Journal of Autoimmunity 44 (2013) 71–81

Contents lists available at SciVerse ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

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

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ARTICLE INFO

Article history:
Received 8 December 2012
Received in revised form
6 March 2013
Accepted 1 April 2013

Keywords:
Autoimmunity
B7-H4
CD4$^+$ T-cell
Costimulatory/co-inhibitory molecule
EAE
Regulatory T-cells

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$^+$ 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$^+$ 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$^+$ 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.

1. Introduction

An important goal in autoimmune disease therapy is development of new treatments re-establishing durable tolerance in self-reactive CD4$^+$ T-cells [1]. MS is an autoimmune disease characterized by Th1 (IFN-γ) and Th17 (IL-17/GM-CSF) CD4$^+$ 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$^+$ T-cell mediated model of MS ideal for characterizing potential tolerance-based immunotherapies and their underlying mechanisms. Full activation of naïve T-cells (CD4$^+$CD62L^{hi}CD25$^-$) 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$^+$ T-cells by APC-expressed B7-family members B7-1 (CD80) and B7-2 (CD86) [6]. Co-stimulatory 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-α, and GM-CSF [10] versus CD4$^+$/FoxP3$^+$ Tregs that are functionally dependent upon TGF-β and/or IL-10 [11–16]. TGF-β in combination with IL-2 is critical for differentiation of Tregs [17] while TGF-β 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$_{35-55}$-induced C-EAE, and treatment of

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

☆ This work was supported by grants from the National Multiple Sclerosis Society Fast Forward Program and from Amplimmune, Inc.

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http://dx.doi.org/10.1016/j.jaut.2013.04.001

wildtype mice with anti-PD-L1 blocking antibody enhances EAE by increasing the number of MOG_{35-55}-specific $CD4^+$ T-cells producing IFN-γ 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 $CD4^+$ 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 $CD4^{\pm}$ 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, $CD4^+$ 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 $CD4^+$ T-cells ($CD4^+$ L-selectin^{hi} 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, $3–5 \times 10^5$ naïve DO11.10 $CD4^+$ T-cells were cultured with an equal number of irradiated BALB/c splenocytes plus $OVA_{323-339}$ (10 μl/ml) in Th0 (200 U/ml IL-2); Th1- (200 U/ml IL-2, 10 ng/ml IL-12, 1 ug/ml anti-IL-4); Th2- (200 U/ml IL-2, 500 U/ml IL-4, 1 ug/ml anti-IFN-γ), Th17- (10 ng/ml TGF-β1, 50 ng/ml IL-6, 1 μg/ml anti-IFN-γ, 1 μg/ml anti-IL-4, 1 μg/ml anti-IL-2), or iTreg- (100 U/ml IL-2, 25 ng/ml TGF-β1, 100 nM retinoic acid) promoting conditions. Peptides ($PLP_{139-151}$, $PLP_{178-191}$, and $OVA_{323-339}$) 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 LiquiChip (Millipore; Billerica, MA).

2.2. Preparation of mouse and human B7-H4Ig

mB7-H4Ig (mouse B7-H4 extracellular domain fused with murine IgG_{2a}) and hB7-H4Ig (human B7-H4 extracellular domain fused with human IgG_1) 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 $CD4^+$ T-cell isolation and culture

Naïve $CD4^+$ T-cells were isolated from total PMBCs (LifeSource; Evanston, IL) of 10–12 healthy donors using AutoMacs cell separation technology. 5×10^5 cells/well were cultured with autologous irradiated total PBMCs (5×10^5 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 $PLP_{139-151}$ 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 $PLP_{139-151}$ 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_{35-55} 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 $PLP_{139-151}$, at a cell density of 8×10^6 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 $PLP_{139-151}$ and $PLP_{178-191}$ 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 (5×10^5 cells/well) in the presence of medium alone, $OVA_{323-339}$, $PLP_{139-151}$ and $PLP_{178-191}$ (20 μg/ml). 24 h post-culture initiation, wells were pulsed with 1 μCi of ^{3}H-TdR and harvested at 72 h and ^{3}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 $PLP_{139-151}$/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 °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 DH_2O to remove O.C.T., and fixed in −20 °C acetone for 10 min. Sections were dried