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## B7-H4Ig inhibits mouse and human T-cell function and treats EAE via IL-10/Treg-dependent mechanisms<sup>☆</sup>

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### Abstract

We evaluated the therapeutic efficacy and mechanisms of action of both mouse and human B7-H4 Immunoglobulin fusion proteins (mB7-H4Ig; hB7-H4Ig) in treating EAE. The present data show that mB7-H4Ig both directly and indirectly (via increasing Treg function) inhibited CD4<sup>+</sup> T-cell proliferation and differentiation in both Th1- and Th17-cell promoting conditions while inducing production of IL-10. B7-H4Ig treatment effectively ameliorated progression of both relapsing (R-EAE) and chronic EAE correlating with decreased numbers of activated CD4<sup>+</sup> T-cells within the CNS and spleen, and a concurrent increase in number and function of Tregs. The functional requirement for Treg activation in treating EAE was demonstrated by a loss of therapeutic efficacy of hB7-H4Ig in R-EAE following inactivation of Treg function either by anti-CD25 treatment or blockade of IL-10. Significant to the eventual translation of this treatment into clinical practice, hB7-H4Ig similarly inhibited the *in vitro* differentiation of naïve human CD4<sup>+</sup> T-cells in both Th1- and Th17-promoting conditions, while promoting the production of IL-10. B7-H4Ig thus regulates pro-inflammatory T-cell responses by a unique dual mechanism of action and demonstrates significant promise as a therapeutic for autoimmune diseases, including MS.

### Keywords

Autoimmunity; B7-H4; CD4<sup>+</sup> T-cell; Costimulatory/co-inhibitory molecule; EAE; Regulatory T-cells

### 1. Introduction

An important goal in autoimmune disease therapy is development of new treatments re-establishing durable tolerance in self-reactive CD4<sup>+</sup> T-cells [1]. MS is an autoimmune disease characterized by Th1 (IFN- $\gamma$ ) and Th17 (IL-17/GM-CSF) CD4<sup>+</sup> T-cell responses to epitopes on myelin basic protein (MBP), proteolipid protein (PLP), and/or myelin-oligodendrocyte glycoprotein (MOG) [2-5]. Experimental autoimmune encephalomyelitis (EAE) is a CD4<sup>+</sup> T-cell mediated model of MS ideal for characterizing potential tolerance-based immunotherapies and their underlying mechanisms. Full activation of naïve T-cells

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(CD4<sup>+</sup>CD62L<sup>hi</sup>CD25<sup>-</sup>) requires interaction of the Ag-specific TCR (signal one) with peptide presented in the context of major histocompatibility complex II (MHC II) on the surface of antigen presenting cells (APCs), and delivery of positive co-stimulatory signals (signal two), i.e., ligation of CD28 on CD4<sup>+</sup> T-cells by APC-expressed B7-family members B7-1 (CD80) and B7-2 (CD86) [6]. Costimulatory interactions serve as potential drug targets, e.g. CTLA-4Ig blocks CD80/CD86-CD28 interaction [1,7-9]. Additionally, both secreted cytokines and cell surface molecules are required for the differentiation of pro-inflammatory Th17 cells secreting IL-17, IL-6, IL-22, TNF- $\alpha$ , and GM-CSF [10] versus CD4<sup>+</sup>/FoxP3<sup>+</sup> Tregs that are functionally dependent upon TGF- $\beta$  and/or IL-10 [11-16]. TGF- $\beta$  in combination with IL-2 is critical for differentiation of Tregs [17] while TGF- $\beta$  in combination with IL-6 drives differentiation of pro-inflammatory Th17 cells [18].

Ligation of T-cell-expressed co-inhibitory receptors, e.g. PD-1 by its ligand PD-L1 (B7-H1), can effectively suppress T-cell responses. We have previously shown that PD-L1 deficient mice exhibit increased severity of MOG<sub>35-55</sub>-induced C-EAE, and treatment of wildtype mice with anti-PD-L1 blocking antibody enhances EAE by increasing the number of MOG<sub>35-55</sub>-specific CD4<sup>+</sup> T-cells producing IFN- $\gamma$  and IL-17 [19]. Similarly, B7-H4 is hypothesized to have immune modulatory function [20-23]. Tumor cell-expressed B7-H4 is suggested to be a mechanism by which these cells evade anti-tumor immune responses [24,25]. Therapeutically, B7-H4Ig treatment modulates the level of inflammatory CD4<sup>+</sup> T-cell function in rheumatoid arthritis, the NOD model of T1D and in allogeneic islet cell transplantation [26-28]. Therefore, the goal of the present study was to determine the efficacy of B7-H4Ig treatment during EAE and more importantly to determine its mechanism(s) of action. We show that B7-H4Ig treatment ameliorates EAE progression via the unique dual mechanisms of inhibiting effector CD4<sup>+</sup> Th1/17-cell activity while concomitantly increasing the number and function of Tregs in both mouse and human T cells.

## 2. Materials and methods

### 2.1. Mice, CD4<sup>+</sup> T-cell isolation and culture

Female SJL/JCrHsd (SJL/J), C57BL/6 (Harlan Labs; Indianapolis, IN), DO11.10, BALB/c (Jackson Laboratories; Bar harbor, ME); SJL Actin/GFP, SJL FoxP3/GFP, and C57BL/6 FoxP3/GFP (bred in-house) were housed under SPF conditions in the Northwestern University Center for Comparative Medicine. Naïve CD4<sup>+</sup> T-cells (CD4<sup>+</sup> L-selectin<sup>hi</sup> cells) were purified using AutoMacs Magnetic Bead cell separation technology (Miltenyi Biotech; Auburn, CA) from total LN cells isolated from unprimed mice with purity ranging from 98 to 99.9%. For *in vitro* activation, 3–5  $\times$  10<sup>5</sup> naïve DO11.10 CD4<sup>+</sup> T-cells were cultured with an equal number of irradiated BALB/c splenocytes plus OVA<sub>323-339</sub> (10  $\mu$ l/ml) in Th0 (200 U/ml IL-2); Th1-(200 U/ml IL-2, 10 ng/ml IL-12, 1  $\mu$ g/ml anti-IL-4); Th2- (200 U/ml IL-2, 500 U/ml IL-4, 1  $\mu$ g/ml anti-IFN- $\gamma$ ), Th17- (10 ng/ml TGF- $\beta$ 1, 50 ng/ml IL-6, 1  $\mu$ g/ml anti-IFN- $\gamma$ , 1  $\mu$ g/ml anti-IL-4, 1  $\mu$ g/ml anti-IL-2), or iTreg- (100 U/ml IL-2, 25 ng/ml TGF- $\beta$ 1, 100 nM retinoic acid) promoting conditions. Peptides (PLP<sub>139-151</sub>, PLP<sub>178-191</sub>, and OVA<sub>323-339</sub>) were purchased from Peptides International (Louisville, KY) and purified by HPLC (purity of 96–99%). After 3–7 d of culture, the T effector cells were isolated and culture supernatants collected and cytokine concentrations determined via multiplex Luminex Liqui-Chip (Millipore; Billerica, MA).

### 2.2. Preparation of mouse and human B7-H4Ig

mB7-H4Ig (mouse B7-H4 extracellular domain fused with murine IgG<sub>2a</sub>) and hB7-H4Ig (human B7-H4 extracellular domain fused with human IgG<sub>1</sub>) fusion proteins were produced in suspension culture in an animal protein-free-adapted CHOK1SV (Lonza Biologics,

Allendale, NJ) cell line utilizing the glutamine synthetase gene expression system, and purified using Amplimmune's protein purification process. The extracellular domain (ECD) of hB7-H4 is 90% identical to the murine sequence.

### 2.3. Human CD4<sup>+</sup> T-cell isolation and culture

Naïve CD4<sup>+</sup> T-cells were isolated from total PMBCs (LifeSource; Evanston, IL) of 10–12 healthy donors using AutoMacs cell separation technology.  $5 \times 10^5$  cells/well were cultured with autologous irradiated total PBMCs ( $5 \times 10^5$  cells/well) and stimulated with anti-CD3 (clone OKT3; eBioscience) and anti-CD28 (Clone CD28.2; eBioscience) (0.5 µg/ml) plus Control-Ig or human B7-H4Ig (10 µg/ml). Cells were cultured in Th0- (100 U/ml IL-2, 1 µg/ml anti-IL-4, 1 µg/ml anti-IFN-γ), Th1- (100 U/ml IL-2, 10 ng/ml IL-12, and 1 µg/ml anti-IL-4), Th2- (100 U/ml IL-2, 10 ng/ml IL-4, 1 µg/ml anti-IFN-γ), Th17- (10 ng/ml TGF-β, 50 ng/ml IL-6, 10 ng/ml IL-1β, 1 µg/ml anti-IFN-γ, 1 µg/ml anti-IL-4), or iTreg- (100 U/ml IL-2, 25 ng/ml TGF-β1, and 100 nM retinoic acid) promoting conditions. After 3–7 d of culture, the T effector cells are isolated and culture supernatants collected and cytokine concentrations determined.

### 2.4. R-EAE, C-EAE, and PLP<sub>139–151</sub> transfer R-EAE

6–7-wk-old female SJL/J or SJL-Actin/GFP mice were immunized s.c. with 100 µl of an emulsion containing 200 µg of *M. tuberculosis* H37Ra (BD Biosciences; San Jose, CA) and 50 µg of PLP<sub>139–151</sub> distributed over three sites on the flank. C57BL/6 mice were immunized s.c. with 100 µl of an emulsion containing 200 µg of *M. tuberculosis* H37Ra and 200 µg of MOG<sub>35–55</sub> distributed over three sites on the flank, and received pertussis toxin on d0 and d+2 post-disease induction via i.p. injection (200 µl of 1 µg/ml). To inactivate Tregs, mice received two injections of anti-CD25 [clone 7D4 at 500 µg/injection i.p. (Bio X Cell; West Lebanon, NH)] on the indicated days. To neutralize IL-10, mice received 6 injections of anti-IL-10 [clone JES5-2A5 at 100 µg/injection i.p. (Bio X Cell; West Lebanon, NH)] on the indicated days [29]. In transfer EAE experiments draining LNs were collected on day 8 post priming, and total draining LN cells were reactivated in the presence of 20 µg/ml of PLP<sub>139–151</sub>, at a cell density of  $8 \times 10^6$  cells/ml for 72 h. After culture  $3–5 \times 10^6$  blast cells were transferred to recipient SJL/J mice. Mice were treated with Control-Ig or B7-H4Ig either at the time of cell transfer or at the onset of remission (approximately day +15–20 for most animals). Individual animals were observed at the indicated time points and clinical scores assessed in a blinded fashion on a 0–5 scale: 0, no abnormality; 1, limp tail; 2, limp tail and hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; and 5, moribund. Data are reported as the mean daily clinical score.

### 2.5. Delayed-type hypersensitivity (DTH) assay and ex vivo recall responses

On d+10 or d+35 post-disease induction, mice were assayed for DTH. Mice were anaesthetized by inhalation of isoflurane and ear thickness measured using a dial thickness gauge. 10 µg of PLP<sub>139–151</sub> and PLP<sub>178–191</sub> in 10 µl of PBS were injected into the left and right ears, respectively. The increase in ear thickness was determined after 24 h. Mice were then sacrificed and single cell suspension of spleens and draining LNs were cultured ( $5 \times 10^5$  cells/well) in the presence of medium alone, OVA<sub>323–339</sub>, PLP<sub>139–151</sub> and PLP<sub>178–191</sub> (20 µg/ml). 24 h post-culture initiation, wells were pulsed with 1 µCi of <sup>3</sup>H-TdR and harvested at 72 h and <sup>3</sup>H-TdR incorporation was detected using a Topcount Microplate Scintillation Counter. Results are expressed as the mean counts per minute (CPM) of triplicate cultures. Cytokine levels were determined via LiquiChip analysis.

## 2.6. Immunohistochemistry

Relapsing-remitting EAE was induced in SJL-FoxP/GFP mice with PLP<sub>139–151</sub>/CFA and mice received either Control-Ig or hB7-H4Ig was decreased above. On the day following the final treatment, mice were anesthetized and perfused with PBS followed by 1% paraformaldehyde, brains and spinal cords were removed and embedded with OCT compound (Sakura Finetek, Trrance, CA), frozen on dry-ice, and stored at  $-80^{\circ}\text{C}$  until processing and analysis. Eight-micrometer sections containing either the lumbar spinal cord or cerebellum were sectioned on the cryostat, sections were air dried at room temperature for 30 min, rinsed in  $\text{DH}_2\text{O}$  to remove O.C.T., and fixed in  $-20^{\circ}\text{C}$  acetone for 10 min. Sections were dried at room temperature for 10 min, washed in PBS  $3 \times 5$  min, blocked in 5% donkey serum in PBS + 0.1% triton x-100 (PBS+) for 60 min. prior to incubation with primary antibodies diluted in PBS + 0.1% Triton x-100 + 5% donkey serum overnight at  $4^{\circ}\text{C}$ , i.e., anti-PLP antibody (clone plpc1) (AbD Serotec; Raleigh, NC) and anti-CD4 antibody (clone GK.15) (eBioscience). After washing in PBS  $3 \times 10$  min, sections were incubated in secondary antibodies in PBS + for 1 h. at room temperature, i.e., Cy3 donkey anti-rat (Jackson ImmunoResearch; West Grove, PA) and FITC anti-rat IgG (Vector Labs; Burlingame, CA) respectively, washed in PBS  $3 \times 10$  min, incubated in DAPI for nuclear staining 5 min, washed  $3 \times 5$  min. in PBS then coverslipped using hard setting Vectamount (Vector Laboratories, Burlingame, CA). Images were taken on a Leica DM 5000B microscope using ImagePro software.

## 2.7. FACS analysis

FACS analysis was performed on spinal cord leukocytes isolated from individual mice (5 per group) perfused with 20 ml of PBS [30]. hB7-H4Ig (PE conjugated) was used for binding analysis of cells spleen and LN cells. Cells were stained with anti-CD4 (clone RM4-5), anti-FoxP3 (clone FJK-16s), anti-Helios (clone 22F6) (eBioscience; San Jose, CA), anti-CD25 (clone PC61), and anti-CD44 (clone IM7) (BD Bioscience; San Diego, CA).  $5 \times 10^5$  viable cells were analyzed per individual sample using a BD Canto II cytometer (BD Bioscience), and the data were analyzed using FlowJo Version 9.5.2 software (Tree Star, Inc.; Ashland, OR).

## 2.8. Statistical analyses

Comparisons of the percentage of animals showing clinical disease were analyzed by  $\chi^2$  using Fisher's exact probability, and two-way ANOVA with a Bonferroni post-test was used to determine statistical differences between mean clinical disease scores. Single comparisons of two means were analyzed by Student's *t*-test.

## 3. Results

### 3.1. Efficacy of mouse and human B7-H4Ig treatment for regulating EAE induction and progression

The present studies were begun to determine the ability of B7-H4Ig to inhibit ongoing R-EAE. To do so, SJL/J mice were primed with PLP<sub>139–151</sub>/CFA and allowed to progress through the acute phase of disease, and treatment with either Control Ig or mB7-H4Ig was initiated during disease remission. The present data show that initiation of mB7-H4Ig treatment during disease remission significantly ameliorated further disease progression (Fig. 1A) decreasing the number of effector  $\text{CD4}^+/\text{CD44}^{\text{hi}}$  cells within the CNS, with a trend toward increasing the number of Tregs (Fig. 1B). Additionally, a significant increase in the number of Tregs was observed in the spleen (data not shown). These findings suggest that mB7-H4Ig treatment may allow for a more tolerogenic environment within the CNS since the ratio of Tregs to Teff-cells was dramatically increased (Control-Ig =  $0.03 \pm 0.0$ ;  $60 \mu\text{g}$ /

dose B7-H4Ig =  $2.89 \pm 1.9$ ; 300  $\mu\text{g}/\text{dose}$  B7-H4Ig =  $3.0 \pm 1.0$ ). While the mB7-H4Ig-induced increase in Tregs within the CNS did not achieve significance in the present experiment, in other experiments (Fig. 6A and B), significance was achieved in both the CNS and the spleen suggesting that the Tregs may be cycling between the spleen and the CNS during various stages of disease. We also demonstrated that treatment beginning on Day +20 with either a low dose (60  $\mu\text{g}/\text{dose}$ ) or high dose (300  $\mu\text{g}/\text{dose}$ ) of mB7-H4Ig or hB7-H4Ig significantly decreased disease progression of MOG<sub>35–55</sub>/CFA-induced C-EAE in C57BL/6 mice. Interestingly, no protection was seen in mice in which treatment was initiated on Day +60 (Fig. 1C and D) although *ex vivo* MOG<sub>35–55</sub>-specific CD4<sup>+</sup> T-cell proliferation was inhibited in both treatment regimens (Fig. 1E).

### 3.2. B7-H4Ig treatment regulates CD4<sup>+</sup> T-cell responses in vivo

To determine if mB7-H4Ig treatment decreases CD4<sup>+</sup> T-cell responses during disease initiation, SJL/J mice were primed with PLP<sub>139–151</sub>/CFA and treated with Control-Ig or mB7-H4Ig beginning on the day of priming. Mouse B7-H4Ig treatment significantly inhibited the PLP<sub>139–151</sub>-specific DTH response as compared to Control-Ig-treated mice (Fig. 2A), and decreased the *ex vivo* PLP<sub>139–151</sub>-specific recall response (Fig. 2B). Relevant to disease therapy, splenocytes from mB7-H4Ig-treated mice exhibited decreased recall responses to both the disease-inducing PLP<sub>139–151</sub> peptide and to PLP<sub>178–191</sub>, the dominant spread epitope, as determined by proliferation (Fig. 2C), and IL-17 (Fig. 2D) and IFN- $\gamma$  secretion (Fig. 2E) demonstrating that mB7-H4Ig can both inhibit effector cytokine production by previously activated T-cells and priming of naïve T-cells.

### 3.3. B7-H4Ig treatment inhibits in vitro differentiation of mouse Th1/Th17 cells

We next examined the ability of B7-H4Ig to modulate the differentiation of naïve DO11.10 CD4<sup>+</sup> T-cells, either containing or depleted of CD25<sup>+</sup> nTregs, and stimulated in Th1- vs. Th17-driving conditions. Mouse B7-H4Ig decreased the levels of IFN- $\gamma$  (Fig. 3A) and IL-17 (Fig. 3C) produced in the absence of Tregs in a dose-dependent manner. Significantly, mB7-H4Ig-induced down-regulation of naïve T-cells containing CD25<sup>+</sup> Tregs under both Th1 (Fig. 3B) and Th17 (Fig. 3D) driving conditions was more robust and associated with a dramatic increase in IL-10 production. Mouse B7-H4Ig-induced inhibition of Th1/Th17 cell differentiation was time-dependent. We next sought to determine if there was a temporal ability of mB7-H4Ig to modulate naïve CD4<sup>+</sup> T-cell differentiation. To do so, naïve DO11.10 CD4<sup>+</sup> T-cells depleted of CD25<sup>+</sup> Tregs were cultured in Th1- or Th17-promoting conditions plus addition or wash out of Control-Ig or mB7-H4Ig at the time of culture initiation ( $t = 0$ ) or 12, 24, 48, or 72 h post-culture initiation. Addition of mB7-H4Ig to the Th1 cell-promoting cultures at any time prior up to 72 h post-culture initiation inhibited IFN- $\gamma$  production. Additionally, wash out of mB7-H4Ig in Th1 cell-promoting conditions before 24 h post-culture initiation, resulted in no decrease in IFN- $\gamma$  production (Fig. 3E). Addition of mB7-H4Ig to the Th17 cell-promoting cultures at any time prior to 48 h post-culture initiation decreased IL-17 production. Wash out of mB7-H4Ig in Th17 cell-promoting cultures before 48 h post-culture initiation, resulted in no decrease in the level of IL-17 produced (Fig. 3F). This time frame is shifted to 12–24 h with regard to the ability of mB7-H4Ig to enhance the level of IL-10 produced by DO11.10 CD4<sup>+</sup> T-cells activated in Th17-promoting conditions (Fig. 3G). Therefore, mB7-H4Ig alteration of naïve T-cell differentiation is time- and cell activation state-dependent.

### 3.4. B7-H4Ig treatment suppresses adoptive transfer R-EAE by PLP<sub>139–151</sub>-specific T cells

The above data show that regulation of pro-inflammatory CD4<sup>+</sup> T-cell function by B7-H4Ig is time-dependent. For the potential clinical development of B7-H4Ig as an autoimmune disease therapeutic, we chose to utilize hB7-H4Ig for the remainder of the studies. Given the

~90% amino acid homology between mouse and human B7-H4 [21], we reasoned that treatment of mice with hB7-H4Ig would be functional. After completing pilot studies to confirm hB7-H4Ig was functionally similar to mB7-H4Ig (data not shown), we next determined the time-dependence of B7-H4Ig binding to CD4<sup>+</sup> T-cells *in vivo*, SJL/J mice were primed with PLP<sub>139-151</sub>/CFA, and binding of hB7-H4Ig to CD4<sup>+</sup> T-cells from the draining inguinal lymph nodes (LNs), spleen, and non-draining cervical LNs was assessed at Days +3, +6 and +10. The data show an increase in hB7-H4Ig binding to T cells in the spleen and draining LNs 6 d post-priming (Fig. 4A). Additionally, hB7-H4Ig binds to an increased percentage of CD4<sup>+</sup> T-cells from PLP<sub>139-151</sub>-primed mice following *ex vivo* peptide re-activation (Fig. 4B). Taking the time-dependency for mB7-H4Ig to modulate the level of cytokine produced with the above hB7-H4Ig binding data suggests that the putative B7-H4 receptor is induced on the surface of CD4<sup>+</sup> T cells in an activation-induced manner.

We next determined if hB7-H4Ig treatment could inhibit adoptive transfer of R-EAE mediated by PLP<sub>139-151</sub>-specific T cells derived from SJL-Actin/GFP Tg mice. Human B7-H4Ig treatment of wildtype SJL/J recipients beginning on the day of cell transfer dramatically decreased disease expression (2/10 mice affected with maximal clinical score of 1.0 each) as compared to Control-Ig-treated mice (10/10 affected) (Fig. 4C). The enhanced efficacy of hB7-H4Ig treatment in the adoptive transfer EAE model compared to active EAE (Fig. 1) likely represents the lack of continued T-cell activation via the peptide/CFA reservoir present in actively immunized mice. Human B7-H4Ig treatment did not result in a significant alteration in the number of donor GFP<sup>+</sup> cells within the spleen (Fig. 4D), but significantly reduced the number of transferred cells within the CNS (Fig. 4E). Furthermore, PLP<sub>139-151</sub>-induced proliferative responses of CNS infiltrating T cells from hB7-H4Ig treated mice were significantly decreased as compared to CNS-infiltrating cells from Control-Ig treated mice (Fig. 4F). These findings indicate that hB7-H4Ig treatment inhibits the CNS transmigration of activated PLP<sub>139-151</sub>-specific CD4<sup>+</sup> T-cells.

We next asked if adoptive R-EAE could be inhibited by addition of hB7-H4Ig to *in vitro* reactivation cultures of PLP<sub>139-151</sub>-sensitized total LN cells. Human B7-H4Ig significantly inhibited production of IFN- $\gamma$ , IL-17, and GM-CSF, while enhancing the levels of IL-10 (Fig. 4G). Functionally, transfer of either  $3 \times 10^6$  blast cells from hB7-H4Ig treated cultures or  $1.5 \times 10^6$  blast cells from Control-Ig treated cultures plus  $1.5 \times 10^6$  blast cells from hB7-H4Ig treated cultures resulted in decreased disease severity as compared to cells from Control-Ig-treated cultures (Fig. 4H). Therefore, hB7-H4Ig decreases the ability of PLP<sub>139-151</sub> sensitized T-cells to induce adoptive R-EAE.

### 3.5. B7-H4Ig treatment decreases the number of effector CD4<sup>+</sup> T cells and increases the number of Tregs in the CNS

The above data clearly show that treatment of mice with B7-H4Ig during both active and adoptive transfer EAE decreases the level of disease severity. The data also show that treatment with hB7-H4Ig decreases the ability of PLP<sub>139-151</sub>-sensitized cells from entering and/or being maintained within the CNS following transfer into an unprimed SJL/J recipients. Additionally, the data from Fig. 1C hinted at a B7-H4Ig treatment-induced decrease in the number of Teff-cells and concomitant increase in the number of Tregs. To further address this later possibility, immunohistochemical analysis was performed on SJL-FoxP3/GFP mice treated with B7-H4Ig during disease remission. Following the last treatment, mice were perfused and histological analysis of the lumbar spinal cord, cerebellum, and the spleen were performed. As shown in Fig. 5A and B, hB7-H4Ig treatment decreased the number of infiltrating CD4<sup>+</sup> T cells present within the white matter tracks of the lumbar spinal cord. Within the cerebellum, lesions were found in both mice treated with Control-Ig and hB7-H4Ig treatment mice, however there was a decreased

number of CD4<sup>+</sup> T cells present within the lesions of hB7-H4Ig treated mice as compared to Control-Ig treated mice (Fig. 5C). Additionally, an increased number of FoxP3<sup>+</sup> cells were found within the lesions of hB7-H4Ig treated mice (indicated by the white arrow in Fig. 5C). In further support of the hB7-H4Ig-induced increase in the number of Tregs, histological analysis of the spleen (Fig. 5D) also showed a significant increase in the number of FoxP3<sup>+</sup> cells within the CD4<sup>+</sup> T cell white pulp regions of the spleen of hB7-H4-Ig-treated mice. Taken together, this analysis shows that hB7-H4Ig treatment decreases the number of CD4<sup>+</sup> T cells present within the CNS and increases the number of Tregs in the both spleen and the CNS.

Our results (Figs. 1C and 5C, and 5D) and others [27] suggest that B7-H4Ig treatment alters the number of Tregs within the target tissue of the ongoing autoimmune response. We thus asked if hB7-H4Ig treatment altered the number of natural Tregs (CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup>/Helios<sup>hi</sup>) versus induced Tregs (CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup>/Helios<sup>lo</sup>) present within the spleen and CNS. The present data show that hB7-H4Ig treatment induced an increase in the number Helios<sup>lo</sup> Tregs within the spleen and a significant increase in the number of both Helios<sup>hi</sup> and Helios<sup>lo</sup> Tregs within the CNS (Fig. 6A, B) arguably indicating drug-induced *in vivo* expansion of both nTregs and iTregs [31,32]. Supporting the ability of B7-H4Ig to induce iTreg expansion *in vivo*, hB7-H4Ig also induced a dose-dependent increase in the numbers of iTregs derived from naïve CD4<sup>+</sup> T-cells stimulated in iTreg promoting cultures (Fig. 6C).

### 3.6. B7-H4Ig treatment increases the number and functional suppressive capacity of Tregs

We next determined if hB7-H4Ig treatment also altered Treg function. Tregs were isolated from unprimed C57BL/6-FoxP3/GFP mice following treatment with either Control-Ig or hB7-H4Ig and co-cultured at various ratios with naïve CD4<sup>+</sup> T-cell plus anti-CD3 (Fig. 6D). FoxP3/GFP<sup>+</sup> Tregs isolated from hB7-H4Ig-treated mice had significantly increased suppressor function, but not Treg number, as compared to Tregs isolated from Control-Ig-treated mice indicating that hB7-H4Ig directly enhances Treg suppressor function in the absence of an inflammatory immune response. To determine if antigen-stimulation is required for the hB7-H4Ig-induced increase in the number of Tregs previously observed (Fig. 6A, B), SJL-FoxP3/GFP mice were primed with PLP<sub>139-151</sub> and treated with Control-Ig or hB7-H4Ig beginning at disease remission and Tregs were sort purified and assayed for suppressive function following the final treatment. Human B7-H4Ig treatment increased both the percentage and number of Tregs recovered and enhanced their *in vitro* suppressive function (Fig. 6E). Lastly, we determined the requirement for Tregs in hB7-H4Ig-mediated regulation of R-EAE *in vivo*. SJL/J mice were primed with PLP<sub>139-151</sub>/CFA and received either two treatments of Control mAb or anti-CD25 at disease remission to inactivate Tregs [33]. Beginning on the day of the second mAb treatment, half of each group was treated with either Control-Ig or hB7-H4Ig. Significantly, functional Tregs must be present at the time of hB7-H4Ig treatment for effective therapeutic regulation of R-EAE as anti-CD25 treatment reversed hB7-H4Ig-induced disease regulation.

We next determined if a mechanistic linkage between the B7-H4Ig-induced increase in the level of IL-10 production by both activated encephalitogenic T cells (Fig. 4G) and by naïve T cells stimulated in either Th1- or Th17-driving conditions (Fig. 3B, D) exists. Therefore, we asked if IL-10 was required for the disease modulating effects of B7-H4Ig treatment. SJL/J mice were primed with PLP<sub>139-151</sub>/CFA and treated with Control mAb or anti-IL-10 in combination with either Control-Ig or hB7-H4Ig beginning at disease remission. The present data show that neutralization of IL-10 reverses the therapeutic effect of hB7-H4Ig treatment clearly demonstrating the IL-10 dependence. Therefore, hB7-H4Ig treatment decreases the level of R-EAE severity in a functional Treg- and IL-10-dependent manner.

### 3.7. Human B7-H4Ig inhibits Th1/Th17 cell differentiation and enhances Th2 cell differentiation of human T cells

The above data show that both mB7-H4Ig and hB7-H4Ig modulate mouse CD4<sup>+</sup> T-cell responses *in vitro* and *in vivo*. We next asked if hB7-H4Ig would similarly modulate human CD4<sup>+</sup> T-cell responses. Naïve human CD4<sup>+</sup> T-cells from 10 to 12 healthy donors were activated in Th0-, Th1-, Th2-, Th17-, or iTreg-promoting conditions as detailed in the Methods. The present data show that hB7-H4Ig treatment decreased cellular proliferation, the level of TNF- $\alpha$  secreted, and the level of lineage-associated cytokine produced in Th1- and Th17-promoting conditions, respectively. Similarly to mouse naïve CD4<sup>+</sup> T cells activated *in vitro*, hB7-H4Ig treatment induced a trend toward increasing IL-10 production in Th0-, Th1-, Th2-, or iTreg-promoting conditions, with a statistically significant increase in the level of IL-10 produced by naïve human CD4<sup>+</sup> T-cells activated in the presence of Th17-promoting conditions (Fig. 7). Thus, hB7-H4Ig modulates inflammatory cytokine production by human CD4<sup>+</sup> T-cells replicating the mouse data. Most importantly, a significant increase in IL-10 production is noted, similar to the pattern seen with mouse cells, when human CD4<sup>+</sup> T-cells are activated in Th17-promoting conditions. Additionally, hB7-H4Ig also significantly increased IL-4 production by human CD4<sup>+</sup> T-cells activated in Th2-promoting conditions.

## 4. Discussion

Our results indicate that short-term treatment of either PLP<sub>139-151</sub>-induced R-EAE in SJL/J mice or MOG<sub>35-55</sub>-induced C-EAE in C57BL/6 mice with either mouse or human B7-H4Ig significantly inhibits disease progression by inhibiting pro-inflammatory Th1/Th17 cell activation/function via Treg activation. The present data are the first to show the functional linkage between the hB7-H4Ig-induced increase in the number of Tregs at the inflammatory site, the hB7-H4Ig-induced increase Treg suppressor function, and the requirement for both functional Tregs and IL-10 at the initiation of B7-H4Ig treatment. This clearly distinguishes the mechanism of B7-H4Ig therapy from the published mechanism of action for CTLA-4Ig treatment that blocks both CD80 and CD86, thereby inhibiting CD4<sup>+</sup> T-cell activation and potentially Treg development [34]. Treg activation was required for regulation of EAE progression as depletion of CD25<sup>+</sup> T-cells prior to B7-H4Ig therapy abrogated clinical protection. As IL-10 was also required for B7-H4Ig-induced regulation of EAE progression, it is likely that the Treg-mediated suppression was mediated via IL-10 production, which is an area of further research within the laboratory. In support of the hypothesis that mouse or human B7-H4Ig induces an inhibitory signal, as opposed to blocking its potential receptor, B7-H4<sup>-/-</sup> mice show increased EAE as compared to wildtype mice [35]. However in contrast to our present data and other studies using B7-H4Ig treatment, the B7-H4<sup>-/-</sup> (B7-X<sup>-/-</sup>) mice did not exhibit a decreased number of nTregs. This may be due to fundamental differences between the two experimental models, i.e., loss of a negative regulatory signal in B7-H4<sup>-/-</sup> mice vs. induction of a regulatory signal following ligation of the B7-H4 receptor following B7-H4Ig treatment. Collectively, our findings suggest that B7-H4Ig cross-links its receptor and induces a regulatory signal within activated target CD4<sup>+</sup> T-cells. While the receptor for B7-H4 is still unknown, our data elucidates the *in vivo* function of B7-H4 on the cellular level, and the signaling pathways that may be involved in B7-H4 receptor-mediated CD4<sup>+</sup> T-cell regulation.

To determine if B7-H4Ig treatment modulated the suppressive function of Tregs *in vivo*, we isolated Tregs from SJL-FoxP3/GFP mice following treatment with Control-Ig or B7-H4Ig during PLP<sub>139-151</sub>-induced R-EAE. Tregs isolated from B7-H4Ig-treated mice indeed showed significantly increased suppressive activity. Regarding the natural biology of B7-H4, published data has shown that alternatively activated macrophages (M2) are able to suppress EAE following transfer [36] and may possess neuroprotective activity [37]. As a



potential innate regulatory effect of B7-H4 signaling, co-culture of IL-10/TGF- $\beta$  treated M2 macrophages expressing B7-H4 decreases CD4<sup>+</sup> T-cell proliferation and induces increased numbers of CD4<sup>+</sup>/FoxP3<sup>+</sup> T-cells [38]. Collectively, our findings indicate that B7-H4Ig treatment induces a tolerogenic microenvironment by increasing the ratio of Tregs to effector CD4<sup>+</sup> T-cells, especially within the CNS, which has been shown to serve as a predictor of disease severity/resolution [39].

Other B7-CD28 family member proteins have been shown to modulate CD4<sup>+</sup> T-cell function *in vitro* via a skewing of the effector response [40]. For example, activation of naïve CD4<sup>+</sup> T-cells in the presence of beads coated with anti-CD3/28 and PD-L1Ig in Th1- or Th17-promoting conditions has been shown to decrease the number of resultant IFN- $\gamma$  and IL-17 producing cells respectively, and to increase the number of CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs [41]. Another B7-family receptor/ligand pair interaction, ICOS/ICOS-L, favors Th2 cell differentiation and IL-4 production [42]. Regarding the ability of an inhibitor molecule to modulate CD4<sup>+</sup> T-cell function, the respective cell must express the correct receptor and the chromatin structure must allow for transcriptional regulation [43]. While the B7-H4 receptor is still unknown, our finding of a time-dependent ability of B7-H4Ig to modulate Th1 cell and Th17 cell differentiation *in vitro* is likely associated with transient B7-H4 receptor upregulation. Our data also show a time-dependent increase in B7-H4Ig binding to activated CD4<sup>+</sup> T-cells within the draining LNs of PLP<sub>139-151</sub>/CFA primed mice. Furthermore, correlating with the time-dependence of B7-H4 receptor expression, is the time-dependency of the transcriptome related to differentiation of effector CD4<sup>+</sup> Th1 and Th17 effector cells (V.K. Kuchroo, personal communication).

Our *in vitro* studies clearly show that mB7-H4Ig has a direct inhibitory effect on the differentiation of both Th1 and Th17 cells in the absence of nTregs, but did not detect any appreciable effect on differentiation of naïve mouse CD4<sup>+</sup> T-cells toward the Th2 phenotype. Similarly, treatment of mice with mB7-H4Ig decreased both the level of cellular proliferation and inflammatory cytokine, i.e., IFN- $\gamma$  and IL-17, by splenocytes reactivated *ex vivo*. Functionally, the decrease in the level of IL-17 produced by splenocytes reactivated with PLP<sub>178-191</sub> peptide supports that hypothesis that mB7-H4Ig decreases the level of epitope spreading that occurs following treatment. However, the data also show that the level of IL-17 secreted by the splenocytes isolated from mB7-H4Ig treated mice activated *ex vivo* with PLP<sub>139-151</sub> peptide does not achieve that same level of significance when compared to the decrease in the PLP<sub>178-191</sub> response. Potential explanations for this finding include the fact that B7-H4Ig treatment was initiated following PLP<sub>139-151</sub> priming and that the bolus of PLP<sub>139-151</sub>/CFA present at the site of injection used to induce R-EAE is still present allowing for continued activation of PLP<sub>139-151</sub>-specific CD4<sup>+</sup> T cells, which would not occur with the PLP<sub>178-191</sub>-specific CD4<sup>+</sup> T cell population which is activated in response to small amounts of endogenously released myelin epitopes.

Significant to its translation to the clinic for autoimmune therapy, hB7-H4Ig similarly inhibited differentiation of naïve human CD4<sup>+</sup> T-cells toward Th1 and Th17 phenotypes while concomitantly inducing IL-10 production perhaps suggesting induction of the recently-described regulatory Th17 phenotype [44]. There was also a trend for increased IL-10 production in iTreg-promoting conditions, and in contrast to the mouse data, hB7-H4Ig also induced increased production of IL-4 and IL-5 when human naïve CD4<sup>+</sup> T-cells were activated in Th2-promoting conditions. Additionally, B7-H4Ig treatment enhanced IL-5 production by human naïve CD4<sup>+</sup> T-cells activated in iTreg-promoting conditions. The later effect may be a consequence of species differences between mouse and human CD4<sup>+</sup> T cells and the identity of the B7-H4 receptor(s), or former FoxP3 expressing cells that have begun to produce Th2-type of cytokines [45]. FoxP3 has been shown to repress inflammatory CD4<sup>+</sup> T-cell-associated genes, while concurrently inducing the expression of

genes associated with Treg function [46,47]. However, the ability to stably induce a Treg phenotype is dependent upon the level and stability of FoxP3 expression [48]. Human Tregs purified based on expression of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>neg</sup> have been shown to be heterogeneous regarding their ability to maintain FoxP3 expression following *in vitro* culture [49]. Loss of FoxP3 expression is associated with a CD45RA<sup>-</sup> phenotype, while CD45RA<sup>+</sup> Tregs maintain FoxP3 expression [50]. It has been recently shown that the CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>neg</sup>CD45RA<sup>-</sup> Tregs that lose FoxP3 following culture upregulate Th2 cell-associated genes as well as secrete IL-4, IL-5, and IL-13, while secreting minimal levels of Th1 cell and Th17 cytokines [45]. To reconcile our present data with the above findings regarding FoxP3 expression stability, ongoing experiments are determining if B7-H4Ig treatment is able to induce a transient low level of FoxP3 expression when naïve CD4<sup>+</sup> T-cells are active in the presence of Th2-promoting conditions.

While our results show that both mouse and human B7-H4Ig possess similar biological activity in EAE, we are concentrating on the use of hB7-H4Ig for potential clinical use. Several important points need to be considered in this context. First, the conformation of the Ig fusion protein needs to be assessed, i.e., whether the desired functional outcome may be increased by the utilization of monomeric, dimeric, or multimeric forms of the fusion protein. Secondly, the role of the fusion protein as an agonist versus an antagonist, for example is the functional activity of the fusion protein inhibited, unaffected, or enhanced by the its binding to host Fc receptors. Lastly, the serum half-life of the respective protein and the longevity of the treatment effect must be considered to optimize its desired efficacy while limiting undesired side effects. In conclusion, signaling via the B7-H4 receptor using B7-H4Ig down-regulates differentiation of mouse (and human) inflammatory Th1/17 cell subsets providing an effective therapy for pre-established EAE which may be highly relevant to the immunoregulation of the pathogenesis MS and other autoimmune diseases. Regulation is mediated by both a direct-cell-intrinsic pathway as well as by Treg-mediated indirect regulation of pro-inflammatory Th1/17 cells activity. Critically, our results show that B7-H4Ig therapy increases both the number and the functional capacity of Tregs and most importantly show the functional relevance of Treg induction to long-lived regulation the progression of relapsing and chronic EAE models.

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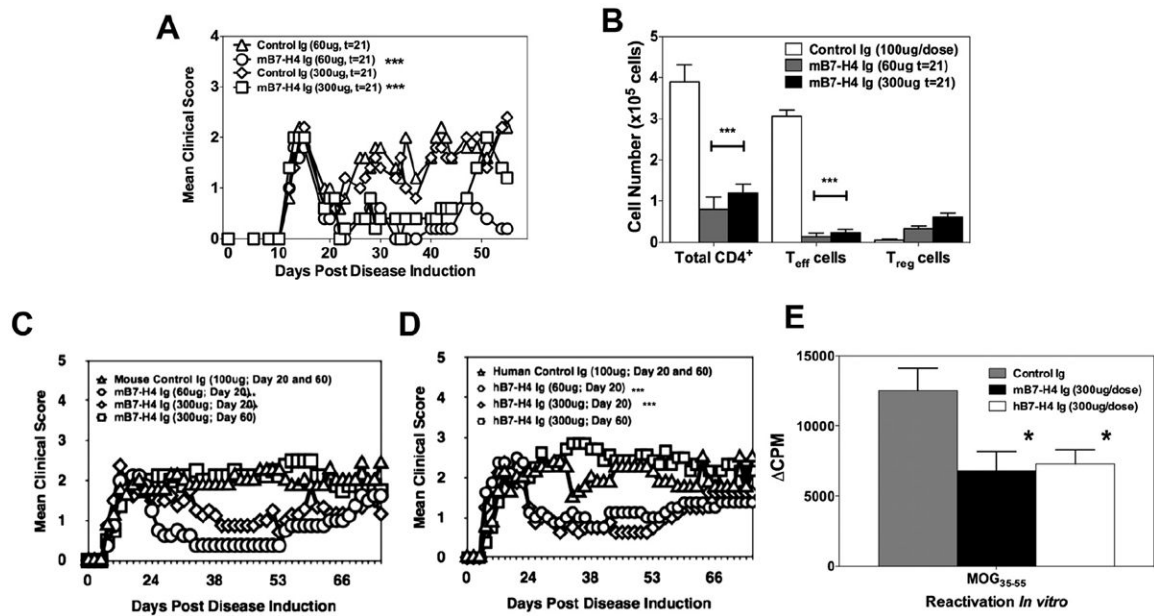
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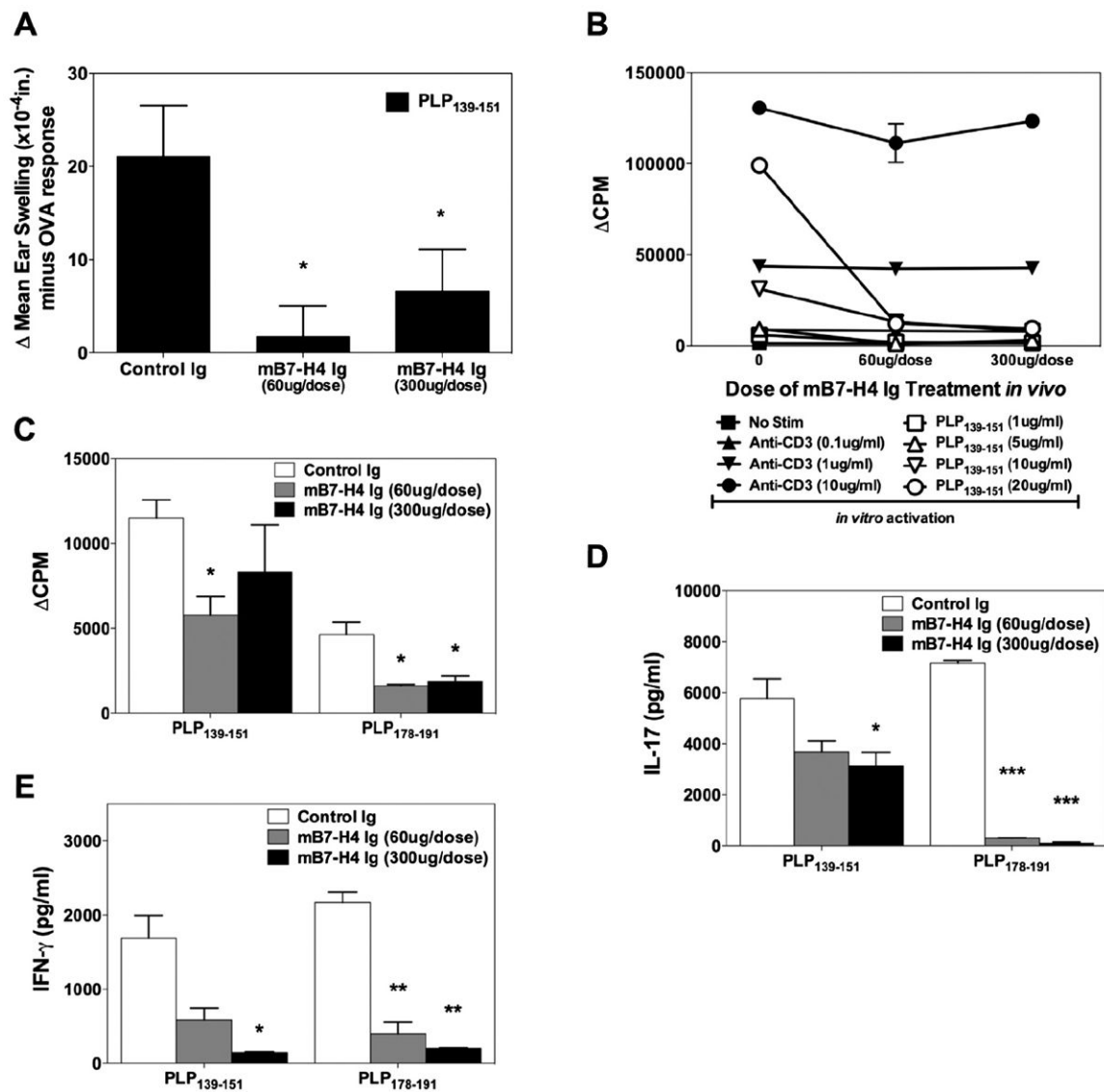
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## Abbreviations

<b>B7-H4Ig</b>	B7-H4-Immumoglobulin fusion protein
<b>hB7-H4Ig</b>	human B7-H4Ig
<b>mB7-H4Ig</b>	mouse B7-H4Ig
<b>C-EAE</b>	chronic experimental autoimmune encephalomyelitis
<b>CNS</b>	central nervous system
<b>CTLA-4</b>	cytotoxic T lymphocyte associated antigen-4
<b>MBP</b>	myelin basic protein
<b>MS</b>	multiple sclerosis
<b>OVA</b>	ovalbumin
<b>PLP</b>	myelin proteolipid protein
<b>R-EAE</b>	relapsing experimental autoimmune encephalomyelitis
<b>T1D</b>	Type I diabetes
<b>TCR</b>	T cell receptor
<b>CFA</b>	complete Freund's adjuvant



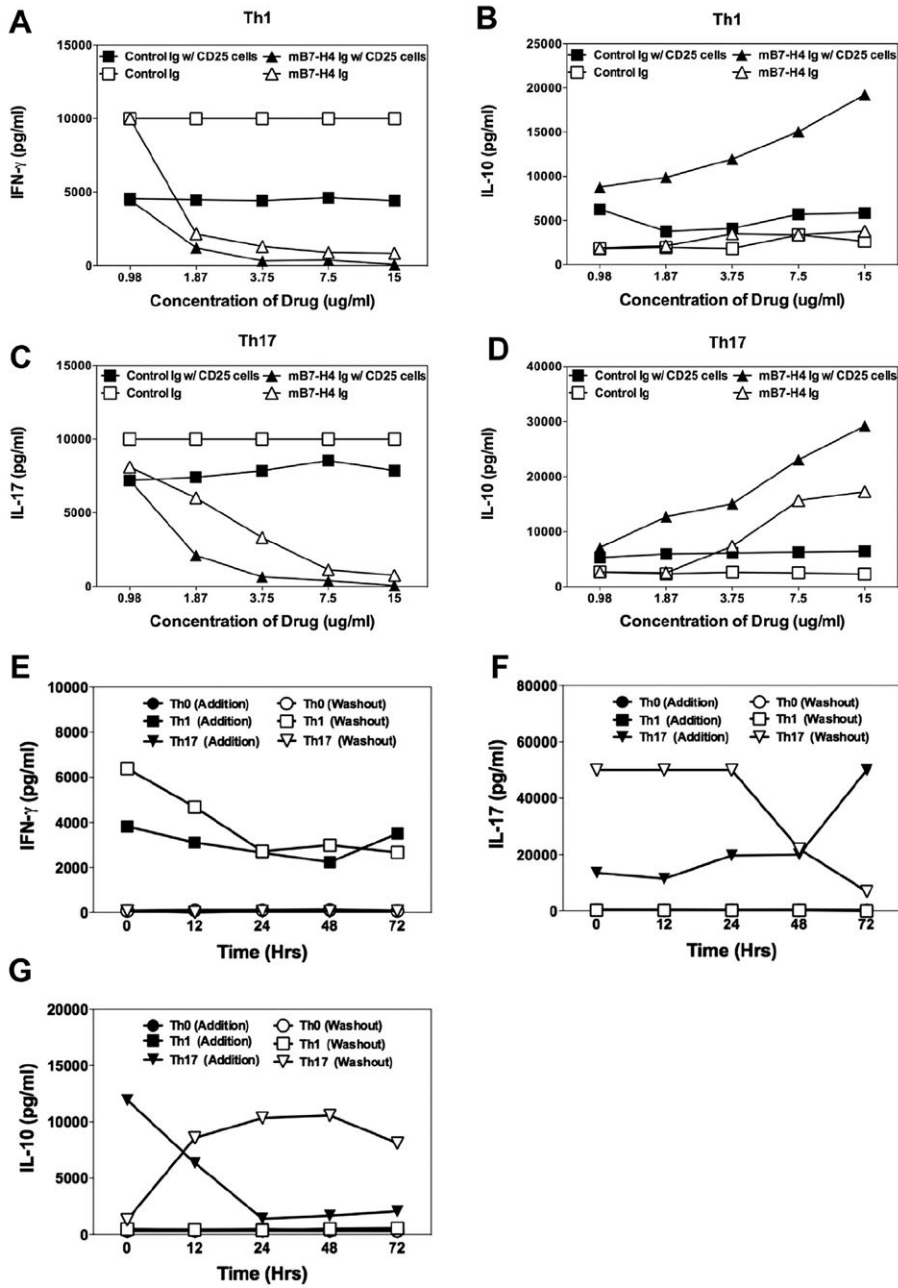
**Fig. 1.** B7-H4Ig decreases EAE disease severity. SJL/J mice ( $n = 10/\text{group}$ ) were primed with PLP<sub>139-151</sub>/CFA and (A) treated with 60 or 300  $\mu\text{g}$  of species and isotype-matched Control-Ig or mB7-H4Ig three-times per week for four weeks beginning on Day +21 ( $t = 21$ ) during disease remission. On Day +35 post-disease induction, (B) the CNS was collected from 3 representative mice per group and the numbers of total CD4<sup>+</sup> T-cells, T<sub>eff</sub> cells (CD4<sup>+</sup>/CD44<sup>hi</sup>/FoxP3<sup>-</sup>), and Tregs (CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup>) were assessed via FACS analysis. C57BL/6 mice ( $n = 10/\text{group}$ ) were primed with MOG<sub>35-55</sub>/CFA and treated beginning on Day +21 ( $t = 21$ ) with 60 or 300  $\mu\text{g}$  of species and isotype-matched Control-Ig or (C) mB7-H4Ig or (D) hB7-H4Ig three times per week for four weeks. The mice were followed for disease and the data are presented as the mean clinical score. Additionally, a subset of the Control-Ig treated mice from panels C and D were treated with mB7-B4Ig or hB7-H4Ig beginning on Day +60 post-disease induction and followed for disease severity. One representative experiment of 2–4 experiments is shown, and disease severity was found to be statistically significant when B7-H4Ig was administered at either the time of disease induction or at Day +20 post disease induction as compared to Control-Ig treated mice ( $p < 0.001$ ). (E) On Day +75 post-priming total splenocytes were reactivated *ex vivo* with MOG<sub>35-55</sub> (20  $\mu\text{g}/\text{ml}$ ), and the level of cellular proliferation was assessed via tritiated-thymidine incorporation. Asterisks (\*, \*\*, \*\*\*) indicate a statistically significant decrease in mean clinical score or in proliferation by cells from B7-H4Ig treated mice in comparison to cells collected from Control-Ig treated mice,  $p < 0.05, 0.01, 0.001$  respectively.



**Fig. 2.** B7-H4Ig decreases myelin-specific T-cell responses. SJL/J mice ( $n = 10$ /group) were primed with PLP<sub>139-151</sub>/CFA and treated with 60 or 300  $\mu$ g of species and isotype-matched Control-Ig or mB7-H4Ig every other day until Day +10 when (A) PLP<sub>139-151</sub> *in vivo* DTH responses and (B) *ex vivo* recall responses of total draining LN cells ( $5 \times 10^5$  cells/well) to increasing concentration of anti-CD3, OVA<sub>323-339</sub>, or PLP<sub>139-151</sub> were determined. Cultures were pulsed with 1  $\mu$ Ci tritiated-thymidine at 24 h and cultures harvested at 72 h. SJL/J mice ( $n = 5$ /group) were primed with PLP<sub>139-151</sub>/CFA and treated with 60 or 300  $\mu$ g of species and isotype-matched Control-Ig or mB7-H4Ig three-times per week for two weeks beginning during disease remission. On Day +35 post-disease induction, total splenocytes were collected from individual mice and activated *ex vivo* ( $5 \times 10^5$  cells/well) with PLP<sub>139-151</sub> or PLP<sub>178-191</sub> (20  $\mu$ g/ml). Two replicate sets of wells were set up such that (C) the first was pulsed with 1  $\mu$ Ci of tritiated-thymidine at 24 h and proliferation and culture supernatants from the second were collected at 72 h and assayed for production of (D) IL-17 and (E) IFN- $\gamma$ . One representative experiment of two-three is presented. Asterisks (\*, \*\*, \*\*\*) indicate a statistically significant decrease in proliferation and cytokine

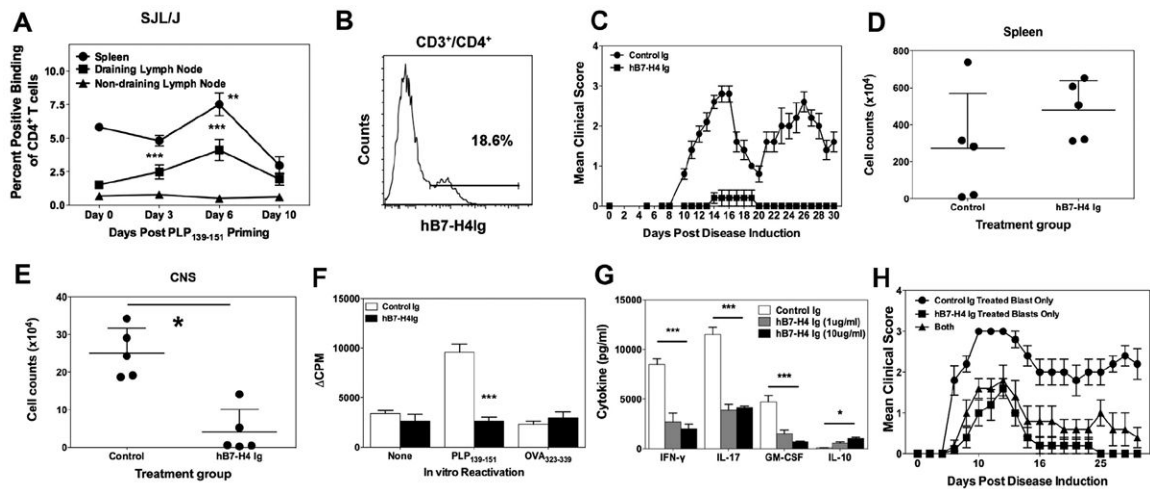
production by cells from B7-H4Ig treated mice in comparison to cells collected from Control-Ig treated mice,  $p < 0.05, 0.01, 0.001$  respectively.





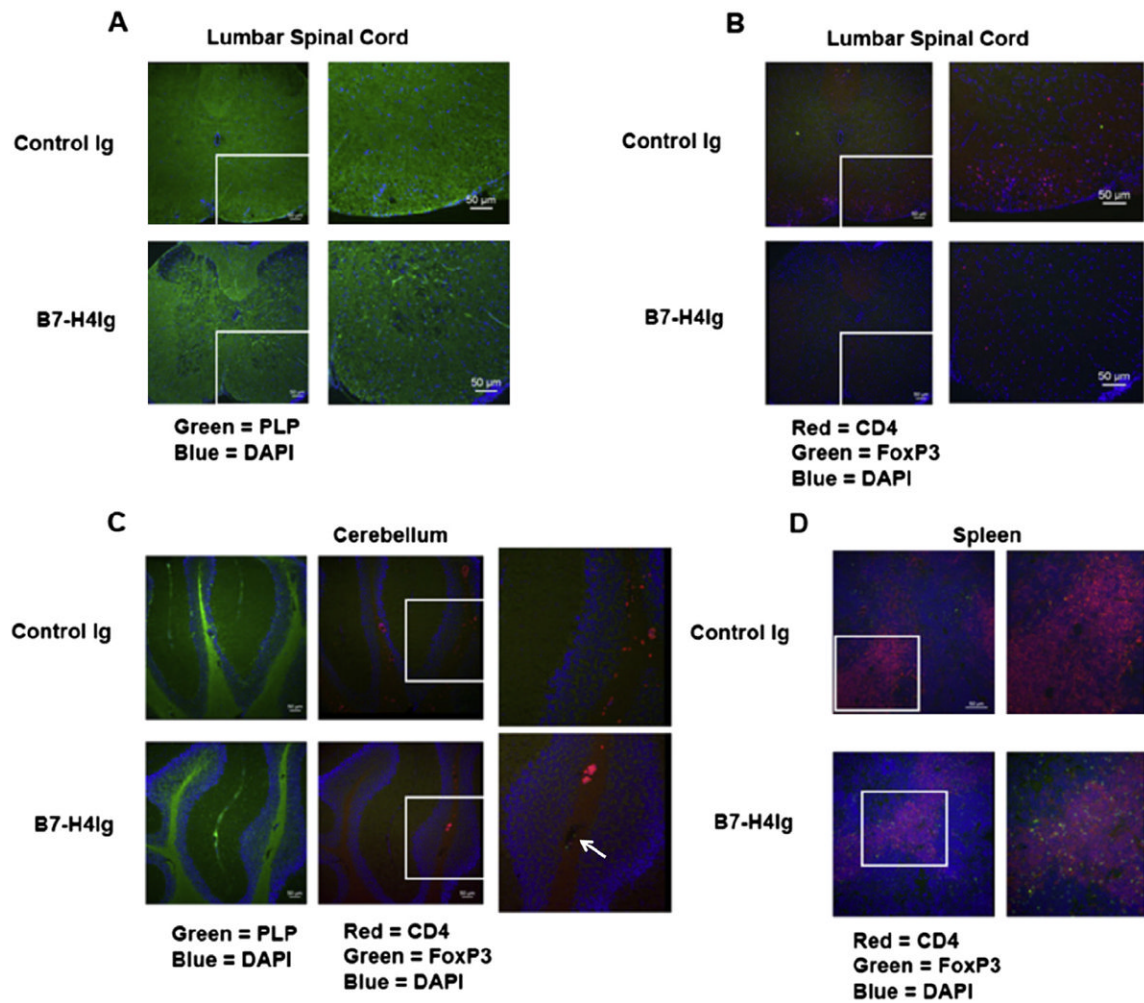
**Fig. 3.** B7-H4Ig inhibits mouse Th1/Th17 cell differentiation. (A–D) CD4<sup>+</sup> T-cells were isolated from naïve DO11.10 mice either specifically excluding CD25<sup>+</sup> nTregs or not (following manufacturer protocol). Cells were cultured ( $5 \times 10^5$  cells +  $5 \times 10^5$  irradiated BALB/c splenocytes + OVA<sub>323–339</sub> (20  $\mu$ g/ml) (A and B) in Th1- [IL-2 (100 U/ml), IL-12 (10 ng/ml), and anti-IL-4 (1  $\mu$ g/ml)] or (C and D) Th17-promoting conditions [TGF- $\beta$  (10 ng/ml), IL-6 (50 ng/ml), IL-23 (10 ng/ml), anti-IL-2, anti-IFN- $\gamma$ , and anti-IL-4 (1  $\mu$ g/ml))] in the presence of increasing concentrations of species and isotype matched Control-Ig or mB7-H4Ig (0–15  $\mu$ g/ml). (E–G) As above, CD4<sup>+</sup> T-cells were isolated from naïve DO11.10 mice specifically excluding CD25<sup>+</sup> cells and cultured in Th0 cell- [IL-2 (100 U/ml), anti-IL-4, and anti-IFN- $\gamma$  (1  $\mu$ g/ml)], Th1 cell-, or Th17 cell-promoting conditions. Control-Ig or mB7-

H4Ig (10  $\mu$ g/ml) was added to the cultures at the various times as indicated or washed out of the cultures at the various times after culture initiation and recultured for the remainder of the 96 h. The levels of IFN- $\gamma$ , IL-17, and IL-10 were assayed via LiquiChip. One representative experiment of two is shown.

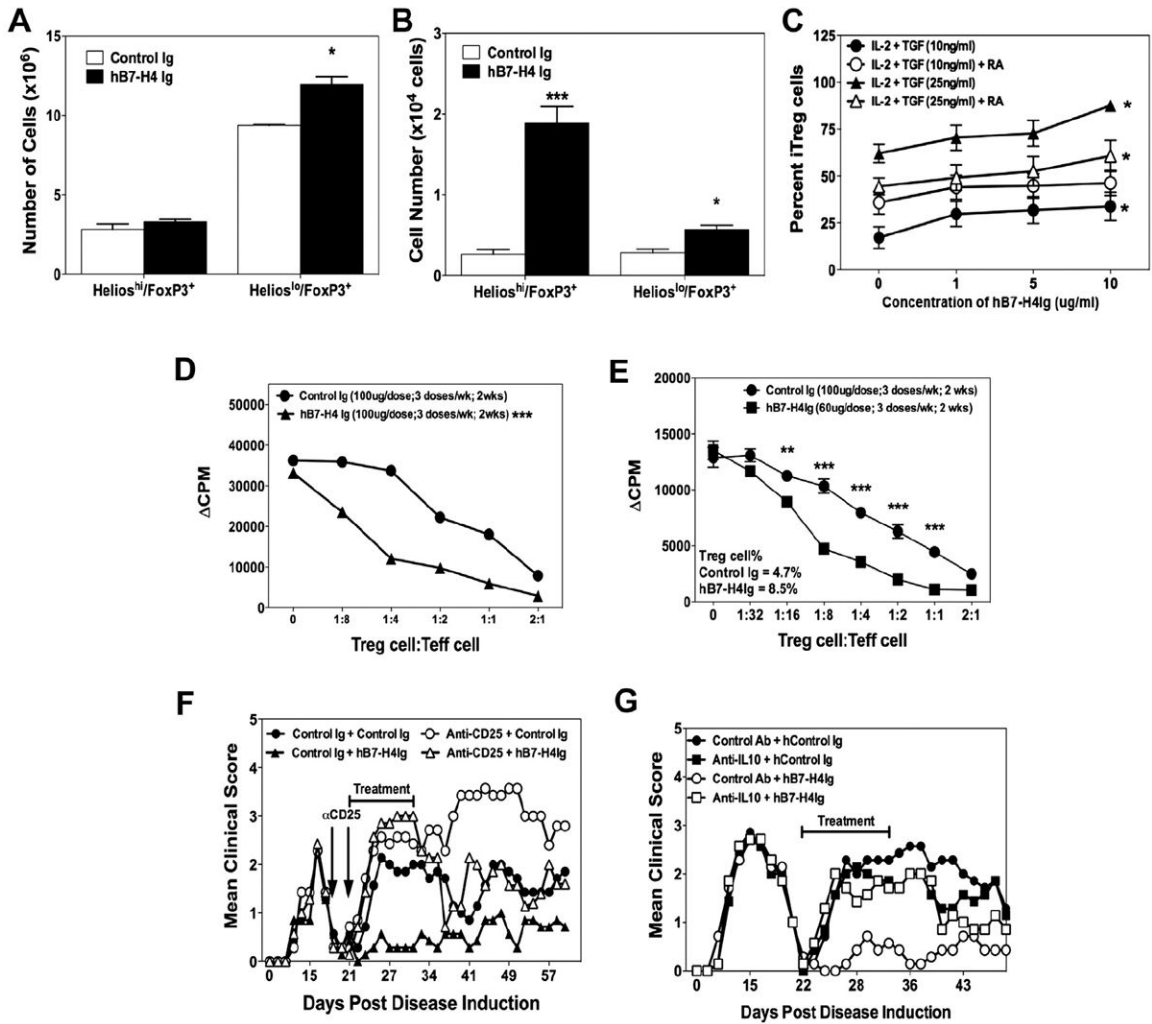


**Fig. 4.**

B7-H4Ig decreases severity of adoptive transfer EAE. (A) Freshly isolated cells from spleens, inguinal LNs (Draining), and cervical LNs (Non-draining) were collected from SJL/J mice primed with PLP<sub>139-151</sub>/CFA on the indicated days and binding of hB7-H4Ig-PE to CD3<sup>+</sup>/CD4<sup>+</sup> T-cells was assessed via FACS analysis. (B) Draining LN cells were collected from PLP<sub>139-151</sub>/CFA-primed SJL/J mice on Day +8, reactivated *in vitro* in the presence of PLP<sub>139-151</sub> (20 μg/ml) for 3 days, and binding of hB7-H4Ig-PE on CD3<sup>+</sup>/CD4<sup>+</sup> T-cells was assessed via FACS analysis. (C) SJL-actin/GFP mice were primed with PLP<sub>139-151</sub>/CFA, draining LNs were collected on Day +8, total LN cells were reactivated *ex vivo* with PLP<sub>139-151</sub> (20 μg/ml), and on Day 3 of culture cell were collected and 5 × 10<sup>6</sup> blast cells were transferred i.v. to naïve recipient SJL/J mice (n = 15/group). Beginning on the day of cell transfer, mice received either three doses per week for two weeks of Control-Ig or hB7-H4Ig (60 μg/dose). The mice were followed for disease severity and the data is presented as the mean clinical score. On Day +10 post-cell transfer, (D) spleens and (E) CNS from the mice treated in Panel C were analyzed via FACS for the presence of transferred cells (n = 5/group). (F) Total cells isolated from individual CNS samples were reactivated *ex vivo* (1 × 10<sup>6</sup> cell/well) with PLP<sub>139-151</sub>, or OVA<sub>323-339</sub> (20 μg/ml). Cultures were pulsed with 1 μCi tritiated-thymidine at 24 h and harvested at 72 h. One representative experiment of 3 is presented. (G) SJL/J mice were primed with PLP<sub>139-151</sub>/CFA, draining LNs were collected on Day +8, and total LN cells were reactivated *ex vivo* with PLP<sub>139-151</sub> (20 μg/ml) plus Control-Ig or hB7-H4Ig (10 μg/ml). On day 3 of culture, cells were collected and supernatants were assessed for cytokine production. (H) 3 × 10<sup>6</sup> blast cells from Control-Ig-treated cultures, 3 × 10<sup>6</sup> blast cells from hB7-H4Ig-treated cultures, or 1.5 × 10<sup>6</sup> blast cells from Control-Ig-treated cultures plus 1.5 × 10<sup>6</sup> blast cells from hB7-H4Ig-treated cultures were transferred i.v. to naïve recipient SJL/J mice (n = 10/group) and mice were followed for disease development. One representative experiment of 3 is presented. Asterisks (\*, \*\*\*) indicate a statistically significant decrease in transferred cells, proliferation and cytokine production following B7-H4Ig treatment in comparison to Control-Ig treatment, p < 0.05, 0.01, 0.001 respectively.

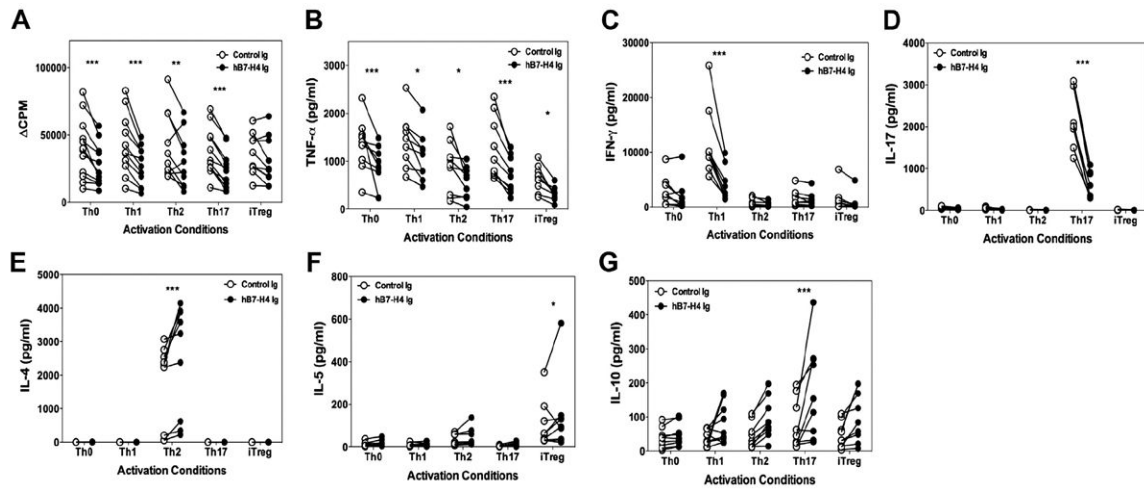


**Fig. 5.** B7-H4Ig treatment decreases the number of effector CD4<sup>+</sup> T cells and increases the number of Tregs in the CNS. SJL-FoxP3/GFP mice (*n* = 5/group) were primed with PLP<sub>139–151</sub>/CFA, and treated with Control-Ig or hB7-H4Ig beginning during disease remission. Following the final treatment mice were perfused, and histological analysis of the lumbar spinal cord, cerebellum, and the spleen. Tissues were frozen in OCT, and 8 μm sections from the lumbar spinal cord were stained for (A) PLP (green) and nuclei (DAPI), and (B) CD4 (red), FoxP3 (green), and nuclei (DAPI). (C) For the sections taken from the cerebellum, similar analysis was completed, and a decrease in the number of CD4<sup>+</sup> T cells was seen with a coordinate increase in the number of FoxP3<sup>+</sup> cells within the lesions of hB7-H4Ig treated mice (white arrow). (D) Spleen sections were analyzed for the presence of CD4<sup>+</sup> cells (red), FoxP3<sup>+</sup> cells (green), and nuclei (DAPI).



**Fig. 6.** B7-H4Ig treatment induces an increase in the number and function of Tregs. SJL/J mice ( $n = 5$ /group) were primed with PLP<sub>139–151</sub>/CFA, and treated with Control-Ig or hB7-H4Ig beginning during disease remission. Following the last treatment (A) spleens and (B) CNS were collected and the number of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup>/Helios<sup>+</sup> versus CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup>/Helios<sup>-</sup> were enumerated from individual mice. (C) CD4<sup>+</sup> T-cells were isolated from unprimed SJL/J mice and cultured ( $2.5 \times 10^5$  cells/well) with anti-CD3 + anti-CD28 (1 g/ml) plus an equal number of irradiated splenocytes in the presence of IL-2 (100 U/ml), TGF- $\beta$ 1 (10 or 25 ng/ml), and retinoic acid (100 nM) plus increasing concentrations of hB7-H4Ig. The cells were collected on Day 4 of culture and the percentage of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> cells determined via FACS analysis. Asterisks (\*) indicate a statistically significant increase in the number of cells in comparison to Control-Ig treated cells,  $p < 0.05$ . (D) Tregs were isolated from unprimed C57BL/6-FoxP3/GFP mice treated with either Control-Ig or hB7-H4Ig (100  $\mu$ g/dose) via MoFlo sorting, co-cultured at various ratios with naïve CD4<sup>+</sup> T-cell plus anti-CD3 (1  $\mu$ g/ml), pulsed with 1  $\mu$ Ci tritiated thymidine at 24 h and cultures harvested at 72 h. (E) SJL-FoxP3/GFP mice were primed as above, and treated with Control-Ig or hB7-H4Ig beginning at disease remission. Following the final treatment splenic Tregs were sort purified and co-cultured at various ratios with  $0.5 \times 10^6$  naïve CD4<sup>+</sup> T-cells plus  $0.5 \times 10^6$  irradiated APCs plus anti-CD3 (1  $\mu$ g/ml). The cultures were pulsed

with 1  $\mu$ Ci tritiated-thymidine at 24 h and harvested at 72 h. Asterisks (\*\*, \*\*\*) indicate a statistically significant decrease in proliferation due to B7-H4Ig treatment as compared to Control-Ig treated cultures ( $p < 0.01$  or 0.001, respectively). (F) SJL/J mice were primed with PLP<sub>139-151</sub>/CFA and treated with Control mAb or anti-CD25 (0.5 mg/dose) on Days +19 and 21 to inactivate Tregs, and then treated with Control-Ig or hB7-H4Ig beginning on Day +21. The mice were followed for disease, and the data is presented as the mean clinical score. Inactivation of Tregs reversed the normal hB7-H4Ig-induced decrease in disease during the period from Days +21–35. One representative experiment of three is shown. (G) To determine the functional relevance of the B7-H4Ig-induced increase in IL-10, SJL/J mice ( $n = 8$  per treatment group) were primed with PLP<sub>139-151</sub>/CFA and treated with Control mAb or anti-IL-10 (0.1 mg/dose) in combination with either a species and isotype-matched Control-Ig or mB7-H4Ig three-times per week for two weeks beginning during disease remission (Day +22 post disease induction). The mice were followed for disease, and data from one experiment is presented as the mean clinical score.



**Fig. 7.**

hB7-H4Ig decreases human Th1/Th17 cell differentiation and increases Th2 cell differentiation. Naive human CD4<sup>+</sup> T-cells were isolated from total PMBCs collected from 10 to 12 healthy donors. The naive CD4<sup>+</sup> T-cells ( $5 \times 10^5$  cells/well) were activated in the presence of autologous irradiated total PBMCs ( $5 \times 10^5$  cells/well) in the presence of anti-CD3 and anti-CD28 (0.5  $\mu$ g/ml) plus Control-Ig or hB7-H4Ig (10  $\mu$ g/ml). Cells were cultured in Th0- [IL-2 (100 U/ml), anti-IL-4, and anti-IFN- $\gamma$  (1  $\mu$ g/ml)], Th1- [IL-2 (100 U/ml), IL-12 (10 ng/ml), and anti-IL-4 (1  $\mu$ g/ml)], Th2- [IL-2 (100 U/ml), IL-4 (10 ng/ml), and anti-IFN- $\gamma$  (1  $\mu$ g/ml)], Th17- [TGF- $\beta$  (10 ng/ml), IL-6 (50 ng/ml), IL-1 $\beta$  (10 ng/ml), anti-IFN- $\gamma$ , and anti-IL-4 (1  $\mu$ g/ml)], or iTreg- [IL-2 (100 U/ml), TGF- $\beta$ 1 (25 ng/ml), and retinoic acid (100 nM)] promoting conditions. Two replicate sets of wells were set up such that one was (A) pulsed with 1  $\mu$ Ci of tritiated-thymidine at 24 h to determine cellular proliferation at 96 h, and for the second set the levels of (B) TNF- $\alpha$ , (C) IFN- $\gamma$ , (D) IL-17, (E) IL-4, (F) IL-5, and (G) IL-10 were determined at 96 h post-culture initiation. Asterisks (\*, \*\*, \*\*\*) indicate a statistically significant alteration in the CPM values or cytokine level due to B7-H4Ig treatment as compared to Control-Ig treated cultures.