhanced T<sub>FH</sub> cell function. Therefore, T cell-mediated humoral immunity during an active immune response is controlled by B7-H1:PD-1 regulation of T<sub>FH</sub> cells.
Materials and Methods:

Mice
C57BL/6J mice were obtained from Jackson Laboratories and were bred in-house. B7-H1⁻/⁻ mice on a C57BL/6 background were as described (12) and bred in-house. Foxp3-GFP mice on a C57BL/6 background (13), IL-4 green-enhanced transcript (4Get) (14), originally on BALB/c background (Bar Harbor ME, USA) and subsequently backcrossed in-house to C57BL/6J. RAG-1⁻/⁻ and Thy1.1⁺ C57BL/6J strain mice purchased from Jackson Laboratories and bred in-house. B7-H1⁻/⁻Foxp3-GFP and B7-H1⁻/⁻4Get reporter mice were generated by inter-crossing. Mice were housed in individually ventilated and filtered cages under positive pressure (Techniplast, Kettering, UK). All animal experiments were performed in compliance with the Irish Department of Health and Children regulations and approved by the Trinity College Bioresources Ethical Review Board.

Parasite infection.
All helminth infections, immunology, parasitology and pathology were as described (15, 16). S. mansoni eggs were isolated and SEA prepared as described (15).

Immunizations
Mice were immunized s.c. in a rear footpad with SEA (50 μg/site) or the tail-base (100 μl each mouse) with 0.25 mg/ml KLH (Calbiochem) emulsified in 0.25 mg/ml complete Freund’s adjuvant (Sigma). Mice were sacrificed on days 0, 7, 14 and 21 following immunization.
**Antibody analysis**

Total and SEA or KLH-specific serum IgG1, IgG2a, IgE and IgM was determined by ELISA (17). In short, medium binding 96-well microplates (Greiner bio-one, Germany) were coated overnight at 4°C with 2 µg SEA or KLH/well or the appropriate anti-mouse capture antibody (BD), blocked with 1% BSA/PBST and incubated with appropriate dilutions of sera. Bound Ig was detected using the appropriate biotin-conjugated rat anti-mouse Ig (BD) followed by streptavidin-HRP conjugate (R&D systems) and O-phenylenediamine dihydrochloride (Sigma Aldrich) substrate. Absorbance at wavelength 490 nm was read using a microplate reader (VersaMax Tunable Microplate Reader, Molecular Devices, CA).

**Flow cytometry**

Single cell suspensions from spleen and mesenteric, inguinal or popliteal LN were prepared and surface marker expression on cells was assessed by flow cytometry. Cells were plated on a 96-well V-bottomed plate (2-10 x 10^6/well) and washed in flow cytometry buffer (PBS with 2% FCS and 0.05% sodium azide) followed by blocking with anti-mouse CD16/32 (clone 93) (eBioscience). The following mAb were used at optimally titrated concentrations: CD1d-PE (IB1), CD4-eFluor 450 (RM4-5), CD-19-eFluor 450 (eBio1D3), CD24-PerCP (M1/69), CD25-APC (PC61.5), CD90.1-APC (HIS51), B220-APC-eFluor 780 (RA3-6B2), BTLA-AlexaFluor 647 (8F4), ICOS-PE-Cy5 (7E-17G9) and PD-1-PE (RMP1-30) (eBioscience): CD5-PE (53-7.3), CD21-APC (7G6), CD23-PE (B3B4), CD95-PECy7 (Jo2), CD138-PE (2B1-2), CXCR5-biotin (2G8), IgD-PE (11-26c.2a), IgM-FITC (11/41), T and B cell activation marker-FITC (GL-7), and streptavidin-PeCy7 (BD). Populations of interest were gated according to appropriate ‘fluorescence minus one’ (FMO) controls (18). Samples were
acquired on a CyAn flow cytometer (Beckman Coulter) and were analyzed with FlowJo software (Tree Star, Inc.).

**BrdU proliferation analysis**

For the cell proliferation assay, WT and B7-H1−/− mice were injected i.p. with 2 mg BrdU (Sigma) immediately prior to immunisation with KLH/CFA. Mice were given further injections of 2 mg BrdU i.p. at 3 day intervals and were sacrificed at day 7, 14 and 21 after KLH immunization. Spleen and inguinal lymph nodes were collected for flow cytometric analysis of TFH cell proliferation. Briefly, cells were surface stained as described (CD4+ B220−PD-1−CXCR5+), washed and suspended in 0.15M NaCl prior to incubation with 95% ice-cold ethanol. Cells were then fixed using the BD Cytofix/Cytoperm kit (BD Biosciences) following the manufacturers instructions, washed and incubated with 50U DNAse 1 (Roche), in 4.2 mM MgCl2 and 0.15 M NaCl for 10 mins. Samples were then stained with anti-BrdU (FITC; BD Biosciences) for 30 mins, washed and re-suspended in flow cytometry buffer.

**Annexin V-FITC/PI staining**

For apoptosis assays, WT and B7-H1−/− mice were immunized with KLH/CFA as described above. At day 21 the mice were sacrificed and the spleens collected for analysis of apoptotic cells. Cells were initially stained using the makers for TFH (CD4+ B220−PD-1−CXCR5+), washed and then stained with the Annexin V-FITC and PI according to manufacturers instructions (BD PharMingen).

**Intracellular cytokine staining**
Intracellular staining for cytokines was performed as described (19). In brief, cell suspensions were restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma Aldrich) for 4 h in the presence of Golgistop (BD Biosciences). After surface staining for CD4⁺ cells or TFH (CD4⁺B220⁻PD-1⁺CXCR5⁺), cells were permeabilized with Cytofix/Cytoperm according to manufacturers instructions (BD Biosciences). Intracellular staining for IL-4-PE (11B11), IL-17-PE (TC11-18H10), IL-21-PE (FFA21) IFN-PE (XMG1.2) (BD Biosciences) and IL-10-PE (JES5-2A5) (Caltag) was performed. Positive cells were selected using appropriate isotype controls for each stain.

**Immunofluorescence Microscopy**

Spleen tissue or inguinal LN from mice were embedded in O.C.T. compound (Sakura Finetek) and snap-frozen in liquid nitrogen. Cryostat sections (6 μm) were fixed in cold 75:25% acetone:ethanol and blocked with 1% BSA (Sigma Aldrich). Sections were incubated with anti-CD4-PE (L3T4) (eBioscience) and anti-GL-7-FITC (GL7) (BD Biosciences). All images were captured at 20× on a LSM 510 laser scanning confocal microscope using LSM 5 software (Carl Zeiss, Inc).

**Cell sorting and RT-PCR**

Spleen and mesenteric LN cells were pooled from mice and CD4⁺ T cells were pre-enriched using a negative selection cocktail (EasySep mouse CD4⁺ T cell enrichment kit, StemCell Technologies Inc, Vancouver, Canada). CD4⁺B220⁻ TFH and non-TFH were further purified on a MoFlo cell sorter (Beckman Coulter) based on their surface expression of CXCR5 and PD-1. Re-analysis of the sorted T cell populations revealed a purity of >97%. For B cell purification, spleen and mesenteric LN cells were pooled from B7-H1⁻ or WT mice, and T
cells were depleted by incubation with anti-Thy.1 mAb (M5/49.4.1; ATCC) and Lo-Tox rabbit complement (Cedarlande, Canada) (11). CD19+ B cells were purified from B cell pre-enriched cells on a MoFlo cell sorter with purity over 98%, as described (20). RNA was isolated using RNeasy and was reverse transcribed using Quantitect reverse transcription kit incorporating a genomic DNA elimination step (QIAGEN). Real-time quantitative PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems) using pre-designed TaqMan gene expression assays specific for murine IL-4 (Mm00445289_m1), BCL6 (Mm00477633_m1) and IL-21 (Mm00517640_m1). Specific gene expression was normalized to 18S ribosomal RNA. Fold expression was calculated using the \( \Delta \Delta CT \) method of analysis (Applied Biosystems).

**CFSE staining and adoptive transfer experiments**

CD4+ T cells were sorted, as above, from Thy1.1+ mice. Purified cells were stained with CFSE at 1 \( \mu \)M (Invitrogen) and CFSE-stained donor cells (~ 4 \( \times \) 10⁶) were injected i.v. into Thy1.2+ WT and B7-H1+ mice who were then immunized s.c. with KLH (0.25 mg/ml) emulsified in CFA (0.25 mg/ml). 7 days later, spleen and inguinal LN cells were analyzed by flow cytometry as above.

For co-transfer experiments, 4 x 10⁶ CD4+ cells from WT mice were transferred into RAG-1−/− mice simultaneously with 4 x 10⁶ CD19+ cells from WT or B7-H1−/− mice. After 14 days mice were immunized with KLH/CFA and animals were killed 14 days later.

**mAb treatment protocol**

Anti-B7-H1 (clone: MIH5), anti-PD-1 (clone: J43) and anti-B7-DC (clone: TY25), prepared as described previously (21). C57BL/6 mice received an i.p. injection of blocking mAb (200
μg/mice) or appropriate control 1 h before immunization with KLH emulsified in CFA (0.25 mg/ml). Mice subsequently received i.p. injection of 200 μg mAb two days after immunization followed by i.p. injection of 100 μg mAb every two days. On day 14 mice were killed and the spleen and inguinal LN cells were removed to be analyzed by flow cytometry.

Statistical analysis

GraphPad Prism and GraphPad Instat software was used to analyse the data. Error bars depict the standard error of the mean (SEM). The paired Student’s t-test or ANOVA were used to determine statistical significance between groups. Values of $P<0.05$ were considered significant.
Results:

CD4\(^{+}\)PD-1\(^{+}\) T cells are significantly increased in the absence of B7-H1 in response to helminth infection.

We have previously demonstrated that *S. mansoni* infection of mice selectively up-regulates B7-H1 on murine macrophages to induce T cell anergy (11). WT or B7-H1\(^{-/-}\) (12) mice were infected with *S. mansoni* cercariae and examined in the acute (week 8) and chronic (week 16) stages of infection. Interestingly, total immunoglobulin (IgE, IgG1 and IgG2a) levels were significantly increased in infected mice in the absence of B7-H1 (Figure 1A). In addition, antigen-specific IgE immunoglobulin levels were similarly enhanced in B7-H1\(^{-/-}\) mice compared to WT mice, with a non-significant increase in both antigen-specific IgG1 and IgG2a (Figure S1A). The elevated immunoglobulin levels corresponded with a greater expansion of germinal center (GC) B cells in the spleen of infected B7-H1\(^{-/-}\) deficient mice compared to WT mice (Figure 1B and 2C). A non-significant increase of GC B cells was also detected in the draining mesenteric LN (data not shown). Furthermore, the total number of B220\(^{-}\)CD138\(^{+}\) plasma cells in the mesenteric lymph nodes was significantly increased in B7-H1\(^{-/-}\) mice compared to WT mice (data not shown). Surface phenotype profiling, as described (20), revealed no basal differences between naive WT and in B7-H1\(^{-/-}\) mice in other B cell populations (data not shown).

The percentage of CD4\(^{+}\) T cells expressing PD-1 was also increased in B7-H1\(^{-/-}\) mice compared to WT mice in both the presence and absence of infection (Figure S1B, C and D). Given the substantial expansion of this PD-1\(^{+}\)CD4\(^{+}\) T cell subset in the absence of B7-H1 following helminth infection, we reasoned that this PD-1\(^{+}\) population might represent a functional subset whose accumulation is normally negatively regulated by the B7-H1:PD-1 pathway in WT mice. Initial studies using intracellular staining demonstrated an increased
expression of Foxp3 in PD-1 cells in B7-H1 mice (data not shown), relevant to recent findings indicating that B7-H1 could regulate the generation of Foxp3 T regulatory (regs) cells (22, 23). In order to address whether Tregs were the expanded CD4PD-1 population, we generated B7-H1/Foxp3-GFP reporter mice. While the percentage of CD4CD25Foxp3 T regulatory cells was unaltered between WT and B7-H1 mice, PD-1 was significantly enhanced on CD4CD25Foxp3 T regulatory cells in B7-H1/Foxp3-GFP mice compared to Foxp3-GFP mice in both the presence and absence of infection (Figure S2). Although further examination of this population is warranted, this Foxp3 population did not account for the majority (>65%) of CD4PD-1 T cells in B7-H1 mice. We therefore sought to define the PD-1CD4 cells that were expanded in the absence of B7-H1 in more detail.

**B7-H1 negatively regulates the expansion of T follicular helper cells in response to helminth infection and antigen immunization.**

Two groups recently demonstrated that helminths, including *S. mansoni* as used herein, induce PD-1Tfh cells (24, 25). As there were elevated levels of immunoglobulin in infected B7-H1 mice (Figure 1), we asked whether an expansion in the Tfh cell compartment might account for the majority of the expanded PD-1CD4 T cell population. CD4B220 T cells that co-expressed PD-1high and CXCR5high were examined by flow cytometry in response to *S. mansoni* infection during the acute phase of infection at week 8 (Figure 2A). In both the spleen and draining mesenteric LN, we demonstrated a significant expansion of both the percentage and total number of Tfh cells in the absence of B7-H1, with further expansion in infected B7-H1 mice (Figure 2B, C and D). Histological analysis confirmed an increased accumulation of CD4 cells within the germinal centre (GL-7 areas in the follicular mantle) of the spleens of B7-H1 compared to WT mice (Figure 2C). This Tfh cell expansion in the
absence of B7-H1 was maintained into the chronic week 16 phase of *S. mansoni* infection (Figure S3A-C).

Consistent with previous reports (26, 27), these T<sub>FH</sub> cell populations expressed high levels of ICOS and BTLA (Figure S4A). Furthermore, T<sub>FH</sub> (CD4<sup>+</sup>B220<sup>-</sup>PD-1<sup>-</sup>CXCR5<sup>+</sup>) cells were found to contain high transcript levels of the canonical T<sub>FH</sub>-associated genes, BCL-6 and IL-21 (Figure S4B) (28). In agreement with recent reports (24, 25), the T<sub>FH</sub> cells induced by helminth infection also contained high levels of IL-4 mRNA compared to CD4<sup>+</sup>B220<sup>-</sup>PD-1<sup>-</sup>CXCR5<sup>-</sup> T cells (Figure S4B). Additionally, dual B7-H1<sup>-</sup>/IL-4eGFP(4Get) mice were generated to confirm that the *S. mansoni*-induced T<sub>FH</sub> cells expressed IL-4 (data not shown), as reported (25). Intracellular cytokine staining of CD4<sup>+</sup>B220<sup>-</sup>PD-1<sup>-</sup>CXCR5<sup>+</sup> T<sub>FH</sub> cells revealed that the frequency of T<sub>FH</sub> cells positive for IFNγ was significantly higher in B7-H1<sup>-</sup>/ mice compared to WT mice in response to infection (Figure S4C). There was no difference in the frequency of IL-4 positive T<sub>FH</sub> cells between WT and B7-H1<sup>-</sup>/ mice, with limited frequency of IL-17 positive cells detected (Figure S4C). Thus, an enhanced percentage of T<sub>FH</sub> cells producing IFNγ in the absence of B7-H1 might represent one mechanism contributing to the enhanced immunoglobulin levels detected in B7-H1<sup>-</sup>/ mice compared to WT mice.

In order to further examine the T<sub>FH</sub> cell expansion in the absence of B7-H1 we immunized the footpads of both WT and B7-H1<sup>-</sup>/ mice with an extract of *S. mansoni* eggs (soluble egg antigens; SEA), a known inducer of T<sub>FH</sub> (25). The draining popliteal LN were examined by flow cytometry for T<sub>FH</sub> cells 14 days later. T<sub>FH</sub> cells were induced following SEA immunization in WT mice (Figure S4D and E). Consistent with the results obtained following infection, immunization with SEA in the absence of B7-H1 resulted in a significantly greater expansion of both the percentage and total number of T<sub>FH</sub> cells in the draining popliteal LN (Figure S4D and E). Collectively, these data demonstrate that T<sub>FH</sub> cells
constitute the dominant population expanded in the absence of B7-H1 both prior to and following helminth infection or after immunization with a helminth-derived antigenic extract. These results are consistent with a model whereby the B7-H1:PD-1 pathway modulates the expansion and function of T_{FH} cells.

**T_{FH} cell expansion and antibody response are negatively regulated by B7-H1.**

To further explore if the changes described above (Figure 1-2, S1-5) were not solely restricted to helminth infection or antigens, we characterized the generation of T_{FH} cells in WT and B7-H1^{+/−} mice in response KLH/CFA, a T-dependent inducer of T_{FH} cells (5). Similar to earlier data above, T_{FH} cells were expanded in the absence of B7-H1 in both the spleen and draining inguinal LN following KLH immunization (Figure 3A). B7-H1^{+/−} mice had significantly more T_{FH} cells, based on both the percentage and total number of cells, in the spleen (Figure 3C) and draining inguinal LN (Figure 3B and D). It is noteworthy that there was a higher basal frequency of T_{FH} cells in PBS-treated B7-H1^{+/−} mice relative to WT animals (Figure 3A-D), reinforcing the concept of unfettered expansion of T_{FH} cells in the absence of B7-H1 regulation. In order to explore whether the alteration in T_{FH} cell homeostasis apparent in B7-H1^{+/−} mice after immunization might impact the immunoglobulin response, as seen in infected B7-H1^{+/−} mice (Figure 1), we measured KLH-specific IgG1 and IgG2a, an isotype induced by CFA (29). We found that B7-H1^{+/−} mice produced significantly more KLH-specific IgG1 and IgG2a over a time course in response to KLH/CFA than the WT counterpart (Figure 3E). IgM levels were not altered between WT and B7-H1^{+/−} mice (data not shown). The spontaneous and infection- or antigen-induced expansion of the T_{FH} population in B7-H1^{+/−} mice indicates this pathway is a homeostatic co-inhibitory mechanism to limit the size and function of this T cell subset and humoral immunity.
B7-H1 expression on B cells regulates TFH cell expansion and antibody responses.

The absence of B7-H1 may enhance the proliferative expansion of TFH cell in vivo thereby explaining their accumulation in response to both infection and immunization. To explore this, we isolated CD4+ T cells from Thy1.1+ mice, labeled them with CFSE and transferred them into Thy1.2+ WT and B7-H1−/− mice. Mice were then immunized with KLH in CFA. The transferred CD4+ T cells differentiated into TFH cells based on their high expression of PD-1 and CXCR5 (data not shown). Furthermore, analysis of the CFSE-stained Thy1.1+ transferred cells revealed significantly greater proliferation of the TFH cells in both the spleen and draining inguinal LN in B7-H1−/− mice compared to WT mice (Figure 4A and data not shown). In order to further characterize the mechanism by which B7-H1 deficiency leads to greater expansion of TFH cells, we determined TFH cell proliferation by examining incorporation of BrdU and apoptosis of TFH cells following KLH/CFA immunization in both WT and B7-H1−/− mice. TFH cells underwent significantly greater proliferation in B7-H1−/− mice compared to WT mice (Figure 4B). In addition, TFH cells from B7-H1−/− mice demonstrated reduced Annexin V-FITC/PI staining relative to WT mice indicating less TFH apoptosis (Figure 4C). This is consistent with the described ability of the B7-H1:PD-1 pathway to lead to cell cycle arrest (30) and suggests that the B7-H1:PD-1 interplay may regulate expansion of TFH cells by limiting cell cycling and enhancing TFH cell susceptibility to apoptosis.

As TFH cell development is dependent on B cells (5, 25, 31), and B cells express high levels of B7-H1 ((8) and Figure 4D), we speculated that this B7-H1:PD-1 B-T cell cross-talk could regulate the expansion of TFH cells. To address this, WT CD4+ T cells were co-transferred with either CD19+ B cells from WT or B7-H1−/− mice into RAG-1−/− mice. After
14 days of rest period, mice were immunized with KLH/CFA. The expansion of T_{FH} cells in KLH-immunized RAG-1^{−/−} mice receiving WT CD4^{+} T cells and B7-H1^{−/−} B cells was significantly elevated in inguinal LN and spleen relative to mice injected with WT T and B cells (Figure 4E, data not shown). Consistent with data from KLH-immunized B7-H1^{−/−} mice (Figure 3D), the greater expansion of the T_{FH} population in mice that received WT T cells and B7-H1^{−/−} B cells corresponded with an enhanced KLH-specific immunoglobulin response compared to control mice (Figure 4F).

Together, these findings extend the co-inhibitory role of the B7-H1:PD-1 pathway in T_{FH} cell expansion to immunization with a classical T-dependent antigen and find that B cells expressing B7-H1 limit the proliferative expansion and survival of T_{FH} cells with concomitant reduction of the antigen-specific antibody response.

**In vivo blocking of B7-H1, but not B7-DC, expands T_{FH} cells and enhances humoral immune responses following immunization.**

Based on the use of B7-H1 deficient mice, we have shown that in the absence of B7-H1 there is a significant expansion of T_{FH} cells and enhanced antibody responses demonstrating a role for B7-H1 in the regulation of humoral immunity. To validate this further and to examine the feasibility of boosting humoral immunity by inhibiting PD-1 interactions, we treated mice with blocking mAbs against B7-H1, PD-1 or B7-DC before and during KLH immunization and monitored the generation of T_{FH} cells and the KLH-specific antibody response. Mice treated with blocking B7-H1 mAbs developed a significantly greater expansion of T_{FH} cells relative to mice that did not receive antibody treatment (Figure 5A); with an increase in both the percentage and total numbers of T_{FH} cells in the spleen (Figure 5B) and inguinal LN (Figure 5C) of KLH/CFA-immunized mice. Consistent with a role for PD-1, which is highly
expressed on T<sub>FH</sub> cells, in their regulation, anti-PD-1 mAb treated mice had a significantly 
(P<0.05) increased expansion of T<sub>FH</sub> cells in the spleen (Figure 5B). In contrast, anti-B7-DC 
mAb treatment did not alter T<sub>FH</sub> cell homeostasis, indicating that the B7-DC:PD-1 pathway 
does not play a regulatory role in T<sub>FH</sub> cell expansion. The expansion following anti-PD-1 
mAb was significant relative to control mice. A recent study using the same anti-PD-1 clone 
(J43) used herein, found that the antibody increased complement-dependent cell death in PD-
1<sup>+</sup> T cells which may explain the reduced T<sub>FH</sub> cell expansion compared to that induced by 
anti-B7-H1 (32). Alternatively, CD80 has also been described as a ligand for B7-H1 (33). We 
have found that T<sub>FH</sub> cells express high levels of CD80 (data not shown). Further studies 
should address whether the interaction of CD80 on T<sub>FH</sub> cells with B7-H1 contributes, in 
association with PD-1, to limiting the accumulation of T<sub>FH</sub> cells.

Similar to data in KLH immunized B7-H1<sup>+/−</sup> mice (Figure 3D), anti-B7-H1 mAb 
treatment significantly enhanced IgG2a levels, whereas anti-B7-DC, had no effect (Figure 
5D) and anti-PD-1 treatment led to a non-significant increase in KLH-specific IgG2a levels 
(Figure 5D). Therefore, mAb-mediated blocking of B7-H1 in vivo led to an expansion in T<sub>FH</sub> 
cells and increased antigen-specific antibody responses.
**Discussion:**

T\textsubscript{FH} cells constitute a distinct T lymphocyte follicle-homing CD4\textsuperscript{+} T lymphocyte population specifically equipped to provide helper signals for B cell antibody production (2). High constitutive surface expression of PD-1 has been used in the phenotypic identification of this subset but the functional significance of this remains to be clarified. We show that in the absence of B7-H1 there is a significant expansion of T\textsubscript{FH} cells and GC B cells resulting in enhanced antigen-specific antibody responses to infection and immunization. These data demonstrate a key role for B7-H1 in the negative regulation of humoral immunity through regulation of T\textsubscript{FH} accumulation.

To date, analysis of the functional significance of PD-1 expression on T cells has primarily focused on its role in tolerance induction (‘exhaustion’) of CD8\textsuperscript{+} T cells (7, 34), while the role of PD-1 expression on CD4\textsuperscript{+} T cells has not been extensively studied. A recent study found that PD-1 expression on CD4\textsuperscript{+} T cells acts to limit activation-induced CD4\textsuperscript{+} T cell expansion and function, but plays no role in the induction of peptide-induced tolerance (35). This suggests that PD-1 has differing roles on CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and is consistent with our data demonstrating that the PD-1:B7-H1 pathway limits the accumulation of CD4\textsuperscript{+} T\textsubscript{FH} cells. With respect to the role of the PD-1:B7-H1 pathway in the generation of Th subsets, it is noteworthy that the percentage of IFN\textgamma-producerng Th1 cells were higher in B7-H1\textsuperscript{-/-} mice compared to WT mice in response to *S.*mansonii infection, consistent with observations shown by another group (36). In addition, we demonstrated no significant difference between WT and B7-H1\textsuperscript{-/-} mice in the percentages of IL-4-producing Th2 or IL-17-producing Th17 cells.

It was previously shown that high PD-1 expression on T\textsubscript{FH} cells is the result of persistent interactions with Ag-presenting GC B cells (37) Thus, it seems likely that an on-
going cross talk between B cells and T<sub>FH</sub> cells results in mutual regulation. The importance of appropriate control of T<sub>FH</sub> cell expansion is illustrated by studies suggesting that aberrant expansion of T<sub>FH</sub> cells can lead to inappropriate antibody production in both autoimmune mouse models and human disease (38, 39). Our data support these findings by demonstrating that PD-1 expression on T<sub>FH</sub> cells is central to a homeostatic co-inhibitory mechanism, mediated by B7-H1-expressing B cells to limit the accumulation of this CD4<sup>+</sup> T cell subset and consequently, the humoral immunoglobulin response. However, our study does not take account for any potential effects of a lack of B7-H1 on APCs on T<sub>FH</sub>, this would need to be addressed by performing transfer of WT and B7-H1<sup>–/–</sup> B and T cells in RAG-1<sup>–/–</sup>B7-H1<sup>–/–</sup> mice in the future.

Despite the elevated antibody responses in <i>S.mansoni</i>-infected B7-H1<sup>–/–</sup> mice, we did not find any major differences in infection-induced immunopathology between WT and B7-H1<sup>–/–</sup> mice (data not shown). In other studies with <i>S.mansoni</i> infected mice that develop different severity of disease, there was no relationship between levels of total IgE or antigen-specific antibody isotypes and the degree of immunopathology (40). While our studies define no major role for B7-H1 in <i>S.mansoni</i> infection, B7-DC expressing DCs have previously been reported to be associated with morbidity in infected mice (41). We did use an antibody to block B7-DC and showed this had no effect on accumulation of T<sub>FH</sub> in response to KLH, however, ideally experiments need to be performed in B7-DC deficient mice to fully define any functional role for the PD-1:B7:H1/B7-DC pathway in helminth infections.

Therapies involving blocking PD-1 and its ligands B7-H1 and B7-DC are being actively explored for clinical use in infectious diseases, such as HIV, autoimmune conditions (42) and certain cancers (43). Our data shows that <i>in vivo</i> blockade of the PD-1:B7:H1 pathway using monoclonal antibodies can significantly alter the humoral immune response,
increasing antigen-specific antibody responses, by promoting the accumulation of antigen-specific CD4+ T<sub>FH</sub> cells. In light of this finding, modulation of the PD-1:B7-H1 pathway in patients would have potential adverse or desirable therapeutic implications depending on the infection or disease addressed. Indeed, a recent study demonstrated that PD-1 blockade evoked a 2-8 fold increase in the SIV-specific antibody titre in a macaque model of human immunodeficiency virus with improved survival (44). Conversely, in vivo blockade with anti-B7-H1 enhanced IgG2a in a murine model of systemic lupus erythematosus (SLE) leading to accelerated nephritis and increased mortality (32). In this paper we provide evidence for an in vivo cellular mechanism which explain previous observations of adverse outcome; whereby modulation of the PD-1:B7-H1 signaling pathway will alter immunoglobulin production through regulation of T<sub>FH</sub> cell expansion. This is consistent with a central role for T<sub>FH</sub> cells in shaping the antibody response as elegantly described recently (45). While data presented are relevant to current trials aimed at examining the feasibility of blocking the PD-1:B7-H1 pathway (46), they highlight the need to consider the potential expansion of the T<sub>FH</sub> cell response and the impact on the heightened antibody response on clinical outcome.

It was recently demonstrated by Shlomchik and colleagues that PD-1 and dual B7-H1/B7-DC interactions function in the positive stimulation of antibody forming cell survival following immunization (47). Our data also demonstrates an expansion of T<sub>FH</sub> cells in the absence of B7-H1/B7-DC signalling, however we describe an enhanced immunoglobulin response as a result of T<sub>FH</sub> expansion and demonstrate a specific role for B cells expressing B7-H1 but not B7-DC in T<sub>FH</sub> regulation and production of antibodies. It is noteworthy that we have seen mouse strain differences in T<sub>FH</sub> and germinal centre B cell induction; while this study used C57BL/6J strain animals, Shlomchik and colleagues used BALB/C strain mice (47). Consistent with the described role of PD-1 and its ligands as a co-inhibitory pathway,
our results demonstrate that the PD-1:B7-H1 pathway functions during the active immune response to restrain T_{FH} expansion, thereby limiting the humoral response. At later stages of the immune response, PD-1 interaction with B7-H1 and/or B7-DC would function as a co-stimulatory molecule to promote long lived antibody-producing cell survival and function. Further study of the relationship between the PD-1:B7-H1 pathway and humoral immunity at different stages of the immune response is warranted.

In summary, this study defines the PD-1:B7-H1 pathway as a new co-inhibitory mechanistic element to the on-going characterization of T_{FH} cell development and function. The data presented herein has implications for targeting the PD-1:B7-H1 pathway as a therapeutic strategy for enhancing humoral immunity for vaccines and the regulation of autoimmune diseases.
Acknowledgements:

The authors are grateful for assistance from Hendrik Nel, Caitriona Walsh, Phil Smith, Bina Mistry, Aoife Smyth and Ann Atzberger.
References:


**Footnotes:**

This work was supported by Science Foundation Ireland (07/IN1/B902), Health Research Board and National Children’s Research Centre.

¹**Corresponding author**

**Mailing address:** Padraic Fallon, Institute of Molecular Medicine, St. James’s Hospital, Trinity College Dublin, Dublin 8, Ireland. **Email:** pfallon@tcd.ie

Phone: 353 1 8963267; Fax: 353 1 8964040

**Abbreviations used in this paper:**

KLH, Keyhole Limpet hemocyanin: SEA, soluble egg antigens: BCL-6, B cell Lymphoma 6.
Figure legends:

Figure 1. Total immunoglobulin levels and germinal center B cells are increased in the absence of B7-H1 following helminth infection.

(A) Serum was obtained from *S. mansoni*-infected (week 8) WT and B7-H1⁻/- mice and examined by ELISA for total IgE, IgG1 and IgG2a. *P<0.05* by student’s t test. ‘NS’ not statistically significant. (B) Gating strategy showing the percentage GC B cells (B220⁺Fas⁺GL-7⁺) in the spleen of uninfected and infected WT and B7-H1⁻/- mice (numbers represent percentages) and (C) bar graphs depicting the percentage and total proportion (×10⁶) of GC B cells in the spleens. Data is mean ± SEM of 6-8 individual mice. *P<0.05* by ANOVA.

Figure 2. T<sub>FH</sub> cell expansion in response to helminth infection is negatively regulated by B7-H1 (A) Representative flow cytometric contour plots of spleen and MLN-derived T<sub>FH</sub> (CD4⁺B200⁻PD-1<sup>high</sup>CXCR5<sup>high</sup>) cells from both uninfected and *S. mansoni*-infected (week 8) WT and B7-H1⁻/- mice. CD4⁺B220⁻ gate is shown. Numbers shown are percentage of T<sub>FH</sub>. (B) Bar graphs are percentage and in the spleens of mice. (C) Confocal images showing CD4⁺ve (red) cells in the GC (GL-7⁺ve green) of the spleens of infected WT and B7-H1⁻/- mice (magnification x20). (D) Bar graphs are percentage and total numbers of T<sub>FH</sub> cells (×10⁶) in the MLN of mice. Data is representative of two separate experiments, with mean ± SEM of 7 individual mice shown. *P<0.05* by student’s t test.

Figure 3. T<sub>FH</sub> cells undergo greater clonal expansion in the absence of B7-H1 in response to immunization with KLH. (A) Representative flow cytometric contour plots depicting spleen and inguinal LN-derived T<sub>FH</sub> cells from WT and B7-H1⁻/- mice 14 days after
immunization with PBS or KLH/CFA. CD4^+ B220^- gate is shown. Numbers in the contour plots show the percentage of T_{FH} cells. (B) Confocal images showing CD4^{+ve} (red) cell in the GC (GL-7^{+ve} green) of inguinal LN of WT and B7-H1^{+/−} mice 21 days after KLH immunization (magnification x20). Graphs show the percentage and total proportion of T_{FH} cells in the spleens (C) and inguinal LNs (D) of immunized mice 7, 14 and 21 days after KLH immunization. (E) ELISA for KLH-specific IgG1 and IgG2a. Data are representative of two independent experiments and presented as mean ± SEM of 6 individual mice. *P<0.05 by student’s t test.

**Figure 4. T_{FH} cell expansion and antibody responses are negatively regulated by B7-H1 and B cells.**

(A) In vivo proliferation of Thy1.1^{+} T_{FH} (CD4^{+} B220^{−} PD-1^{high} CXCR5^{high}) 14 days after transfer of CFSE-labelled Thy1.1^{+} CD4^{+} cells into Thy1.2^{+} WT and B7-H1^{+/−} mice and immunization with PBS (shaded) or KLH/CFA (open). Numbers in the histograms show the percentage of Thy1.1^{+} T_{FH} cells that have diluted their CFSE. (B) BrdU incorporation into T_{FH} cells (percentage) in inguinal LNs of untreated and KLH/CFA-immunized WT and B7-H1^{+/−} on day 7, 14 and 21. (C) Percentage of Annexin V-FITC^{+} T_{FH} cells on day 21 post-immunization. (D) Representative flow cytometric contour plots depicting inguinal LN-B220^{+} B cells that express B7-H1 from WT and B7-H1^{+/−} mice 14 days after immunization with PBS or KLH/CFA. Numbers show the percentage of B cells expressing B7-H1. (E) Representative flow cytometric contour plots depicting T_{FH} cells in the inguinal LN from KLH-immunized RAG-1^{+/−} mice injected with WT CD4^{+} cells and CD19^{+} B cells from WT or B7-H1^{+/−} mice. Numbers show the percentage of T_{FH}. CD4^{+} B220^{−} gate is shown. (F) Bar graphs are the total proportion of T_{FH} cells in the inguinal LNs. (G) Anti-KLH IgG2a responses in immunised RAG-1^{+/−} mice. Data are representative of two independent experiments and presented as
mean ± SEM of 4-8 individual mice. *P<0.05 by student’s t test. ‘NS’ not statistically significant.

**Figure 5. Blockade of B7-H1 but not B7-DC induces in vivo expansion of T_{FH} cells and a corresponding increase in the specific immunoglobulin response.** WT mice immunized with KLH/CFA were treated with anti-B7-H1, anti-B7-DC and anti-PD-1 mAbs and examined on day 14 as outlined in materials and methods. (A) Representative flow cytometric contour plots of spleen and inguinal LN-derived T_{FH} cells. Numbers in the contour plots show the percentage of T_{FH}. CD4⁺B220⁻ gate is shown. A different anti-PD-1 mAb clone was used in flow cytometry than *in vivo* blocking. Bar graphs are the percentage of total proportion of T_{FH} cells in the spleens (B) and inguinal LNs (C) of KLH/CFA immunized mice. (D) ELISA for KLH-specific IgG2a. Data are from two separate experiments with mean ± SEM of 3 to 4 individual mice shown. *P<0.05 by ANOVA. ‘NS’ not statistically significant.