

PD-1 ligands, negative regulators for activation of naïve, memory, and recently activated human CD4⁺ T cells[☆]

Guifang Cai^a, Arnon Karni^b, Enedina M.L. Oliveira^a, Howard L. Weiner^b,
David A. Hafler^a, Gordon J. Freeman^{c,d,*}

^a Laboratory of Molecular Immunology, Department of Neurology, Brigham and Women's Hospital, Boston, MA 02115, USA

^b Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115, USA

^c Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, USA

^d Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

Received 21 May 2004; accepted 14 September 2004

Available online 23 November 2004

Abstract

We examined the role of the PD-1 pathway on the activation of naïve, memory, and recently activated human CD4⁺ T cells to test whether they responded differently. PD-1 ligand blockade modestly enhanced the percentage of responding T cells and production of IFN- γ in a primary response to myelin basic protein (MBP) in normal donors. PD-1 ligand blockade strongly enhanced proliferation and cytokine production by memory or recently activated T cells (tetanus toxoid and MBP). Blockade of PD-L1 alone had more effect than PD-L2, consistent with its higher expression on ex vivo dendritic cells; furthermore, anti-PD-L1 plus anti-PD-L2 resulted in the greatest enhancement. Moreover, PD-L1-Ig inhibited anti-CD3 induced activation of naïve, memory, and recently activated CD4⁺ T cells. Together, our data demonstrated PD-1 functioned as a negative regulatory pathway on naïve T cells during a primary response, and more potently, on memory or recently activated T cells during a secondary response.

© 2004 Elsevier Inc. All rights reserved.

Keywords: PD-1; PD-1 ligand; Costimulation; Dendritic cells; Human T cells; Naïve T cells; Memory T cells; Myelin basic protein

1. Introduction

Previous studies have demonstrated that optimal T cell activation requires two signals: an antigen-specific signal through the T cell receptor (TCR) and a costimulatory signal [1,2]. Ligation of CD28 on T cells through binding its ligands, B7-1 (CD80) and B7-2 (CD86), on the surface of antigen presenting cells (APC) delivers a

potent costimulatory signal [3–6]. PD-1,¹ a new member of the CD28 family, is transcriptionally induced in activated T cells and B cells [7–9] and contains ITIM and ITSM motifs [10–12]. The ligands for PD-1 (PD-L1 [13] and PD-L2 [14], also known as B7-H1 [15] and B7-DC [16]) are structurally related to the B7 family, and can be induced in monocytes, dendritic cells, endothelial cells, keratinocytes, and B cells [13–17].

Previous studies demonstrated that BALB/c mice deficient in the PD-1 gene developed an early onset autoimmune dilated cardiomyopathy associated with

[☆] This work was supported by NIH Grants AI39671 and CA84500 (to G.J.F.), and NS24247 and NIHP01AI39671 (to D.A.H.). G. Cai is a recipient of a research fellowship from the National Multiple Sclerosis Society.

* Corresponding author. Department of medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115. Fax: +1 617 632 5167.

E-mail address: gordon_freeman@dfci.harvard.edu (G.J. Freeman).

¹ Abbreviations used: PD-1, programmed death-1; PD-L, PD-1 ligand; PBMC, peripheral blood mononuclear cell; DC, dendritic cells; TT, tetanus toxoid; MBP, myelin basic protein; SD, standard deviation.

auto-antibody against heart tissue [18]. In C57BL/6 mice, disruption of the PD-1 gene led to a late onset of a systemic lupus erythematosus (SLE) [19], and polymorphisms in the human PD-1 gene are associated with development of SLE, rheumatoid arthritis, and diabetes [20–22]. Blockade of the PD-1 ligand during EAE or diabetes exacerbate the diseases [23,24]. Furthermore, like CTLA4, ligation of PD-1 on human T cells suppresses their activation including proliferation and cytokine production [13,14,25,26]. Given the importance of PD-1 as an immunosuppressive pathway and the loss of peripheral tolerance in autoimmune disease, we investigated the role of the PD-1 ligand/PD-1 pathway during activation of human T cells. Previous work has shown that blockade of PD-1 ligand on dendritic cells (DC) could stimulate alloresponses [17,27] but no examination of human soluble antigen-specific responses has been reported.

Moreover, the role of PD-L1 and PD-L2 is controversial with some studies supporting a stimulatory role [15,16,28], particularly on naïve T cells [16]. Since PD-1 is expressed following T cell activation, the possibility exists that PD-L1 and PD-L2 might engage a stimulatory receptor on naïve T cells but an inhibitory receptor, PD-1, on memory T cells. Consequently, we examined the effects of PD-L1-Ig and PD-1 ligand blockade on naïve, memory, and recently activated T cells. Using specific antigens (TT or MBP), our studies revealed that blockade of PD-1 ligand slightly enhanced naïve T cell activation in a primary response, and had a much stronger enhancing effect on memory and recently activated T cells during a secondary response. Our data also demonstrated that blockade with PD-L1 and/or PD-L2 mAbs enhanced T cell activation in response to anti-CD3 stimulation. Conversely, PD-L1-Ig inhibited T cell activation of naïve, memory, and recently activated CD4⁺ T cells in response to anti-CD3 or anti-CD3/CD28. In summary, our data demonstrated a negative role for PD-1 pathway during both primary and secondary stages of T cell activation.

2. Materials and methods

2.1. Subjects

Peripheral venous blood was obtained from normal donors after informed consent under IRB approval.

2.2. Antibodies and Ig fusion proteins

Monoclonal antibodies to human PD-L1 (29E.2A3, mIgG2b) and PD-L2 (24F.10C12, mIgG2a) were prepared as previously described [14,17] and have been shown to block the interaction with PD-1 [17]. Anti-CD3 (UCHT1), mouse IgG2a, IgG2b, CD4-PE, Lineage

cocktail 1, CD11c-PE, and IgG1-PE were purchased from PharMingen (San Diego, CA). Biotinylated PD-L1 and PD-L2 were prepared as previously described [29] and used for FACS analysis. Anti-CD3 (OKT3) was from American Type Culture Collection (Manassas, VA). Anti-CD28 (3D10) was a generous gift of Dr. Mary Collins (Wyeth Research, Cambridge, MA). PD-1-PE was purchased from eBioscience (San Diego, CA). Tetanus toxoid was purchased from Massachusetts Biological Laboratory (University of Massachusetts Medical Center, Jamaica Plain, MA). Human PD-L1-murine IgG2a fusion protein (PD-L1-Ig) [9,13] was a generous gift of Dr. Mary Collins (Wyeth Research, Cambridge, MA). Human MBP was extracted from brain as previously described and its purity is greater than 99% [30].

2.3. FACS staining

For staining of PD-1 ligand expression on PBMCs, freshly isolated PBMCs were stained with Lineage cocktail 1 (Lin1) (containing FITC-conjugated antibodies against CD3, CD14, CD16, CD19, CD20, and CD56), PE-conjugated anti-CD11c, and biotin-conjugated anti-PD-L1 or anti-PD-L2 on ice for 30 min, cells were then washed twice with FACS buffer (1% BSA, 0.02% sodium azide, in 1× PBS), followed by another 15 min incubation on ice with streptavidin-CyChrome (BD PharMingen). Cells were then washed twice with FACS buffer before FACS analysis on a FACScan (BD PharMingen). For PD-1 expression, MBP-specific T clones (Ob.1A12, Ob.20b [31]) were stimulated with plate bound anti-CD3 (UCHT1, 0.5 µg/ml) plus anti-CD28 (1 µg/ml) for 48 h. Cells were then harvested, washed, and stained with PE-conjugated mouse anti-human PD-1.

2.4. Purification of CD45RA⁺RO⁻, CD45RA⁻RO⁺, and CD45RA⁺RO⁺ CD4⁺ T cells

CD4⁺ T cells were purified from PBMCs of normal healthy donors using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA) as described by the company. Briefly, PBMCs were resuspended at 10⁸/ml in cold buffer (PBS containing 0.5% bovine serum albumin, 0.5 mM EDTA) and incubated with hapten-conjugated antibodies (anti-CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and glycophorin A). Cells were incubated with beads conjugated with anti-hapten antibody followed by passage through a magnetic column (Miltenyi Biotec, Auburn, CA). CD4⁺ T cells will flow through the column while CD4⁻ cells are retained. Purified CD4⁺ T cells (purity >95%) were then washed twice followed by incubation with either CD45 RA beads or CD45RO beads and negative selection through a LD magnetic column (Miltenyi Biotec, Auburn, CA). FACS analysis revealed that CD45RA⁻ cells contained >95% CD45RA⁻RO⁺ CD4⁺ T cells and CD45RO⁻ cells

contained >95% CD45RA⁺RO⁻ CD4⁺ T cells. For the purification of CD45RA⁺RO⁺ T cells, CD4⁺ T cells were stained with CD45RA-FITC and CD45RO-CyChrome antibodies (PharMingen, CA) and sorted by FACS Vantage SE (BD Bioscience, San Jose, CA). The purity of CD45RA⁺RO⁺ CD4⁺ T cells was >99%.

2.5. *In vitro* stimulation of PBMCs with anti-CD3 and tetanus toxoid

PBMCs were purified by Ficoll/Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden), and 1.5×10^5 cells/well were plated into 96-well U-bottomed plates. Soluble anti-CD3 (OKT3) or tetanus toxoid (TT) was added followed by 5 days culture. Cytokines in the supernatant were measured by ELISA and proliferative responses were assessed by the incorporation of [³H]thymidine during the last 24 h. To measure TT responses by CD45RA⁺RO⁻, CD45RA⁻RO⁺, and CD45RA⁺RO⁺ CD4⁺ T cells, 5×10^4 cells per well were stimulated with 0, 0.1 or 1 flocculation units/ml (Lf/ml) TT for 7 days in the presence of irradiated autologous PBMCs as APC. On day 7, cytokines were measured by ELISA and proliferative responses were assessed by the incorporation of [³H]thymidine during the last 24 h.

2.6. Effect of PD-L1-Ig fusion protein during T cell activation

To determine the effect of PD-L1-Ig fusion protein during T cell activation in response to anti-CD3 and/or anti-CD28, purified subsets of CD4⁺ T cells (CD45RA⁺RO⁻, CD45RA⁻RO⁺, and CD45RA⁺RO⁺) were stimulated with plate bound anti-CD3 (UCHT1, PharMingen, CA) and/or anti-CD28 (3D10), in combination with 0, 2 or 5 µg/ml plate bound PD-L1-Ig for 5 days in 96 U-bottomed plates (5×10^4 cells/well). Plates were coated with 0, 2 or 5 µg/ml PD-L1-Ig, 1 µg/ml anti-CD3 and/or 1 µg/ml anti-CD28 for 2 h at 37°C in 100 µl PBS solution. Mouse IgG2a was added as needed to keep the amount of total protein constant at 7 µg/ml. Plates were washed twice with PBS before cell culture was initiated. Cytokines in the supernatant were measured on day 5. Proliferation was measured on day 6 by pulsing with [³H]thymidine during the last 24 h.

2.7. 11-day assay for primary T cell activation

PBMCs from normal donors were prepared as described. Briefly, blood from healthy adult donors was centrifuged at 1000 rpm for 5 min and autologous plasma at the top was harvested and used to constitute media after heat inactivation at 56°C for 30 min. Peripheral blood mononuclear cells were separated by Ficoll/Hypaque density gradient centrifugation (Amersham, Uppsala, Sweden) and were washed three times

thereafter. Cells were plated in 96-well U-bottomed plates at 1.5×10^5 /well and cultured in media (RPMI-1640), 2 mM glutamine, 1000 U/ml penicillin, 10 µg/ml streptomycin, 10 mM Hepes, 1 mM sodium pyruvate, and 1% (vol/vol) non-essential amino acids (all from Gibco, Grand Island, NY) containing 5% autologous plasma, with or without 40 µg/ml human MBP in combination with 10 µg/ml anti-human PD-L1 and or anti-PD-L2 or isotype controls (mouse IgG2a or mouse IgG2b) for 11 days. Hundred microliters of supernatant was removed to measure cytokines on day 11. Cells were then pulsed with 1 µCi/well [³H]thymidine (NEN Life Science Products, Boston, MA) in 100 µl fresh media for another 24 h. Cells were then harvested on a Tomtec cell harvester (Tomtec, Orange, CT), and incorporation of [³H]thymidine was determined using liquid scintillation counter (1205 Betaplate counter; LKB-Wallac). Positive wells were defined as having CPM or cytokines greater than the mean plus two standard deviations of the 30 isotype control wells cultured without MBP.

2.8. Cytokine ELISA

Cytokines in culture supernatants were assayed by ELISA using Abs to human IL-10, IL-13 (BD PharMingen), or IFN-γ (Endogen, Woburn, MA) according to the manufacturer's instructions.

2.9. Secondary T cell responses

PBMCs were stimulated with or without 40 µg/ml human MBP for 11 days in media with 5% autologous plasma. Cells were then rested for 2 days, changing media each day, and each well was then split into four conditions into a 96-well round-bottomed plate and cultured with 10^5 irradiated autologous PBMCs as APC, with or without 40 µg/ml human MBP, in the presence of 10 µg/ml anti-PD-L1 plus anti-PD-L2 or isotype controls for 3 days. Each condition was performed in duplicate. On day 3, cytokines in the supernatant were measured by ELISA, and cells were pulsed with 1 µCi [³H]thymidine per well and proliferation was measured. A positive well was defined by SI >2 and ΔCPM >2500 (SI = (CPM of the wells with MBP)/(CPM of the wells without MBP)), ΔCPM = (CPM of the wells with MBP) – (CPM of the wells without MBP). The control wells used to calculate SI or ΔCPM were cells originating from the same primary culture well and treated with the same antibodies but without MBP.

2.10. Statistics

Paired Student's *t* test was performed using INSTAT software (GraphPad Software, San Diego, CA). A *p* < 0.05 was considered significant.

3. Results

3.1. PD-L1 is preferentially expressed on ex vivo dendritic cells compared to monocytes

Previous studies have demonstrated PD-L1 and to a lesser extent, PD-L2, expression on in vitro cultured dendritic cells (DC) could be upregulated by IFN- γ [13–17,27]. IL-4 has been shown to strongly enhance PD-L2 and modestly upregulate PD-L1 expression on macrophages and dendritic cells [32–34], possibly in a STAT6 dependent manner [34]. IL-10 has been shown to upregulate PD-L1 expression on monocytes and immature DC but modestly downregulate expression on mature DC [17,27]. To phenotype PD-1 ligands expression in our system, we examined expression on ex vivo Lin1[−]CD11c⁺ DC in freshly isolated PBMCs compared to monocytes (Lin1⁺CD11c⁺ cells). Six of seven normal donor's Lin1[−]CD11c⁺ DC expressed low levels of PD-L1 but little PD-L2 (Figs. 1A and B), and the percentage of PD-L1 positive cells was significantly higher than PD-L2 positive cells ($p=0.013$, paired Student's t test). In contrast, monocytes from most donors expressed little PD-L1 but three of seven donors had moderate levels of PD-L2, though the expression levels of PD-L1 and PD-

L2 on monocytes cells were not significantly differently ($p=0.16$). Nevertheless, the percentage of PD-L1⁺ Lin1[−]CD11c⁺ DC was higher than PD-L1⁺ monocytes (22.5 ± 14 and 3.7 ± 2.8 , respectively, $p=0.0008$), and the percentage of PD-L2⁺ Lin1[−]CD11c⁺ DC was lower than that of PD-L2⁺ monocytes (5.7 ± 5.5 and 12.1 ± 16.2 , respectively, $p<0.05$). Thus PD-L1 was preferentially expressed on ex vivo Lin1[−]CD11c⁺ DC while PD-L2 was expressed moderately on CD11c⁺ monocytes, suggesting PD-L1 might have a more prominent role in regulating T cell activation by DC. The expression of PD-1 on resting and activated MBP-specific T cell clones was also examined. PD-1 was not expressed on two resting MBP-specific T cell clones but was strongly induced at 48 h after stimulation with plate bound anti-CD3 plus anti-CD28 (Fig. 1C).

3.2. PD-1 ligand/PD-1 pathway plays an inhibitory role during T cell activation in response to anti-CD3 stimulation

We investigated the functional role of PD-1 ligand/PD-1 interaction in T cell activation by blocking the PD-1 ligand/PD-1 pathway using mAbs against PD-L1 and/or PD-L2. The effects of blocking PD-1 ligand on regulating T cell activation in response to soluble anti-CD3 in cultures of PBMCs were examined (Fig. 2). Our data showed that blockade of PD-1 ligand enhanced T cell proliferation and cytokine production. Blockade of both PD-L1 and PD-L2 ligands had the strongest enhancing effect on T cell proliferation and production of IFN- γ , IL-13, and IL-10 with 3-fold and 4-fold increases in proliferation and IFN- γ production, respectively, at 0.1 μ g/ml anti-CD3. Blockade of either PD-L1 or PD-L2 alone also enhanced T cell activation in response to anti-CD3 stimulation, but to a lesser extent than the combination of both mAbs. PD-L2 blockade had less effect compared to blockade of PD-L1 alone and showed considerable donor-to-donor variability ranging from none to modest effect. Together, these data demonstrated that blocking PD-1 ligands enhanced T cell activation, indicating an inhibitory role during T cell activation.

3.3. PD-L1-Ig inhibits proliferation and cytokine production in both memory and naïve CD4⁺ T cells

Since PD-1 is not expressed in naïve or resting T cells and is upregulated following T cell activation, other studies have proposed an alternative receptor for PD-1 ligands that could mediate a stimulatory rather than an inhibitory signal for T cell activation. Consequently, we examined whether PD-L1-Ig had differential effects on the responses of CD45RA⁺RO[−] (naïve), CD45RA⁺RO[−] (memory), and CD45RA⁺RO⁺ (recently activated) CD4⁺ T cells [35] upon stimulation with plate bound anti-CD3 or anti-CD3 plus anti-CD28 (Fig. 3B).

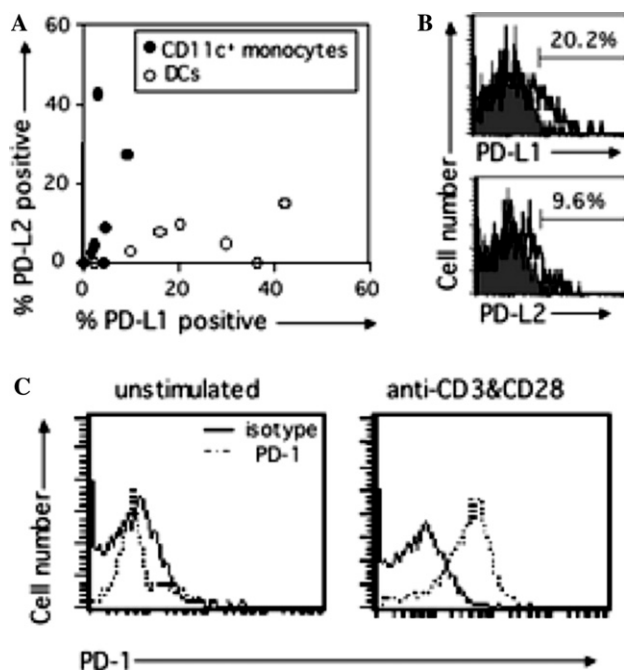


Fig. 1. (A) PBMCs from seven normal donors were stained with anti-PD-L1 or anti-PD-L2 and anti-Lin1 as described. Data shown are gated on Lin1[−]CD11c⁺ for dendritic cells (open circles) or CD11c⁺Lin1⁺ for monocytes (filled circles). Each dot represents one individual. (B) An example of FACS analysis of DC. Shaded is isotype control, bold and unshaded is specific mAb for PD-L1 or PD-L2. (C) PD-1 expression on activated T cell clones. MBP-specific clones were stimulated with anti-CD3 and anti-CD28 or isotype control for 48 h, and cells were then harvested and stained for PD-1. Data shown are representative of two different clones.

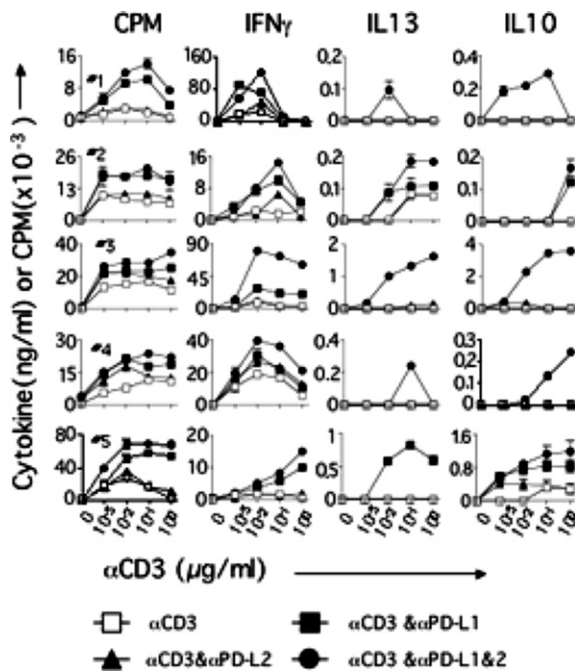


Fig. 2. Blockade of PD-L1 or PD-L2 enhanced T cell activation in response to anti-CD3. PBMCs from normal donors were plated at 1.5×10^5 /well in 96-well U-bottomed plates and stimulated with the indicated concentrations of soluble anti-CD3 for 5 days. Proliferation was measured on day 5 by pulsing with [^{3} H]thymidine for another 24 h. Cytokine levels in the supernatant were measured by ELISA. Open squares are isotype control mAb, filled squares are anti-PD-L1, closed triangle are anti-PD-L2, and filled circles are anti-PD-L1 plus anti-PD-L2. Donor number is indicated in the upper left.

Memory and recently activated T cells proliferated more strongly in response to anti-CD3 compared to naïve T cells ($p < 0.05$). However, this difference was no longer observed when anti-CD28 was added ($p = 0.2$), confirming the importance of CD28 for activation of naïve T cells. Our data showed that naïve T cells did not produce cytokines upon stimulation with anti-CD3 alone, while addition of anti-CD28 led to low levels of IFN- γ , IL-13 and IL-10, which were completely suppressed by PD-L1-Ig at 2 μ g/ml ($p < 0.01$). Memory and recently activated CD4 $^+$ T cells produced high levels of IFN- γ and these levels were greatly reduced by PD-L1-Ig at 2 μ g/ml ($p < 0.05$) and 5 μ g/ml of PD-L1-Ig further suppressed cytokine production compared to 2 μ g/ml PD-L1-Ig ($p < 0.01$). Furthermore, memory CD4 $^+$ T cells produced high levels of IL-10 and IL-13 and these levels were strongly inhibited by PD-L1-Ig ($p < 0.05$). These results suggest that PD-1 or other receptor for PD-L1 function to inhibit activation of naïve, memory, and recently activated CD4 $^+$ T cells (see Fig. 3).

3.4. Role of blockade of the PD-1 pathway on memory T cell responses to TT

To further examine the role of the PD-1 pathway during antigen-specific responses, we examined the effect of

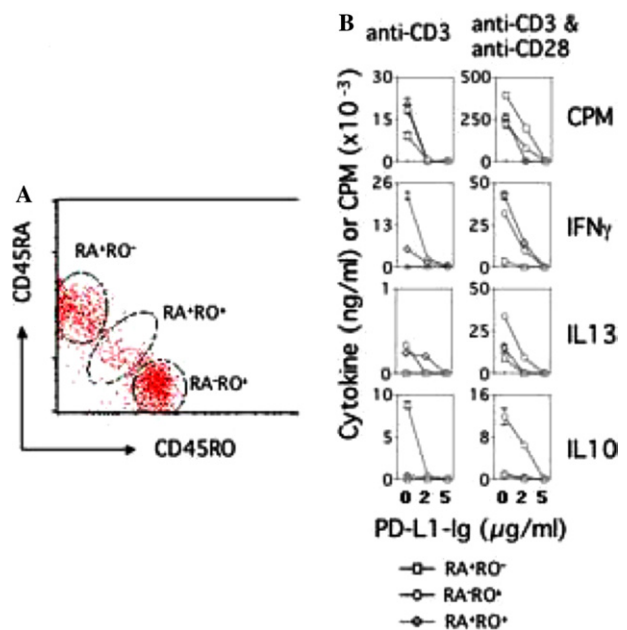


Fig. 3. PD-L1-Ig inhibited proliferation and cytokine production by subsets of CD4 $^+$ T cells (CD45RA $^+$ RO $^-$, CD45RA $^+$ RO $^+$, and CD45RA $^+$ RO $^+$) in response to plate bound anti-CD3 or anti-CD3 plus anti-CD28. (A) Sorting gate for CD45RA $^+$ RO $^+$ CD4 $^+$ T cells. (B) 96 U-bottomed plates were coated with 0, 2 or 5 μ g/ml PD-L1-Ig, 1 μ g/ml anti-CD3 and/or 1 μ g/ml anti-CD28 for 2 h at 37°C in 100 μ l PBS solution. mIgG2a was added as needed to keep the amount of Ig constant at 7 μ g/ml. Plates were washed twice with PBS before cell culture was initiated. Cytokines in the supernatant were measured on day 5. Proliferation was measured on day 5 by pulsing with [^{3} H]thymidine for another 24 h. Data shown are one of three experiments with similar results and statistical analysis used paired Student's t test comparing means from all experiments.

PD-1 ligand blockade on memory T cell responses using tetanus toxoid, a well-characterized antigen for recall responses. PBMCs were cultured with varying concentrations of TT and proliferation and cytokine responses were determined (Fig. 4A). Blockade of PD-L1 enhanced proliferation at low TT antigen concentration (0.1 Lf/ml, $p < 0.01$, paired Student's t test) and had less enhancing effect at higher antigen concentration (1 Lf/ml, $p = 0.07$). However, blockade of PD-L1 significantly enhanced cytokine production at either low or high antigen concentration compared to antigen alone ($p < 0.01$ for both 0.1 and 1 Lf/ml). Blockade of PD-L2 significantly enhanced IL-13 and IL-10 production at both 0.1 and 1 Lf/ml of TT ($p < 0.01$), however, blockade of PD-L2 significantly enhanced IFN- γ and proliferation only at low antigen concentration ($p < 0.01$ at 0.1 Lf/ml and $p = 0.16$ at 1 Lf/ml). Combined blockade of PD-L1 and PD-L2 significantly enhanced proliferation and cytokine production at both low and high antigen concentration ($p < 0.01$), and there was an additive enhancing effect. In general, PD-L1 blockade contributed more than PD-L2 blockade but there was individual variation. Since PD-1 ligands can be induced on both APC and T cells, we tested whether blocking on APC or T cell was more

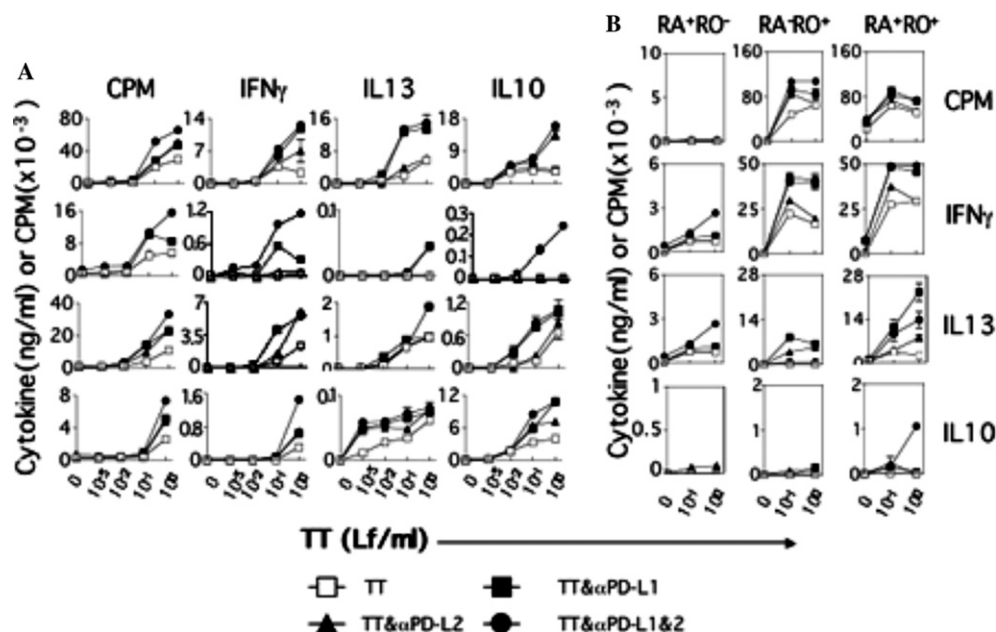


Fig. 4. Blockade of PD-L1 or PD-L2 enhanced T cell activation in response to tetanus toxoid (TT). (A) PBMCs from normal donors were prepared as described, and plated at 1.5×10^5 /well in 96-well U-bottomed plates, and were stimulated with various concentration of TT for 7 days. Cytokine levels and proliferation were assessed as described. Each row indicates an independent donor. (B) CD45RA⁺RO⁻, CD45RA⁺RO⁺, and CD45RA⁺RO⁺ CD4⁺ T cells (purity >95%) were prepared as described in Section 2, and cultured with irradiated autologous PBMCs as APC. T cells were cultured in the presence of various concentrations of TT, anti-PD-L1 (10 μ g/ml) and/or anti-PD-L2 (10 μ g/ml) or isotype controls for 7 days. Proliferation and cytokine levels in the supernatant were measured as described. Data shown are one of three experiments with similar results and statistical analysis used paired Student's *t* test comparing means from all experiments.

important. APC or T cells were preincubated with mAb, washed, and then cultured together. Preincubation of APC with mAb enhanced T cell activation but preincubation of T cells had no effect (data not shown, TT or anti-CD3), suggesting that expression of PD-1 ligands on APC was more important in our system.

We also examined whether PD-1 ligand blockade had differential effects on the TT responses of CD45RA⁺RO⁻ (naïve), CD45RA⁺RO⁺ (memory), and CD45RA⁺RO⁺ (recently activated) CD4⁺ T cells (Fig. 4B). Naïve CD4⁺ T cells did not significantly proliferate in response to TT, consistent with their being naïve T cells that have not seen antigen previously. Nevertheless, the blockade of both PD-1 ligands enhanced production of IFN- γ and IL-13 by naïve T cells, particularly at high antigen dose ($p < 0.01$). The blockade of PD-1 ligand had a more potent enhancing effect on proliferation or cytokine production of memory or recently activated T cells compared to naïve CD4⁺ T cells in response to TT. Briefly, memory and recently activated CD4⁺ T cells both proliferated vigorously to TT, and recently activated CD4⁺ T cells proliferated modestly even in the absence of antigen stimulation, consistent with their recent activation status [35]. Proliferation of both memory and recently activated T cells was moderately enhanced by blockade of PD-L1 and PD-L2 ($p < 0.05$), and IFN- γ production was increased 2-fold ($p < 0.01$) with PD-L1 blockade accounting for most of the enhancement. Blockade of PD-L2 enhanced prolifera-

tion and IFN- γ production by memory and recently activated T cells only at low concentration of TT (0.1 Lf/ml, $p < 0.01$ for both IFN- γ and proliferation) but not at high concentrations of TT (1 Lf/ml TT, $p > 0.1$). IL-13 and IL-10 production were also enhanced by PD-1 ligand blockade but there was more individual variation in the importance of PD-L1 or PD-L2 on production of IL-13 and IL-10 than on proliferation and IFN- γ production, where PD-L1 was more significant. The enhancement was dependent on the presence of antigen (TT).

3.5. Effects of blocking PD-L1 and PD-L2 during primary responses to MBP by human PBMCs from normal healthy donors

In order to examine the role of PD-1 ligands in naïve T cell responses, PBMCs from normal healthy donors were stimulated with 40 μ g/ml human MBP for 11 days, and proliferation and IFN- γ production were determined (Fig. 5). An 11-day culture was optimal for detecting T cell responses to MBP and 30 wells were analyzed for each condition. As expected for naïve T cell responses to MBP as a primary response, only a few wells showed a positive response (Fig. 5) and we thus analyzed only wells with positive responses (Fig. 5), defined as wells with CPM or IFN- γ greater than the mean plus 2 SD of the isotype control. For proliferative responses, there was a small increase in the percentage of positive wells when

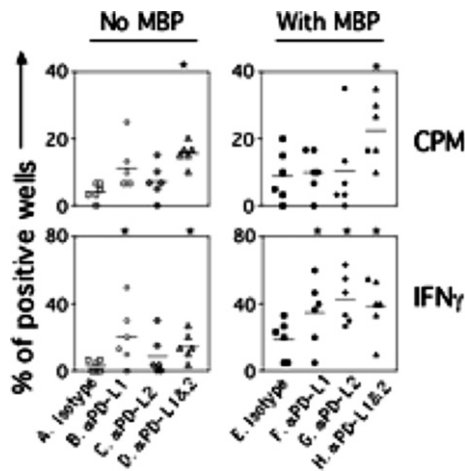


Fig. 5. Effect of PD-1 ligand blockade on T cell proliferation and IFN- γ production during a primary response to MBP. PBMCs from normal donors were stimulated with 40 μ g/ml MBP along with 10 μ g/ml anti-PD-L1 and/or anti-PD-L2 or isotype controls for 11 days. IFN- γ in the supernatant was measured by ELISA, and proliferation was measured by [^{3} H]thymidine incorporation on day 11. Each experiment was performed with PBMCs from a single healthy donor and six experiments are shown. For all six donors, the data were analyzed by calculating the percentage of positive wells, which equals the number of positive wells divided by total numbers of wells (= 30) and multiplied by 100. Proliferation and IFN- γ positive wells were defined as described in Section 2.7. Each symbol indicates the value for one donor. Solid horizontal lines indicate averages of six donors for each condition (* p < 0.05).

MBP was added to the culture, consistent with a primary response of normal donors to MBP, but this was not statistically significant (compare A and E, p = 0.22, paired Student's t test, Fig. 5). The addition of either anti-PD-L1 or anti-PD-L2 alone resulted in a small increase in positive wells in the absence or presence of MBP, but this was not statistically significant (A and B, A and C, E and F, E and G, p > 0.05). However, the combination of anti-PD-L1 and anti-PD-L2 significantly increased the percentage of positive wells in the absence or presence of MBP (p = 0.0007 for A and D, p = 0.013 for E and H, respectively). The increase in proliferation in the absence of MBP but presence of anti-PD-L1 and anti-PD-L2 suggests that PD-1 ligand blockade can also enhance the response of other T cells in the population, making it difficult to observe an MBP-specific effect on proliferation. To eliminate the possibility of this background proliferation being due to the presence of foreign antigens, these experiments were performed in the presence of autologous serum. These results suggest that blockade of PD-L1 or PD-L2 might lead to enhanced T cell activation in response to self-peptide in the context of self-MHC. This background increase could be inhibited by anti-DR antibodies (data not shown), indicating the response is MHC class II-restricted. In the presence of MBP, blockade of PD-L1 and PD-L2 led to a further small increase in the percentage of positive wells but this was not statistically significant (p = 0.20 for D and H).

The enhancing effect of blockade of PD-L1 and/or PD-L2 on IFN- γ production was more significant than the effect on proliferation. The addition of MBP to the culture moderately increased the percentage of IFN- γ positive wells compared to its absence (p = 0.02 for A and E). The addition of anti-PD-L1 had more effect than anti-PD-L2 on increasing the percentage of positive wells in the absence of MBP (p < 0.05 for A and B, p = 0.43 for A and C), and the combination of both antibodies had the strongest effect (p < 0.01 for A and D). Furthermore, there was a significant further increase in IFN- γ production when MBP was present and PD-L2 or PD-L1 and PD-L2 were blocked (p < 0.01 for C and G; D and H). Together, our data showed that blockade of both PD-1 ligands had a stronger enhancing effect than either alone. Moreover, the enhancement of IFN- γ production was more significant than proliferation during a primary response to MBP by normal donors.

3.6. Blockade of PD-1 ligand enhanced MBP-specific responses during secondary T cell responses

The results presented in Fig. 5 showed that PD-1 ligand blockade enhanced the cytokine production of T cells in a primary response to MBP. We also examined the effect of PD-1 ligand blockade during secondary stimulation of recently activated MBP-specific T cells. Briefly, after an 11-day primary stimulation with MBP in the absence of PD-1 ligand blockade and a 2-day rest, each culture well was split into four conditions and T cells were stimulated for 3 days with irradiated autologous PBMCs as APC with or without MBP, in combination with isotype control or anti-PD-L1 plus anti-PD-L2 mAbs. A 3-day culture is long enough to assay the recall response of already activated MBP-specific T cells and short enough to avoid an increase in background proliferation of non-MBP-specific T cells. Fig. 6A showed T cell proliferation and IFN- γ , IL-13, and IL-10 production in wells that had an MBP-specific response (defined as stimulation index of CPM > 2 and Δ CPM > 2500 in response to MBP). By comparing the Δ CPM or Δ cytokine (Δ = proliferation or cytokine levels with MBP minus the value without MBP) between isotype and anti-PD-L1 plus anti-PD-L2 (Fig. 6B), our data showed that PD-1 ligand blockade significantly increased proliferation and IFN- γ production of MBP-specific T cells in a secondary stimulation (p < 0.05 for both CPM and IFN- γ production, paired Student's t test). PD-1 ligand blockade had a variable effect on production of IL-13 (3 decrease, 1 increase, and 8 not detectable) and IL-10 (2 decrease, 4 increase, and 6 not detectable), perhaps as a consequence of the increased IFN- γ production (Fig. 6B). Previous studies in mice have found that blockade of PD-L2 during at the time of allergen challenge in asthma led to enhanced IL-5 and IL-13 production and modestly decreased IFN- γ production [33], while studies by Curiel

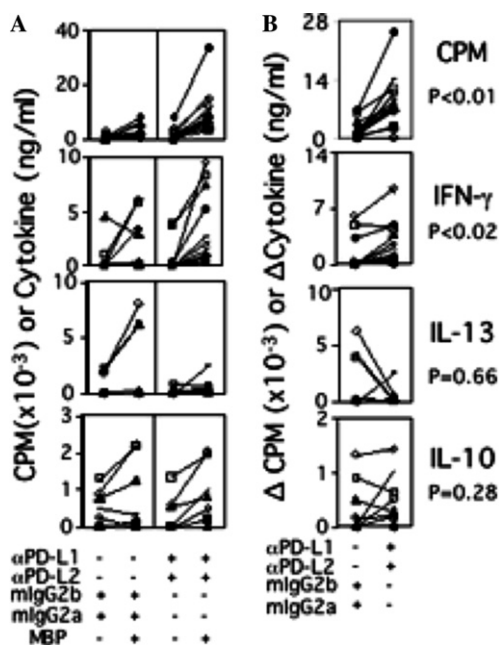


Fig. 6. PD-1 ligand blockade enhances MBP-specific responses during secondary T cell stimulation. (A) PBMCs from normal donors were cultured with 40 μ g/ml human MBP for 11 days in a total of 30 wells. Cells were then rested for an additional 2 days by culturing in new media. Secondary stimulation was performed by dividing each culture well into four groups and culturing with 10⁵ irradiated autologous PBMCs as APC in the presence or absence of 40 μ g/ml human MBP, in combination with 10 μ g/ml anti-PD-L1 and anti-PD-L2 or isotype controls as indicated, in duplicate. Supernatants were harvested on day 3 for determination of cytokine production and T cell proliferation was determined by [³H]thymidine incorporation on day 4. Data shown are for MBP-specific wells, defined by Δ CPM > 2500 and SI > 2. (B) The change (Δ) in CPM or cytokine production in (A) was calculated by subtracting CPM or cytokine levels of antibody without MBP from the same antibody with MBP. Paired Student's *t* test was performed to calculate the *p* value for Δ CPM or Δ cytokine production. A uniform symbol is used for the four conditions from each primary well. There were 12 positive culture wells in total and data were pooled from four different experiments (with 30 wells in each experiment).

et al. [36] showed that blockade of PD-L1 modestly decreased IL-10 production and enhanced IL-2 and IFN- γ production during immune response against human tumor antigen. Thus, the PD-1 pathway is more like an attenuating regulator rather than a regulator of Th1/Th2 differentiation. Together, our results showed that PD-1 ligand blockade also increased secondary T cell responses, particularly proliferation and IFN- γ production, suggesting that the PD-1 pathway might be one of the specific targets for immunoregulation of previously activated antigen-specific T cells.

4. Discussion

Previous studies have demonstrated that PD-1 is a member of the CD28 family and like CTLA-4 could

deliver a negative signal for T cell activation [13,14,25,26]. PD-1 has two ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) that are members of the B7 gene family [13–17]. PD-1 is expressed on activated T cells and might be a target for immunoregulation for various diseases. However, studies by other groups have suggested that PD-1 ligands might deliver a positive signal for T cell activation, particularly for naïve T cells [16]. This raises the question whether the PD-1/PD-1 ligand pathway has different regulatory roles depending on T cell activation status and the relative importance of the PD-1/PD-1 ligand pathway during primary and secondary responses. In order to answer these questions, we examined the effect of PD-1 ligand blockade during primary and secondary responses to antigen-specific stimulation (MBP and TT). We also examined the effect of PD-1 ligand blockade or PD-L1-Ig on purified naïve, memory or previously activated CD4⁺ T cells. Blockade of the PD-1 ligand/PD-1 interaction with monoclonal antibodies against PD-L1 and/or PD-L2 enhanced T cell proliferation and IFN- γ , IL-10, and IL-13 production by PBMCs from normal donors in response to tetanus toxoid or anti-CD3, consistent with a negative regulatory role for the PD-1 ligand/PD-1 pathway. In response to TT or MBP, the inhibitory role of the PD-1 pathway was more prominent in recently activated and memory T cells during the secondary response compared to primary responses, consistent with its expression on activated T cells but not naïve T cells. PD-L1-Ig was able to inhibit activation of naïve as well as recently activated and memory T cells following anti-CD3 or anti-CD3/CD28 stimulation. Together, our studies show that PD-1 plays a modest inhibitory role during primary T cell responses and a strong inhibitory role during secondary T cell responses.

Using blocking antibodies against PD-L1 or PD-L2, our studies showed that blockade of PD-L1 or PD-L2 enhanced T cell activation in response to anti-CD3, TT or MBP. Blockade of PD-L1 had more effect on enhancing T cell activation than PD-L2, consistent with our finding that PD-L1 was expressed at higher levels than PD-L2 on ex vivo isolated DC. This is in contrast to studies with in vitro cultured, GM-CSF/IL-4 stimulated DC in which PD-L2 blockade had a stronger effect [17]. In different DC populations, the relative importance of PD-L1 or PD-L2 will likely depend on their relative levels of expression and this will reflect the prior cytokine milieu of the DC.

The role of the PD-1 ligand/PD-1 pathway has been controversial with some groups reporting stimulatory effects [15,16,28] and other groups an inhibitory effect [13,14,17,27,37]. One possibility to explain these conflicting results might be that different T cell populations respond differently to PD-1 ligand, perhaps because of differing expression of PD-1, alternative receptors, or adapter proteins. Consequently, we examined the effect

of PD-1 ligand stimulation or blockade on the activation of naïve, memory, and recently activated CD4⁺ T cells. Our data showed that PD-L1-Ig strongly inhibited proliferation of naïve T cells in response to plate bound anti-CD3 or anti-CD3 plus anti-CD28. Cytokine production by naïve CD4⁺ T cells was low but was also inhibited by PD-L1-Ig. Using MBP antigen and normal blood donors as a means to probe the response of antigen-specific naïve CD4⁺ T cells [38,39], we found that PD-1 ligand blockade slightly enhanced the percentage of responding T cells for proliferation and production of IFN- γ . Similarly, when recently activated CD4⁺ T cells (CD45RA⁺RO⁺) or memory T cells (CD45RA⁺RO⁺) were treated with PD-L1-Ig, T cell proliferation and cytokine production were inhibited in both populations. Consistent with this, when PD-1 ligands were blocked with mAbs, T cell proliferation and cytokine production of recently activated T cells or memory T cells were enhanced in either antigen-specific responses (TT and MBP) or stimulation with anti-CD3 or anti-CD3/CD28. Thus the conflicting results do not seem to be due to differing responses of T cell populations.

PD-L1 or PD-L2-Ig fusion proteins have been shown to stimulate [15,16,28] or inhibit T cell activation [13,14,27,37] and these conflicting results may be due to the Ig fusion proteins acting as agonists in some situations and as antagonists in others [40]. The differing results with Ig fusion protein might be explained by preparations having differences in efficacy of ligand engagement leading to productive engagement versus receptor blocking. Differences in efficacy of ligand engagement might be accounted for by different effective multimericities of the Ig fusion protein preparations. Indeed, studies with angiopoietin-Ig fusion proteins have shown that the dimeric form binds and blocks the Tie2 receptor but higher order multimers bind and stimulate the receptor [40]. Alternatively, there may be a second receptor for PD-1 ligand which might stimulate T cell responses as suggested by Dong et al. [15,41] and Tseng et al. [16].

All investigators have found that antibodies to PD-1 ligands enhance T cell responses [13,14,27,37]; however, some groups have suggested this is due to the mAb transmitting an activating signal through cross-linking of PD-L1 into an activated T cell [42] or through PD-L2 into a dendritic cell [43]. The mAbs used in our work have been shown to block the interaction between PD-1 ligand and PD-1 and to have the same enhancing effect when used as monomeric Fab or whole antibody [17,27]. Consequently, we do not believe the enhancing effects are due to cross-linking.

The effect of PD-1 ligand blockade was most pronounced during secondary stimulation of T cells, consistent with the expression of PD-1 on activated T cells. Releasing this inhibition may contribute to the development of autoimmunity. Indeed, polymorphisms in human PD-1 are associated with susceptibility to lupus

erythematosus, rheumatoid arthritis, and diabetes [20–22]. C57BL/6 mice deficient in PD-1 develop a late onset lupus-like arthritis with glomerulonephritis, which is accelerated by the introduction of the *lpr* (CD95, fas) mutation [19]. Studies with blocking mAbs by Salama et al. and Ansari et al. also demonstrated a negative regulatory role of the PD-1 pathway during T cell activation against self-antigen in two different mouse models (EAE and Type I diabetes) [23,24]. Together, these studies strongly suggest that PD-1 has an important inhibitory pathway for peripheral tolerance.

Together, our data demonstrated that the PD-1 ligand/PD-1 pathway functioned as a negative regulatory pathway for naïve T cell activation during a primary response to MBP or TT, and more importantly, on previously activated T cells or memory T cells during the secondary response. These results indicate that T cell activation is a summation of positive signals provided by such signals as TCR avidity and number, B7-1 and B7-2/CD28 mediated costimulation, ICOS/ICOS-ligand, and other positive costimulatory signals balanced against negative signals provided by PD-1 ligand/PD-1, B7-1 and B7-2/CTLA-4 as well as other inhibitory signaling pathways. The PD-1 ligand/PD-1 pathway antagonizes the activation of T cells including autoreactive cells, thereby assisting in the maintenance of peripheral tolerance. Immune inhibition via PD-1 might be a good candidate for immunotherapy since this pathway is most active in previously activated T cells.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.celimm.2004.09.004](https://doi.org/10.1016/j.celimm.2004.09.004).

References

- [1] R.H. Schwartz, *Cell* 71 (1992) 1065–1068.
- [2] P.A. Bretscher, *Proc. Natl. Acad. Sci. USA* 96 (1999) 185–190.
- [3] A.H. Sharpe, G.J. Freeman, *Nat. Rev. Immunol.* 2 (2002) 116–126.
- [4] C.A. Chambers, M.S. Kuhns, J.G. Egen, J.P. Allison, *Annu. Rev. Immunol.* 19 (2001) 565–594.
- [5] B. Salomon, J.A. Bluestone, *Annu. Rev. Immunol.* 19 (2001) 225–252.
- [6] B.M. Carreno, M. Collins, *Annu. Rev. Immunol.* 20 (2002) 29–53.
- [7] Y. Agata, A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita, T. Honjo, *Int. Immunol.* 8 (1996) 765–772.
- [8] R. Vibhakkar, G. Juan, F. Traganos, Z. Darzynkiewicz, L.R. Finger, *Exp. Cell Res.* 232 (1997) 25–28.
- [9] L. Carter, L.A. Fouser, J. Jussif, L. Fitz, B. Deng, C.R. Wood, M. Collins, T. Honjo, G.J. Freeman, B.M. Carreno, *Eur. J. Immunol.* 32 (2002) 634–643.
- [10] M.L. Thomas, *J. Exp. Med.* 181 (1995) 1953–1956.
- [11] E. Vivier, M. Daeron, *Immunol. Today* 18 (1997) 286–291.
- [12] J.M. Chemnitz, R.V. Parry, K.E. Nichols, C.H. June, J.L. Riley, *J. Immunol.* 173 (2004) 945–954.

- [13] G.J. Freeman, A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, T. Honjo, *J. Exp. Med.* 192 (2000) 1027–1034.
- [14] Y. Latchman, C.R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A.J. Long, J.A. Brown, R. Nunes, E.A. Greenfield, K. Bourque, V.A. Boussiotis, L.L. Carter, B.M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A.H. Sharpe, *G.J. Freeman, Nat. Immunol.* 2 (2001) 261–268.
- [15] H. Dong, G. Zhu, K. Tamada, L. Chen, *Nat. Med.* 5 (1999) 1365–1369.
- [16] S.Y. Tseng, M. Otsuji, K. Gorski, X. Huang, J.E. Slansky, S.I. Pai, A. Shalabi, T. Shin, D.M. Pardoll, H. Tsuchiya, *J. Exp. Med.* 193 (2001) 839–846.
- [17] J.A. Brown, D.M. Dorfman, F.R. Ma, E.L. Sullivan, O. Munoz, C.R. Wood, E.A. Greenfield, G.J. Freeman, *J. Immunol.* 170 (2003) 1257–1266.
- [18] H. Nishimura, T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, T. Honjo, *Science* 291 (2001) 319–322.
- [19] H. Nishimura, M. Nose, H. Hiai, N. Minato, T. Honjo, *Immunity* 11 (1999) 141–151.
- [20] L. Prokunina, C. Castillejo-Lopez, F. Oberg, I. Gunnarsson, L. Berg, V. Magnusson, A.J. Brookes, D. Tentler, H. Kristjansdottir, G. Grondal, A.I. Bolstad, E. Svenungsson, I. Lundberg, G. Sturfelt, A. Jonssen, L. Truedsson, G. Lima, J. Alcocer-Varela, R. Jonsson, U.B. Gyllenstein, J.B. Harley, D. Alarcon-Segovia, K. Steinsson, M.E. Alarcon-Riquelme, *Nat. Genet.* 32 (2002) 666–669.
- [21] S.C. Lin, J.H. Yen, J.J. Tsai, W.C. Tsai, T.T. Ou, H.W. Liu, C.J. Chen, *Arthritis Rheum.* 50 (2004) 770–775.
- [22] C. Nielsen, D. Hansen, S. Husby, B.B. Jacobsen, S.T. Lillevang, *Tissue Antigens* 62 (2003) 492–497.
- [23] M.J. Ansari, A.D. Salama, T. Chitnis, R.N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S.J. Khoury, H. Auchincloss Jr., M.H. Sayegh, *J. Exp. Med.* 198 (2003) 63–69.
- [24] A.D. Salama, T. Chitnis, J. Imitola, H. Akiba, F. Tushima, M. Azuma, H. Yagita, M.H. Sayegh, S.J. Khoury, *J. Exp. Med.* 198 (2003) 71–78.
- [25] F. Bennett, D. Luxenberg, V. Ling, I.M. Wang, K. Marquette, D. Lowe, N. Khan, G. Veldman, K.A. Jacobs, V.E. Valge-Archer, M. Collins, B.M. Carreno, *J. Immunol.* 170 (2003) 711–718.
- [26] M. Ishida, Y. Iwai, Y. Tanaka, T. Okazaki, G.J. Freeman, N. Minato, T. Honjo, *Immunol. Lett.* 84 (2002) 57–62.
- [27] N. Selenko-Gebauer, O. Majdic, A. Szekeres, G. Hoffer, E. Guthmann, U. Korthauer, G. Zlabinger, P. Steinberger, W.F. Pickl, H. Stockinger, W. Knapp, J. Stockl, *J. Immunol.* 170 (2003) 3637–3644.
- [28] L.T. Nguyen, S. Radhakrishnan, B. Ciric, K. Tamada, T. Shin, D.M. Pardoll, L. Chen, M. Rodriguez, L.R. Pease, *J. Exp. Med.* 196 (2002) 1393–1398.
- [29] J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, *Current Protocols in Immunology*, Wiley, New York, 1994.
- [30] G.E. Deibler, R.E. Martenson, M.W. Kies, *Prep. Biochem.* 2 (1972) 139–165.
- [31] K. Ota, M. Matsui, E.L. Milford, G.A. Mackin, H.L. Weiner, D.A. Hafler, *Nature* 346 (1990) 183–187.
- [32] S.C. Liang, Y.E. Latchman, J.E. Buhlmann, M.F. Tomczak, B.H. Horwitz, G.J. Freeman, A.H. Sharpe, *Eur. J. Immunol.* 33 (2003) 2706–2716.
- [33] T. Yamazaki, H. Akiba, H. Iwai, H. Matsuda, M. Aoki, Y. Tanno, T. Shin, H. Tsuchiya, D.M. Pardoll, K. Okumura, M. Azuma, H. Yagita, *J. Immunol.* 169 (2002) 5538–5545.
- [34] P. Loke, J.P. Allison, *Proc. Natl. Acad. Sci. USA* 100 (2003) 5336–5341.
- [35] J.M. LaSalle, D.A. Hafler, *Cell. Immunol.* 138 (1991) 197–206.
- [36] T.J. Curiel, S. Wei, H. Dong, X. Alvarez, P. Cheng, P. Mottram, R. Krzysiek, K.L. Knutson, B. Daniel, M.C. Zimmermann, O. David, M. Burrow, A. Gordon, N. Dhurandhar, L. Myers, R. Berggren, A. Hemminki, R.D. Alvarez, D. Emilie, D.T. Curiel, L. Chen, W. Zou, *Nat. Med.* 9 (2003) 562–567.
- [37] T. Okazaki, A. Maeda, H. Nishimura, T. Kurosaki, T. Honjo, *Proc. Natl. Acad. Sci. USA* 98 (2001) 13866–13871.
- [38] C. Scholz, K.T. Patton, D.E. Anderson, G.J. Freeman, D.A. Hafler, *J. Immunol.* 160 (1998) 1532–1538.
- [39] A.E. Lovett-Racke, J.L. Trotter, J. Lauber, P.J. Perrin, C.H. June, M.K. Racke, *J. Clin. Invest.* 101 (1998) 725–730.
- [40] S. Davis, N. Papadopoulos, T.H. Aldrich, P.C. Maisonpierre, T. Huang, L. Kovac, A. Xu, R. Leidich, E. Radziejewska, A. Rafique, J. Goldberg, V. Jain, K. Bailey, M. Karow, J. Fandl, S.J. Samuels, E. Ioffe, J.S. Rudge, T.J. Daly, C. Radziejewski, G.D. Yancopoulos, *Nat. Struct. Biol.* 10 (2003) 38–44.
- [41] H. Dong, S.E. Strome, D.R. Salomao, H. Tamura, F. Hirano, D.B. Flies, P.C. Roche, J. Lu, G. Zhu, K. Tamada, V.A. Lennon, E. Celis, L. Chen, *Nat. Med.* 8 (2002) 793–800.
- [42] H. Dong, S.E. Strome, E.L. Matteson, K.G. Moder, D.B. Flies, G. Zhu, H. Tamura, C.L. Driscoll, L. Chen, *J. Clin. Invest.* 111 (2003) 363–370.
- [43] S. Radhakrishnan, L.T. Nguyen, B. Ciric, D.R. Ure, B. Zhou, K. Tamada, H. Dong, S.Y. Tseng, T. Shin, D.M. Pardoll, L. Chen, R.A. Kyle, M. Rodriguez, L.R. Pease, *J. Immunol.* 170 (2003) 1830–1838.